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THE ROLE OF CORTICOSTERONE IN THE LOSS OF

IMMUNE FUNCTION IN THE ZINC DEFICIENT A/J MOUSE

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PAULA DEPASQUALE-JARDIEU

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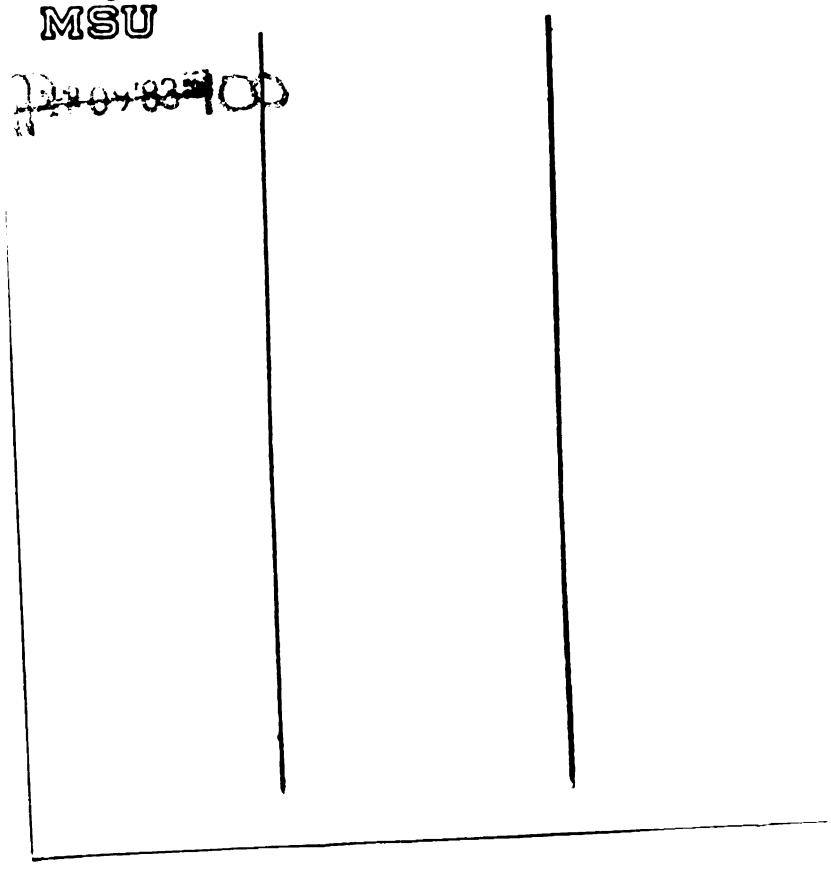

THE ROLE OF CORTICOSTERONE IN THE LOSS OF IMMUNE FUNCTION IN THE ZINC DEFICIENT A/J MOUSE

By

Paula DePasquale-Jardieu

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ABSTRACT

THE ROLE OF CORTICOSTERONE IN THE LOSS OF
IMMUNE FUNCTION IN THE ZINC DEFICIENT A/J MOUSE

By

Paula DePasquale-Jardieu

To date, no explanation has been offered for the preferential involution of the thymus and subsequent immune dysfunction which occurs as a result of dietary deficiencies. It has been suggested that nutritional deficiencies constitute a stress on the animal leading to stimulation of the adrenal cortex and a rise in serum glucocorticoids, proven thymolytic hormones.

The purpose of this research was to determine if the immune impairment resulting from zinc deficiency was stress induced. The data indicate that markedly elevated levels of plasma corticosteroids were present in the zinc deficient mouse during the final stages of zinc deficiency. Although 50% of the immune impairment occurred prior to corticosterone elevation, an additional significant reduction in T-cell function occurred shortly after the appearance of elevated levels of corticosterone. Removal of the steriod via adrenalectomy offered only a modest protection (20%) against this further drop in immune capacity. Thus, these data argue against a major role for corticosterone in the immunosuppression of zinc deficiency and further demonstrate the importance of zinc to lymphocyte function.

ACKNOWLEDGEMENTS

The author wishes to express her sincerest appreciation to Dr. Pamela Fraker for her guidance, understanding, and friendship throughout these studies. Special thanks to the members of her guidance committee, Dr. S. Aust and Dr. G. Riegle, and to Dr. R. Luecke for his assistance with these experiments.

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OBJECTIVE

The primary goal of this research was to determine the cause of the severe thymic atrophy and T-cell impairment brought about during zinc deficiency. Since thymus derived lymphocytes (T-cells) constitute the cell mediated arm of the immune system and also act in conjunction with B cells (as T-cell helpers) in most antibody mediated responses, any disruption of thymic integrity would result in a reduction in immune capacity. Thus, because of the significance of T-cells to immune function, it is important to determine the mechanism whereby zinc deficiency causes rapid involution of the thymus and subsequent loss in immune function.

Based on observations of adrenal hypertrophy in zinc-deficient animals, this study was undertaken to determine if zinc deficiency might constitute a chronic stress resulting in reactions mediated via the hypothalmus-pituitary-adrenocortical axis. If zinc deficiency activated this stress axis, it would result in a rise in serum glucocorticoids which could account for the observed decrease in immune capacity.

LITERATURE SURVEY

It is becoming increasingly more apparent that the ability to combat infection is greatly compromised by nutritional deficiencies. The partnership of infection and malnutrition is firmly established in many of the world's underdeveloped populations. A host of dietary deficiencies have been shown to cause preferential wasting of the lymphatic organs compared to other body organs (1, 2). The thymus, the site of maturation of T lymphocytes, which are critical to both cellular and antibody mediated immunity, is often the most severely atrophied organ in deficient animals (3, 4). As a result, host defense mediated via the cellular immune mechanism is usually more severely impaired than that mediated through the humoral immune mechanism (5). It is not surprising then that diseases associated with depressed cellular immunity, such as measles, smallpox, and other viral infections, are leading causes of death in malnourished populations (6).

Based on these observations, a number of studies have been initiated to determine the effects of various dietary deficiencies on the immune response. Thus far, diets deficient in specific vitamins (7), amino acids (8), trace elements (9), protein (10), or essential fatty acids (11), have all been shown to impair immune function. This section will serve to review the results of these investigations.

There seems to be little or no disagreement among investigators that protein calorie malnutrition (PCM) as seen in humans (12) and other animals (13) alters cell mediated immunity but does not appear to adversely effect the humoral arm of the immune system (14). A study

by Smythe (5) in 1971 examined the capacity of protein-calorie malnourished children to respond in the delayed type hypersensitivity reaction, a measure of cell mediated immunity. Seventeen children with PCM were tested against nineteen normal children for their ability to react to skin application of dinitrochlorobenzene (DCB). Twelve of the PCM children failed to react and none reacted severely. In contrast, all of the normal children were able to mount some response, while thirteen children developed a severe reaction to DCB. In this same study, lymphocytes from thirty PCM infants and nine normal infants were tested for their ability to respond to the T-cell mitogen phytohemagglutinin (PHA). Lymphocytes from normal children had a fourfold higher mitotic index than lymphocytes from PCM children. From these results, Smythe concluded that T-cell dependent cell mediated immunity was impaired in PCM children. In another study by Gautam, (15), skin allograft survival, another measure of T-cell function, was increased from nine days to sixteen days in mice maintained on diets containing inadequate levels of protein.

Neumann et al (16) investigated the effects of PCM on the humoral arm of the immune system of PCM children. Immunization with keyhole limpet hemocyanin (KLH) or pneumococcal polysaccharide, two B-cell dependent antigens, revealed no difference in immune responsiveness between PCM children and normal children. In addition, various tests of humoral responses of protein calorie deficient animals support these findings (17).

Other investigators have defined the effects of specific vitamin deficiencies on the immune response. Rats deficient in vitamin B_6 had an increased success in skin transplants (18). Vitamin B_6

deficient guinea pigs had a reduced delayed type hypersensitivity response to purified protein derivative (19).

Jose (20) examined the effects of deficiencies of various essential amino acids on the immune response. Cell mediated immunity, as measured by the in vitro cytotoxic assay, was depressed when amino acid levels were decreased to 10% of required levels. However, a depression in humoral response was also detected in these studies.

Several researchers have shown that metal deficiencies may result in depressed immunity. In a study of iron deficient children, Chandra (21) found no difference in IgG levels or antibody titers to tetanus toxoid when the responses of these children were compared to normal children, but cell mediated immunity (CMI) as measured by delayed type hypersensitivity to Canida Albicans was impaired. Also, magnesium deficiency in rats depressed the hemolysin titer to sheep red blood cells (SRBC) (22).

Studies designed to define the consequences of zinc deficiency on the immune system have suggested a similiar pattern of immune dysfunction. In a major study on zinc deficiency in humans provided by Prasad (23), he found indications of lymphocytopenia, poor wound healing, and increased susceptability to disease accompanied the deficiency.

A genetic defect which results in decreased absorption of zinc has recently been identified. This disease, acrodermatitis enteropathica, is transmitted as an autosomal recessive trait (24). Patients with this disease have low levels of immunoglobulin and death often results from infection with Canida Albicans. Post mortem studies on a child with this disease revealed no germinal centers in the spleen, lack

of lymph nodes, and a thymus which consisted of epithelial cells with few thymocytes (25).

Published reports on experimentally induced zinc deficiency have explored the relationship between immune function and the deficiency. Luecke had repeatedly observed that the thymus was the most severly atrophied organ in zinc-deficient pigs (26) and mice (27). In an initial study by Fraker (28) on A/J female mice made zinc deficient for 4 weeks, then immunized with sheep red blood cells, the deficient mice produced only 10% as many plaque forming cells (PFC) as controls. Reconstitution of the deficient mice with thymocytes from normal mice, prior to immunization with SRBC, restored the response to near normal levels. This indicated a preferential impairment of T-cell function. These observations have been confirmed by Fernandas (29) using A/J males. In addition, he showed a defective development of T-killer lymphocytes, after in vitro sensitization with tumor cells, as well as a defective development of natural killer (NK) lymphocytes in zinc-deficient mice. Pekerak, et al, (30) showed that the antibody response to live Francisella vaccine was intact in the zinc-deficient rat if the animals were immunized prior to being made zinc deficient. This agrees with Frakers (27) studies which revealed that mice primed with a hapten-carrier prior to being made zinc deficient were able to respond as well as zinc-adequate mice when given a second injection of the antigen. Thus, mature antigen activated lymphocytes appear to be more resistant to the absence of zinc than are virgin lymphocytes. Fraker (31) also demonstrated that T-cells involved in the delayed type hypersensitivity reaction were also depressed in zinc-deficient mice.

Thus, it appears that zinc deficiency, as well as other nutritional deficiencies, produces a rapid and seemingly preferential wasting of the thymus as well as greatly impaired T-cell dependent processes. The question arose as to whether or not some common mechanism was responsible for these findings which is only indirectly related to the nutritional element in question. One possible explanation hinges on the observation of Luecke (32) and Quarterman (33) that the adrenal gland was enlarged in zinc-deficient animals. This finding has led to the suggestion that zinc deficiency might constitute a chronic stress on the animal resulting in adrenal hypertrophy (33, 34, 35). Hypertrophy of the adrenal gland could result in increased synthesis and secretion of the adrenal steroids, including the glucocorticoids. Since the injurious effects of glucocorticoids on thymocytes have been well documented (36), a rise in serum glucocorticoids could account for the loss in immune capacity characterized in zinc-deficient animals.

This hypothesis is not without precedent since it has been established that a variety of stressful stimuli can induce reactions that are mediated by the hypothalmus-pituitary-adrenocorticoid system which ultimately result in increased levels of adrenal steroids. In the early 50's Selye (37) noted what he termed a "general alarm syndrome" in response to a variety of systemic stresses. Using a number of physical, as well as dietary stresses, he noted two general characteristics in the experimental animals: (a) adrenal hypertrophy and (b) thymic atrophy. More recent work indicates that stress, in whatever form, causes a discharge of the hypothalmic releasing factor which in turn invokes pituitary adrenocorticotropic hormone (ACTH) secretion. ACTH acts on

the adrenal cortex via adenylate cyclase to promote synthesis and secretion of the glucocorticoids. The release of glucocorticoids normally facilitate adaptive changes which allow the organism to survive noxious stimuli, possibly by diminishing the peripheral uptake of glucose and amino acids which can then be conserved for use by the vital organs. It is this function of the glucocorticoid which merits further emphasis.

Clearly, histological (38) and immunological evidence (39) show that elevated levels of serum glucocorticoids cause lysis of immature cortical T-cells and wasting of the thymic cortex with the mature, cortical lymphocytes being more resistant. By destroying immature thymocytes, glucocorticoids interfere with the normal process of replenishing the peripheral lymphocyte population. The mechanism whereby the glucocorticoids destroy lymphocytes is open to controversy. Glucocorticoids have been shown to inhibit DNA, RNA, and protein synthesis (40). These effects are all believed to result from hormonal inhibition of glucose uptake. It has been suggested that the hormone promotes the synthesis of a protein that inhibits transport of glucose into the cell, thus imparing the ability of the carbohydrates to provide ATP (41). The inhibition of glucose uptake and subsequent reduction of carbohydrate dependent ATP production may then be responsible for the lysis of immature T-cells.

Further evidence of the effects of stress on immunological processes is provided by studies employing mechanically induced stresses. Gisler (42) has determined that exposure of mice to stresses, such as cold, ether, and noise, result in a loss in immune capacity as measured by in vitro responsiveness to sheep red blood cells.

Noting the adrenal hypertrophy and rapid wasting of the thymus observed in zinc deficiency, it was not unreasonable to assume that zinc deficiency, and possibly other nutritional deficiencies, might constitute a chronic stress on the animal leading to manifestations of the alarm syndrome. Thus, this thesis, using zinc deficiency as a model, investigated the possible role of glucocorticoids as the common mechanism responsible for the immune dysfunction resulting from nutritional deficiencies.

Chapter I

A POSSIBLE ROLE FOR CORTICOSTERONE IN THE IMMUNE DYSFUNCTION OF ZINC DEFICIENCY

Abstract

Previous investigations from our laboratory have shown that zinc deficiency causes rapid atrophy of the thymus with subsequent loss of T-cell helper function in the young adult A/J mouse. The purpose of this investigation was to determine if zinc deficiency constituted a chronic stress on the mouse leading to the elevation of glucocorticoid levels which is known to destroy thymic lymphocytes.

The results of these experiments indicated that zinc-deficient mice indeed have increased levels of plasma corticosterone (115 ug/100 ml plasma) compared to mice fed zinc-adequate diets (40 ug/100 ml plasma). A significant reduction in T-cell helper function, which occurred four days after the rise in steroid concentration, suggested that corticosterone might contribute to the loss in immunity; however, about half of the total loss in T-cell helper function occurred prior to the increase in plasma corticosterone and appeared to be due to other factors associated with the lowered zinc levels.

Introduction

While the immune impairment resulting from zinc deficiency had been well characterized by our laboratory (28,31), the actual destructive mechanism(s) evoked during the deficient state remained to be defined. Besides the various known and unknown zinc-dependent biochemical processes that could be adversely effected by dietary zinc deficiency, there was also evidence to suggest that elevated levels of corticosteroid could be present in the zinc-deficient mouse. Results of the literature survey suggested that zinc deficiency might constitute a chronic stress resulting in stimulation of the adrenal cortex and a rise in serum glucocorticoids. If zinc deficiency activated the stress axis, it would result in a rise in the level of serum corticosteroids which might account for the observed loss in numbers of functional thymocytes. The purpose of this study was to determine if indeed zinc deficiency was stressful to the mouse, since the injurious effects of glucocorticoids on thymocytes and thymic integrity have been well documented both *in vivo* (43) and *in vitro* (44).

Materials and Methods

Animals and Diets

Twenty-one ± 2 day old A/J female mice (Jackson Laboratory, Bar Harbor, Maine) weighing 12 ± .9g were used in this experiment. To minimize exposure to environmental zinc, and prevent reabsorption of zinc from body wastes, the mice were housed in stainless steel cages with mesh bottoms. Feed containers and water bottles were washed with 4N HCl and rinsed with deionized water to remove all residual zinc. The mice were fed ad libitum a biotin-fortified egg white diet which contained either deficient (<0.7 ug Zn/g) or adequate (>10 ug Zn/g) levels of zinc as determined from previous studies (45). Diet consumption was measured 3 times per week, and the mice were weighed at least once a week. All mice had free access to deionized-distilled water (<0.2 ug Zn/g).

Zinc Assay by Atomic Absorption Spectroscopy

A known weight of sample to be analyzed for zinc content was added to a preweighted acid washed flask. Twenty-five ml of 32N HNO_3 and 5 ml of 11.7N $HClO_4$ were added to the flask and the sample was slowly evaporated to near dryness. The residue remaining was diluted to a known weight with 10% HCl. Flasks containing only the acid reagents served as blanks.

Zinc concentration of the digested samples was determined with a Varian AA-175 atomic absorption spectrophotometer (Varian Techtron, Springvale, Australia). Absorption values were determined at 214 nm. A

standard curve for zinc absorption was obtained by dilution of a commercial zinc standard solution in 10% HCl.

Collection of Plasma for Corticosteroid Assay

Twenty-one day-old A/J female mice were divided into twenty-four groups consisting of nine mice per group. Twelve groups were fed zinc-deficient diets (<1 ug Zn/g) and twelve were maintained on zinc-adequate diets (50 ug Zn/g) ad libitum. On day zero, twelve mice were bled to determine basal levels of corticosterone. Every other day, beginning with day seven, and every day, from day twelve to nineteen, nine zinc-deficient and nine control mice were randomly selected to be bled. Each mouse was bled only once during the course of the experiment. Mice were bled at 0900 hours each day when the level of corticosterone was at its lowest point in the diurnal cycle. (Data supporting this observation will not be presented in this thesis.) Entrance to the animal room was prohibited for a 3-hour period prior to the bleeding in order to maintain basal levels of corticosterone. Zinc-deficient and control mice were selected at random for bleeding and immediately etherized. Blood was collected from each mouse by retroorbital puncture within 1-minute of removal of the mouse from its cage. Mice from the same cage were removed simultaneously. Approximately 0.15 ml of blood was taken from each mouse and collected in heparinized tubes. The plasma was separated for use in the corticosterone assay.

Corticosterone Assay

A spectrofluorometric method for measuring unconjugated cortisol and corticosterone was adapted from the methods of Sweat (46), Glick (47), and Martin and Martin (48) to allow quantitation on a micro

scale of the steroid present in the small amount of plasma recoverable from mice. The experimental protocol is outlined in Figure 1.

All glassware used in this assay was washed in 16 N HNO_3 overnight and rinsed with distilled H_2O to minimize nonspecific fluorescense. Fifty ul of heparinized plasma were extracted by shaking for 2 minutes with 0.6 ml methylene chloride. After centrifugation, the aqueous layer was discarded and the solvent layer washed with 0.1 ml of 0.1N NaOH. Following centrifugation, the alkaline phase was discarded and the extract was evaported to dryness using a nitrogen evaporator. Double distilled water (0.1 ml) was then added to the residue. The water layer was shaken for 2 minutes with 0.6 ml carbon tetrachloride to extract the corticosterone. Following centrifugation, the aqueous phase was transferred to a clean test tube and the CCl_4 layer was set aside for determination of corticosterone. Cortisol was removed from the remaining aqueous layer by extraction with 0.6 ml methylene chloride for 2 minutes. After centrifugation, the aqueous layer was discarded and the solvent fraction was assayed for cortisol. Half ml aliquots of the solvent layer from each tube containing either cortisol or corticosterone were then pipetted into tubes containing 200 ul of the fluorescent mixture which consisted of 3 parts 32N H_2SO_4 to 1 part absolute ethanol. The tubes were shaken for 2 minutes to develop the fluorescence, centrifuged, and the solvent layer was then removed by aspiration. The acid layer from each tube was transferred to a microcuvette and after 30 minutes the amount of fluorescence was determined at 475 nm (1°) and 525 nm (2°) on a spectrofluorimeter (American Instrument Co., Silver Springs, Maryland). Standards containing 0.05 to 0.1 ug corticosterone and cortisol (Applied Science Laboratories, State College, Pennsylvania)

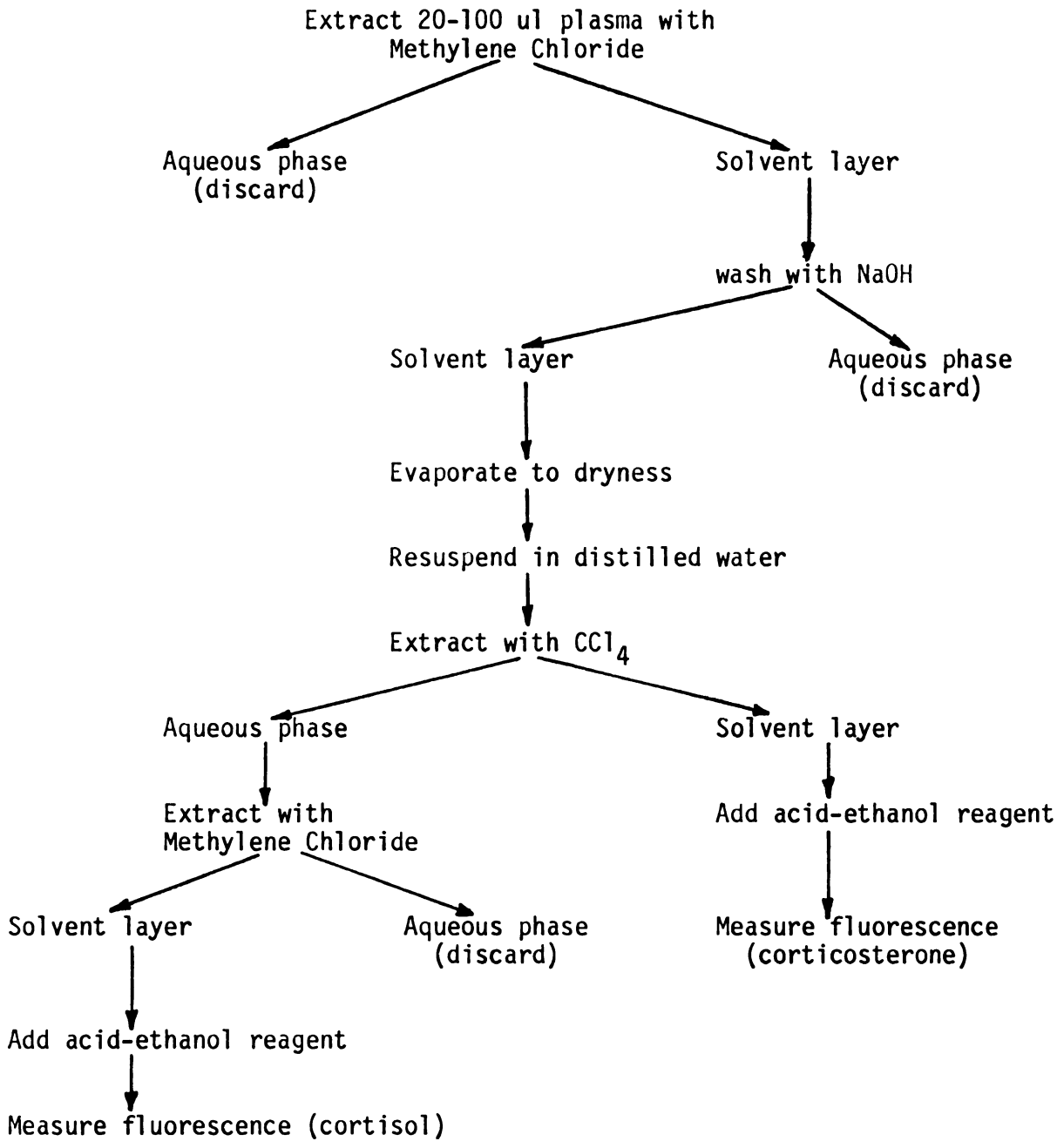

Figure 1.

Flow chart for determination of cortisol and corticosterone in blood plasma.

were carried through the same procedure. Water blanks were used to correct for background fluorescence.

This method was found to give a linear relationship between fluorescence and concentration of standard over a range of 0.01 to 0.2 ug of corticosterone (Figure 2). Related steroids, such as progesterone and 11-deoxycortisol (Pflatz and Bauer, Stanford, Connecticut), gave fluorescent readings of less than 1% that of corticosterone. The Martin and Martin (48) method of oxime formation was used to determine approximate values for nonspecific fluorescence generated by contaminating fluorogens. Based on multiple trials, this method yielded fluorescent readings equal to 1-7 ug/100 ml of plasma. Determination in triplicate of standard amounts of corticosterone added to plasma of known corticosterone concentration revealed that 85% of the steroid was recovered by this method.

Detection of Antibody Producing Cells

A modification of the Jerne plague assay was used to examine the humoral response of the mice to SRBC. In this assay, 5 days after immunization, the splenic lymphocytes from each mouse were mixed with a population of SRBC in an agarose layer. Anti-SRBC antibody produced by the lymphocytes will bind to SRBC in the region surrounding the plasmocyte. Upon addition of complement, the sensitized SRBC will lyse and produce a clear circular plaque in the agar which when counted will yield the number of antibody producing cells per mouse spleen.

In this assay, SRBC stored at 10°C in Alsever solution (Gibco Diagnostics, Grand Island, New York), were washed and suspended in phosphate buffered saline at a concentration of 2×10^9 cells/ml.

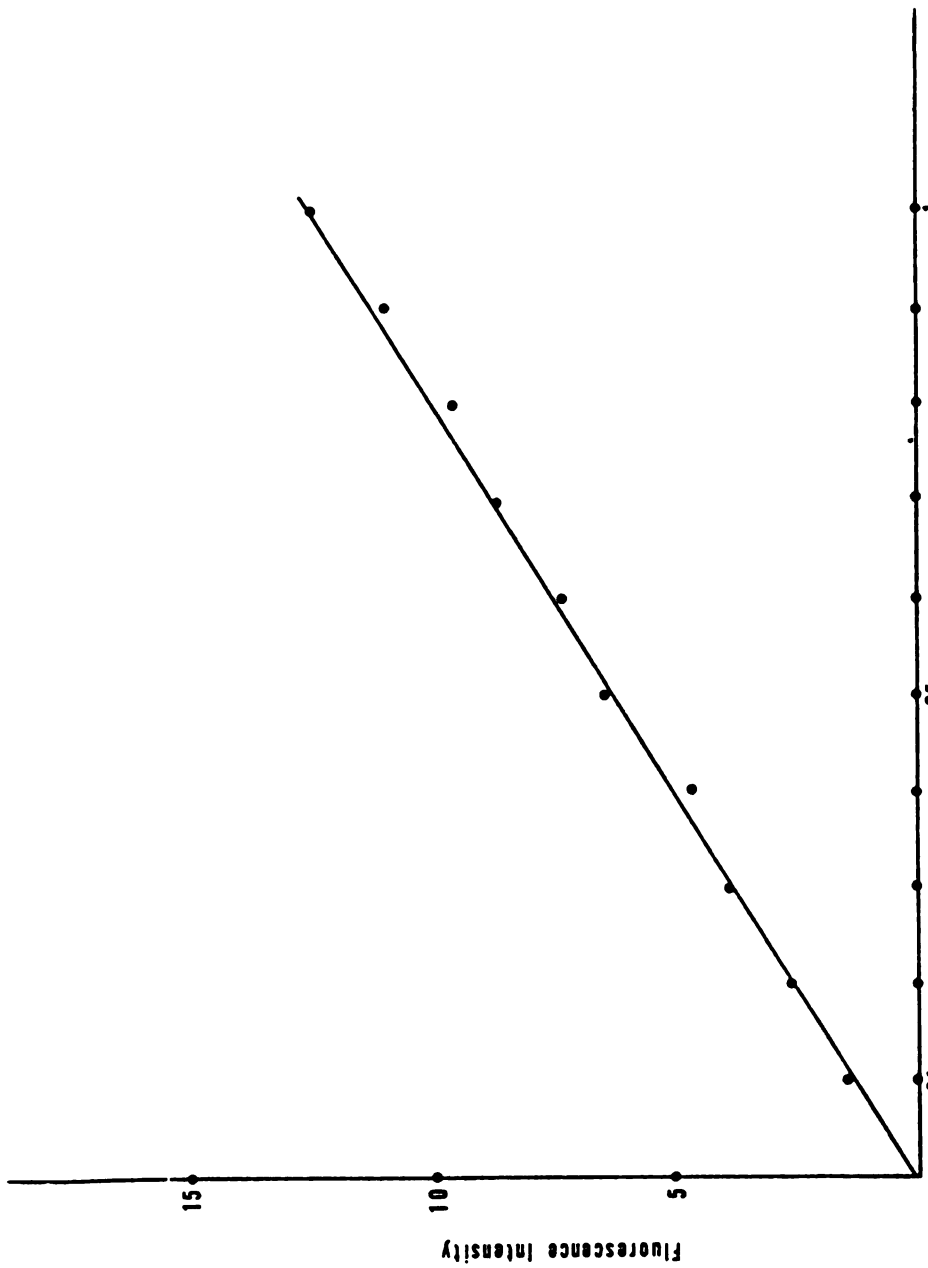

Figure 2. Standard curve for fluorimetric determination of plasma corticosterone.

Sterile minimal essential medium (MEM) containing Earles salts (Gibco Diagnostics, Grand Island, New York) and penicillin was used to maintain the lymphocytes. The mice were etherized and their spleens removed. After being cut into several pieces, the spleens were pressed through an 80 mesh stainless steel screen to produce a single cell suspension. The suspension was washed and resuspended in a 1 ml volumn. One-tenth ml of splenocytes and 0.1 ml SRBC were added to 0.8 ml of MEM containing 0.6% agarose maintained at 53°C. This mixture was poured onto a 60 mm petri dish containing a bottom layer of 0.6% agarose-MEM. Each lymphocyte suspension was plated in duplicate. The petri dishes were incubated for 1.5 hours in a humidified 37° tissue culture chamber. Direct plaques, resulting from IgM producing plasmocytes, can be visualized by addition of 0.5 ml of nonhemolytic guinea pig complement. To develop the indirect or IgG plaques, it was necessary to increase the complement fixation efficiency by a 1/2 hour incubation with 0.5 ml of rabbit anti-mouse IgG prior to addition of complement.

Unimmunized A/J female mice produced 30-60 plaques/spleen. Correction was made for these background plaques and for the small number of IgM plaques which appear on the IgG plates. All data is expressed as average plaque number produced per spleen.

Previously, it had been determined that a 6-hour recuperation period prior to immunization was adequate to eliminate any effect of the stress of bleeding on the Jerne response.

Histology

Immediately following etherization, the thymuses of the zinc-deficient and control mice were removed, weighed, and placed in Bouins

fixative for 72 hours (49). The tissues were dehydrated in a series of dioxane-water washes (Dioxane:H_2O, 1:4, 1:2, 1:1, 2:1) and embedded in Paraplast (Sherwood Medical Industries, St. Louis, Missouri). Sections 5 u thick were mounted on glass slides and stained using the Masson-Trichrome procedure (49) which allowed for discrimination of the medulla from the cortex. Mid-sections of each thymus were examined by light-microscopy. A grid was superimposed over micrographs of these sections to allow quantitation of medulla and cortical areas.

Statictical Methods

All data were examined by analysis of variance with statistical significance of treatment differences being determined by Student's t-test (50).

Results

Determination of Corticosterone Levels

The results of the fluorescent assay for plasma corticosterone levels are shown in Figure 3. A significant elevation in corticosterone occurred in the zinc-deficient mice on day 14 and increased through day 19. At the peak of the response, plasma from the zinc-deficient mice averaged 115 ug of corticosterone per 100 ml. The control mice maintained a basal level of approximately 40 ug corticosterone per 100 ml of plasma. In addition, the adrenal glands were enlarged (33%) relative to body weight in the zinc-deficient mice.

Fluorescence in the cortisol fraction was also calculated for both dietary groups and found to equal 5-10 ug/100 ml of plasma based on the fluorescence of the cortisol standard. [The question has recently arisen as to whether or not cortisol is produced by the rodent (51). The small amount of fluorescence detected in these fractions from whatever source did not contribute significantly to the date. Henceforth, only data for corticosterone will be reported.]

Effect of Corticosterone on 1° Response to SRBC

It was necessary to determine what role, if any, this elevation in steroid levels played in the impairment of T-cell helper function in the zinc-deficient mice. To resolve this question, it was essential to relate the elevation in corticosterone levels to a concomitant reduction in immune capacity. To examine this, the antibody-mediated response of the control and the zinc-deficient mice was also assessed throughout

Figure 3.

Determination of corticosterone levels in the A/J mice. Fluorometric determination of plasma corticosterone levels were made on plasma that was collected each day at 0900 hours from the zinc-deficient (.------.) and control mice (.——.) (for method, see text). Each point represents the mean ± SEM of nine mice. Arrow (↓) indicates the significant elevation (P = <0.01 or less) in corticosterone level of zinc-deficient mice compared to controls.

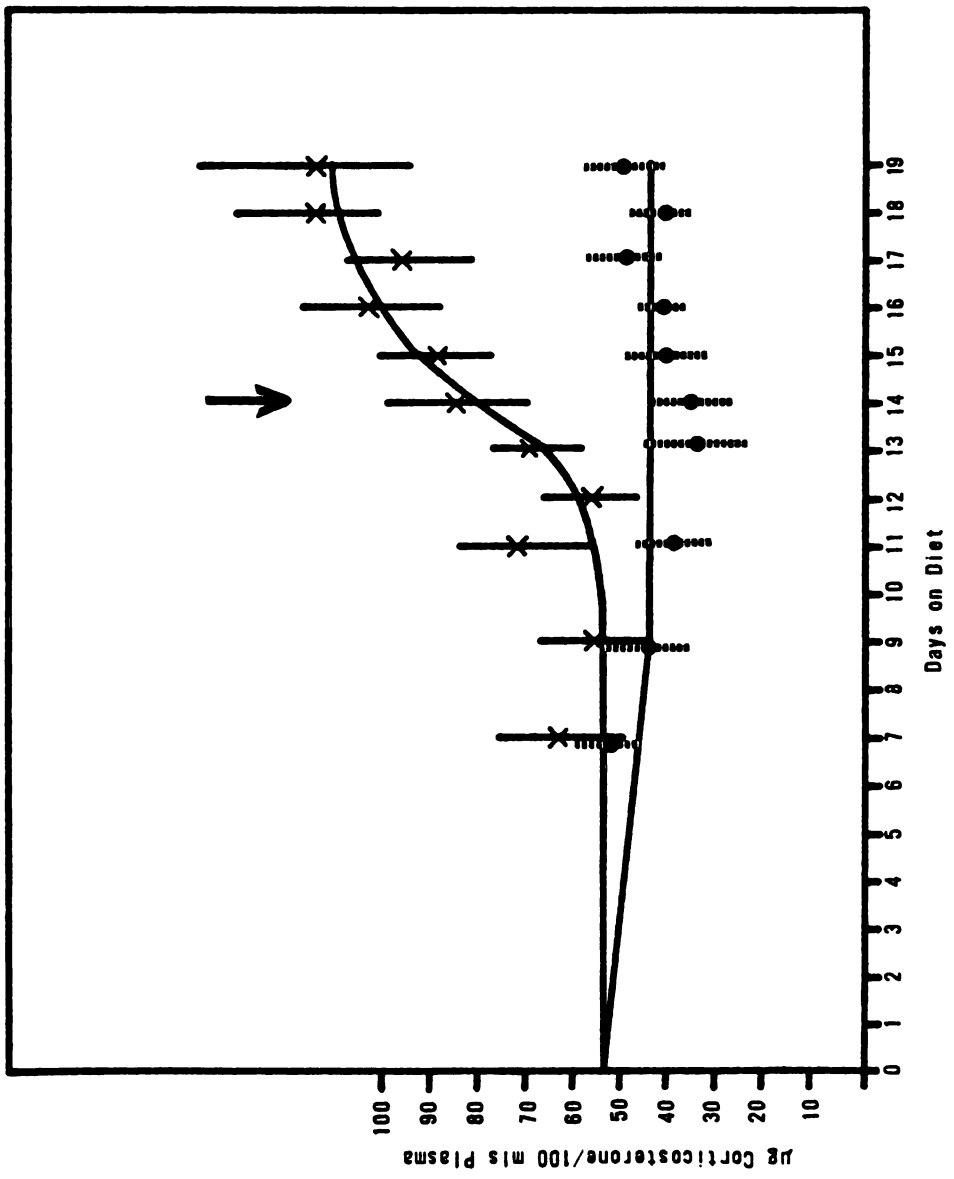
µg Corticosterone/100 mls Plasma
100
90
80
70
60
50
40
30
20
10
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
Days on Diet

Figure 3.

the experiment by means of the Jerne plaque assay. The number of direct and indirect PFC/spleen are shown in Figures 4 and 5, respectively.

The IgM response of the zinc-deficient mice remained almost constant throughout the first 19 days of the experiment (11,000 PFC/ spleen). At this time, the response began to fall and by day 24 it had decreased to about 1/2 this value to an average of 6000 IgM PFC/spleen. It should be noted that the ability of the control mice to respond to antigen increased with age and reached a plateau of approximately 25,000 PFC/spleen by day 19 as is normal during development (52). Zinc deficiency apparently prevented further development of plaquing capacity. An additional decrease in the direct response was observed from day 19 to day 24. Thus, as the mice aged, the difference in plaquing ability of the zinc-deficient mice compared to the control mice broadened. This is best illustrated by expressing the direct response of the zinc-deficient mice as % of controls as shown in Figure 4.

The indirect response of the control mice also showed a similar increase with age, escalating from an initial response of 10,000 PFC/ spleen to a mean value of 35,000 PFC/spleen (Figure 5). On day 14, prior to significant elevation in glucocorticoids, the IgG response of the zinc-deficient mice was 60% of the control response ($p < 0.02$). However, an additional 25% drop in the indirect response of the zinc-deficient mice occurred between days 17 and 19. This second major decrease occurred 4 days after a significant elevation in plasma corticosterone levels. Throughout days 19 to 24, the indirect response continued to decline in the presence of high steroid levels reaching a value of 15% of the control response at the termination of this experiment. It should be noted that the serum zinc level remained constant

Figure 4.

IgM PFC response of zinc-deficient and control A/J mice to SRBC as determined by Jerne plaque assay. PFC/per spleen response of controls and zinc-deficient mice immunized 5 days previously with 1×10^8 SRBC. Each bar represents the mean $\pm$ SEM of nine mice. Arrow (↓) indicates appearance of significant elevation in corticosterone levels in the plasma of the zinc-deficient mice compared to control mice. $P = <0.01$ or less as calculated by the Student's t-test.

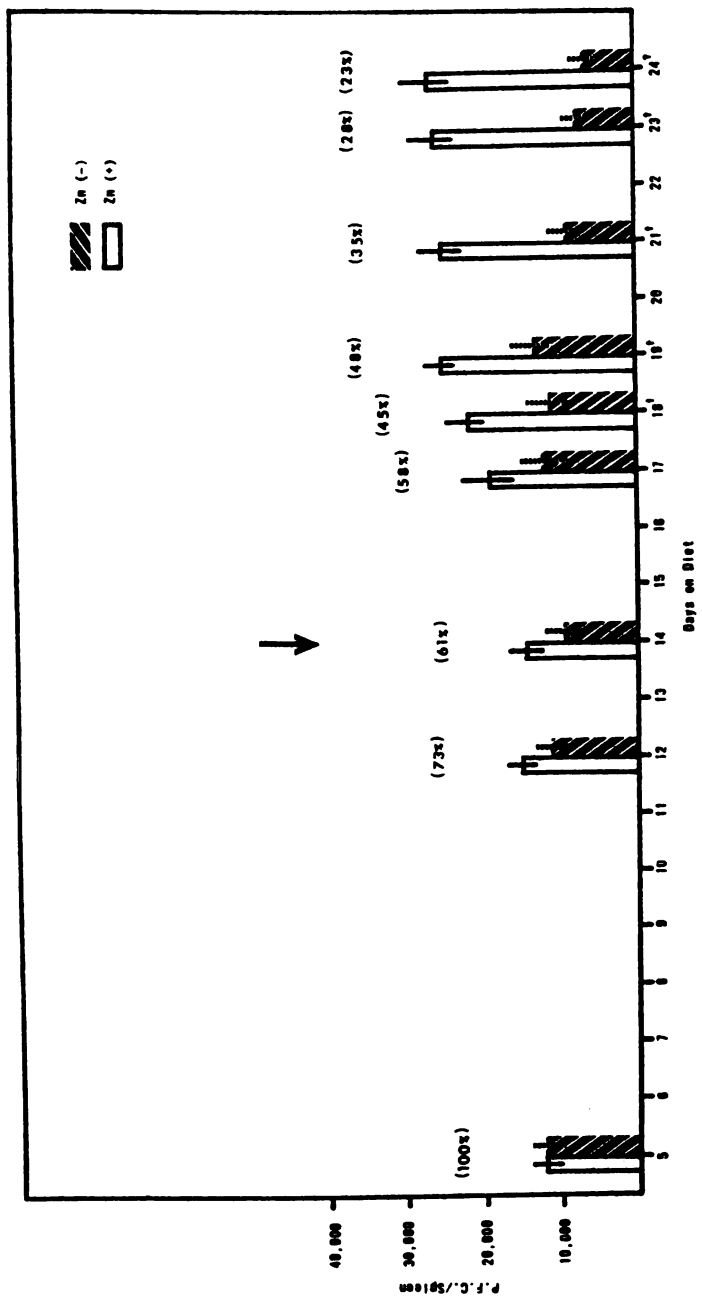
Zn (-)
Zn (+)
(100%)
(73%)
(61%)
(58%)
(45%)
(48%)
(33%)
(28%)
(23%)
40,000
30,000
20,000
10,000
P.F.C./Spleen
Days on Diet

Figure 4.

Figure 5.

IgG PFC response of zinc-deficient and control A/J mice to SRBC. The number of indirect PFC spleen were determined by the Jerne plaque assay for control and zinc-deficient mice. Each bar represents the mean ± SEM of nine mice. Arrow (↓) indicates appearance of elevated levels of corticosterone in the plasma of the zinc-deficient mice compared to controls. $P = <0.01$. $P = <0.02$ or less as calculated by the Student's t-test.

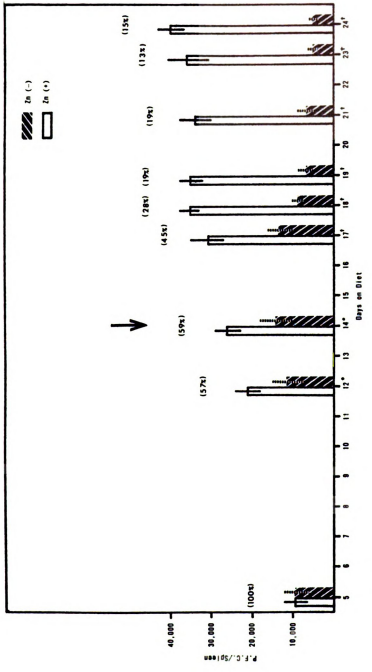
Zn (-)
Zn (+)
(100%)
(57%)
(59%)
(45%)
(28%)
(19%)
(19%)
(13%)
(15%)
40,000
30,000
20,000
10,000
Days on Diet

Figure 5.

throughout this second drop in immune function at 35 ug Zn/100 ml plasma compared to a control value of 96 ug Zn/100 ml plasma.

Effect of Zinc Deficiency on Thymic Integrity

By day 14, the wet weight of the thymuses of the zinc-deficient mice had decreased to 48% of the control mice (Table 1). This reduction occurred before a significant rise in glucocorticoid level was observed. For the remaining 10 days of the experiment, thymic involution continued with the thymus diminishing to 16% of control weight in the presence of the high levels of corticosterone generated during this time period. Representative sections of the thymuses taken during the experiment are found in Figure 6.

Figure 6d represents the thymus of a control mouse taken on day 13 of the experiment. This organ has a large, darkly stained cortex packed with lymphocytes surrounding a lighter staining medulla. Figure 6a represents a thymus taken from a zinc-deficient mouse on day 12 of the deficiency prior to elevation in corticosterone levels. The thymus had undergone considerable atrophy with preferential involution of the cortex over the medulla. With elevation of corticosterone (Figure 6b) the cortex continued to involute until on day 16 it appeared as a dark-staining ring surrounding the medulla. By this time, some atrophy of the medulla had also occurred. At the termination of the experiment, the thymuses of the zinc-deficient mice were severely involuted (Figure 6c). The cortex was completely indistinguishable, and the medulla had also undergone considerable atrophy with few thymocytes visible.

TABLE 1

Variation With Time of Body and Thymus Weight of A/J Mice Fed Zinc-Deficient or Zinc-Adequate Diet[1]

Experimental Day	Dietary GrouP	Body Weight	Thymus Weight
5	-Zinc	(95%)[3]11.9±0.6[2]	(89%)22.8±3.7
	+Zinc	12.5±0.9	25.7±2.8
12	-Zinc	(87%) 11.5±0.6	(47%) 9.2±1.7[4]
	+Zinc	13.2±0.7	19.6±2.8
14	-Zinc	(82%) 12.1±0.7	(48%) 9.7±1.0[4]
	+Zinc	14.7±0.8	19.7±1.8
16	-Zinc	(75%) 11.9±0.8[4]	(39%) 7.2±1.0[4]
	+Zinc	15.8±0.3	19.9±0.8
18	-Zinc	(71%) 11.4±0.5[4]	(31%) 7.2±0.9[4]
	+Zinc	16.0±0.6	21.1±2.8
20	-Zinc	(78%) 11.6±0.6	(30%) 7.7±1.6[4]
	+Zinc	14.7±0.7	25.7±3.9
22	-Zinc	(72%) 11.3±0.8[4]	(17%) 4.8±7.0[4]
	+Zinc	15.7±0.8	28.2±2.9
24	-Zinc	(68%) 11.2±0.7[4]	(16%) 4.9±1.0[4]
	+Zinc	16.5±0.4	31.1±3.9

[1]On the day of each Jerne assay, the body and thymus weights of the control and zinc-deficient mice were determined to assess the effect of the deficiency on these parameters. [2]Mean ± SEM,n = 9. [3]Ratios are a comparison of weight of the zinc-deficient mice (-Zinc) to control mice (+Zinc). [4]P = <0.01 or less as calculated by Student's t-test.

Figure 6.

Light micrographs of thin sections of representative thymuses of zinc-deficient and control A/J mice. A. Thymus of a zinc-deficient mouse taken on day 12 of the experiment prior to corticosterone elevation. Note the preferrential involution of the thymic cortex. B. Thymus of a zinc-deficient mouse taken on day 17 after significant elevation of corticosterone--cortex has continued to involute. C. Thymus of a zinc-deficient mouse on day 19 of the deficiency--with prolonged deficiency cortical medullary border indistinguishable with few thymocytes remaining in the organ. D. Thymus of a control mice taken on day 12 of the experiment. Note the large, darkly stained cortex packed with lymphocytes surrounding the lighter staining medulla. Masson Trichrome (X20).

A
B
C
D

Discussion

The adrenal hypertrophy observed in zinc-deficient mice suggested that adrenal steroids might be responsible for the rapid thymic atrophy and subsequent reduction in immune capacity characterized in these animals. The results of these experiments indicate that zinc deficiency did indeed produce a significant elevation in glucocorticoid levels in the A/J female mouse. To examine the possible role of this rise in steroid level on T-cell functionality, the indirect Jerne response was measured since it is more dependent on T-cell helper function than the direct response (53). The observed decline in the indirect response of the zinc-deficient mice occurred in two separate phases. The first significant drop in response (60%) was seen prior to elevation in plasma corticosteroid levels and, therefore, was due to other factor(s) associated with the absence of zinc itself. However, the second reduction in the T-cell helper-dependent response occurred 4 days after a significant rise in the corticosterone level. Since the life span of a mature SRBC helper T-cell is estimated to be 4 days (54), such a decrease would be expected if indeed precursor T-cells were lysed by the corticosterone.

While a direct relationship between the rise in glucocorticoids and the second decline in immunity remained to be firmly established, there was evidence to suggest that the two phenomena were indeed related, and that elevated levels of adrenal steroids produced during zinc deficiency might contribute to the observed loss in immune capacity.

Since modification of the immune response by stress is implied by the results of this first experiment, ways were sought to more quantitatively relate the amount of T-cell impairment caused by the depletion in zinc itself to that which may result from the presence of high levels of corticosterone.

Chapter II

EFFECT OF ADRENALECTOMY ON THE IMMUNE DYSFUNCTION RESULTING FROM ZINC DEFICIENCY IN THE A/J FEMALE MOUSE

Abstract

Previous studies from this laboratory have shown that zinc-deficient mice had significantly elevated levels of plasma corticosterone compared to mice fed zinc adequate diets. The purpose of this investigation was to determine the relationship, if any, between the increase in glucocorticoid levels and the rapid thymic atrophy and consequent loss in T-cell helper function observed in zinc-deficient mice.

The results of experiments employing adrenalectomized mice indicate about 1/2 the loss in T-cell helper function in the zinc-deficient mice occurs prior to any elevations in serum corticosterone (12 ug/100 ml) and is therefore due to other factors associated with the depletion in zinc. Nevertheless, a second reduction in T-cell helper function did occur shortly after the appearance of significant quantities of glucocorticoids in the serum of the intact or nonadrenalectomized mice (82 ug/100 ml). The adrenalectomized zinc-deficient mice which still had minimal levels of serum corticosterone (7 ug/100 ml plasma) were not significantly protected by this treatment having only marginally better humoral immune capacity (20%) than the intact deficient mice. Further, the loss in T-cell function in the adrenalectomized zinc-deficient mice occurred despite the absence of thymic involution. The data presented here argue against a major role for corticosterone in

the immune dysfunction observed in zinc-deficient mice and further demonstrate the importance of zinc to lymphocyte function.

Introduction

Although data from the previous experiment suggested that corticosterone might contribute substantially to the decline in immune function resulting from zinc deficiency, a direct relationship between the two remained to be firmly established.

To further quantitate and evaluate the role of elevated levels of corticosterone in the immune dysfunction observed in zinc-deficient mice, a series of experiments were attempted. Using a variety of means of exogenous administration of corticosterone to mice, it was not possible to simulate with any kind of accuracy the proper physiological levels of corticosterone produced by the deficient mice. Injection of metopirone, an 11-B-hydroxylase inhibitor (55), caused a persistant loss of balance and led to a reduced feed intake in these mice which made meaningful dietary experiments impossible. Failure of these methods to produce an adequate system for study led to the initiation of a series of experiments using adrenalectomized mice. Adrenalectomy permitted examination of the effects of zinc deficiency on immunity in the absence of high levels of circulating glucocorticoids. The data to be reported here indicate that corticosterone appears to play only a minor role in the loss of immune capacity resulting from zinc deficiency.

Material and Methods

Mice and Diets

Since the death rate following adrenalectomy was prohibitably high with three week old mice, six week old A/J female mice were used in this experiment. Mice were housed in cages constructed of polycarbonate and stainless steel and maintained in a temperature and light regulated environment. Cages, feed jars, and water bottles were washed as previously outlined in Chapter I. Mice were fed ad libitum a biotin fortified egg white diet containing either deficient (< 0.8 u Zn/g) or adequate (> 50 ug Zn/g) levels of zinc. The zinc content of the diet was determined as described in Experiment I. Diet consumption was measured 3 times per week. All intact mice had free access to deionized distilled water (< 0.2 ug Zn/g). Adrenalectomized mice were provided with 1% NaCl in deionized distilled water for drinking (< 0.3 ug Zn/g).

Adrenalectomy

All surgical procedures were done under ether anesthesia using a nose cone. Bilateral adrenalectomies were performed using a dorsal approach. Skin and muscle incisions were made; the adrenals were located, grasped with forceps and moved to a position suitable for excision. Following removal of the gland, the fascia was closed with 6-0 Ethilon (Ethicon, Inc., Somerville, New Jersey) sutures while stainless steel clamps were used to close the incision. All of the mice were given a 5-day stabilization period prior to use in the experiment.

Collection of Plasma for Zinc and Corticosterone Assay

Zinc-deficient and control mice were selected for bleeding and immediately etherized. Blood was collected from each mouse by retro-orbital punctures within one minute of initial cage disturbance with all mice from a cage being removed simultaneously. Approximately 0.20 ml of blood was taken from each mouse by insertion of acid washed capillary tubes into the eye socket. Blood was collected in acid washed, heparinized tubes. Plasma was separated and used for corticosterone and zinc determinations.

Mice were bled at 0900 hours when the corticosterone level is at its lowest point in the diurnal cycle (56). Entrance to the animal room was prohibited for a 3 hour period prior to bleeding in order to maintain basal levels of corticosterone in the control mice. In addition, mice from each group were bled only once during the experiment to prevent measurement of erroneously high corticosterone levels resulting from the stress of multiple bleedings.

Plasma Zinc Assay

Plasma zinc levels were determined by flameless atomic absorption spectrophotometry. Twenty ul of plasma were diluted in 10 mM HNO_3 and analyzed using a Varian 175 Carbon Rod Atomizer. Concentrations were determined by comparison to standard zinc solution also prepared in HNO_3.

Corticosterone Assay

The spectrofluorometric assay for measuring unconjugated corticosterone in mouse plasma was discussed in detail in Chapter I.

Detection of Antibody Producing Cells

Mice were immunized intraperitoneally with a 1 x 10^8 sheep red blood cells (SRBC) in sterile phosphate buffered saline. A modification of the Jerne plaque assay, previously described in Chapter I, was used to determine the total number of direct and indirect plaque forming cells (PFC) produced per mouse spleen.

Histology

At autopsy, the thymuses of all mice were removed, weighed, and prepared as described in Chapter I.

Results

In the previous study of zinc deficiency using A/J female mice, it was determined that elevated levels of serum corticosterone were produced in the latter stages of the deficiency. Thus, to quantitate the possible effect of elevated glucocorticoid levels on the immune impairment resulting from zinc deficiency, the antibody response of zinc-deficient intact mice was compared to the antibody response of adrenalectomized zinc-deficient mice.

To this end, 42 $\pm$ 2 day old A/J female mice were divided into 4 groups; zinc-deficient adrenalectomized, zinc-deficient intact, zinc-adequate adrenalectomized, and zinc-adequate intact. Each of the four groups contained 36 mice. Body weight, diet consumption, plasma zinc and corticosterone levels, as well as humoral immune capacity of the mice, were assessed after 3, 4, and 6 weeks on the synthetic diets.

Weight Gain and Food Consumption

Throughout the experiment, adrenalectomized and intact zinc-deficient mice weighted significantly less than their respective controls (Table 2) with a 5 gram weight differential between zinc-deficient and zinc-adequate mice present by week 6. Conversely, there was no significant difference in body weight between the intact and adrenalectomized mice, thus indicating that adrenalectomy itself had little effect on weight gain.

On the average, both groups of adrenalectomized mice consumed significantly less diet than the nonadrenalectomized mice [an observation which is consistent with the literature on adrenalectomized

TABLE 2

Body Weight and Diet Consumption of Intact and Adrenalectomized A/J Mice Maintained on Zinc-Adequate or Deficient Diets

Body Weight

Weeks	Zinc-Deficient Adrenalectomized (g)	Zinc-Deficient Intact (g)	Zinc-Adequate Adrenalectomized (g)	Zinc-Adequate Intact (g)
0	15.7 ± 0.2 +	16.1 ± 0.6	15.8 ± 0.4	17.1 ± 0.4
3	16.7 ± 2.0[a]	16.5 ± 0.8[b]	19.3 ± 0.8	18.6 ± 0.8
4	16.9 ± 0.8[c]	16.3 ± 0.5[d]	20.1 ± 0.5	20.0 ± 0.4
6	15.8 ± 1.0[e]	16.0 ± 0.7[d]	20.5 ± 1.0	21.0 ± 0/9
Food Consumption g/mouse/day	1.8 ± 0.1[f]	2.2 ± 0.1	2.0 ± 0.2[b]	2.3 ± 0.1

+ means ± SEM of 7 to 9 mice

[a] $p < .05$ as compared to zinc-adequate adrenalectomized mice

[b] $p < .05$ as compared to zinc-adequate intact mice

[c] $p < .01$ as compared to zinc-adequate adrenalectomized mice

[d] $p < .001$ as compared to zinc-adequate intact mice

[e] $p < .001$ as compared to zinc-adequate adrenalectomized mice

[f] $p < .05$ as compared to zinc-deficient intact mice

animals (57)]. However, as seen from the body weight data, this decrease in food consumption did not appear to interfere significantly with weight gain.

Assessment of Plasma Corticosterone Levels

At 3, 4, and 6 weeks, 9 mice from each of the 4 groups were bled and their plasma was assayed for corticosterone. The results of these determinations are shown in Table 3.

After 3 weeks on the deficient diet, the nonadrenalectomized zinc-deficient mice showed no elevation in glucocorticoid levels; however, by week 4 significant levels of corticosterone were present in the zinc-deficient intact mice (82 ug corticosterone/100 ml plasma). The zinc-adequate intact mice maintained a level of approximately 40 ug corticosterone/100 ml plasma, while the adrenalectomized zinc-deficient and adrenalectomized zinc-adequate mice had a level of approximately 12 ug corticosterone/100 ml of plasma through weeks 3 and 4. By week 6 the corticosterone level of the intact zinc-deficient mice remained significantly elevated (101 ug/100 ml plasma). The plasma corticosterone levels of the adrenalectomized zinc-deficient and adrenalectomized zinc-adequate mice remained at 10 ug/100 ml of plasma, while intact zinc-adequate mice had risen slightly to 55 ug/100 ml of plasma by week 6.

Determination of Plasma Zinc Levels

The results of weekly determinations of plasma zinc levels of these mice are found in Table 4. Adrenalectomy appeared to have no effect on plasma zinc levels throughout the experimental period. No significant differences in plasma zinc was seen between zinc-deficient

TABLE 3

Plasma Corticosterone Levels of Intact and Adrenalectomized A/J Mice Maintained on Zinc-Adequate or Deficient Diets

Weeks	Zinc-Deficient Adrenalectomized	Zinc-Deficient Intact	Zinc-Adequate Adrenalectomized	Zinc-Adequate Intact
	ug/100 ml plasma	ug/100 ml plasma	ug/100 ml plasma	ug/100 ml plasma
3	7.0 ± 3.6[+]	12.0 ± 3.6[a]	8.0 ± 2.5	11.0 ± 1.0
4	12.1 ± 1.6	81.8 ± 9.5[c]	13.0 ± 4.0	43.3 ± 8.2
6	5.4 ± 2.0	101.0 ±15.0[c]	5.2 ± 1.8	55.1 ± 9.0

Plasma Corticosterone were measured by spectrofluorometric assay (for method see text). [+] Means ± SEM of 7 to 9 mice

[a] NS as compared to zinc-deficient adrenalectomized level

[b] NS as compared to zinc-adequate intact level

[c] $p < .01$ or less as compared to zinc-adequate intact level

TABLE 4

Plasma Zinc Concentrations of Intact and Adrenalectomized A/J Mice Maintained on Zinc-Adequate or Deficient Diets

Weeks	Zinc-Deficient Adrenalectomized	Zinc-Deficient Intact	Zinc-Adequate Adrenalectomized	Zinc-Adequate Intact
	ug/100 ml plasma	ug/100 ml plasma	ug/100 ml plasma	ug/100 ml plasma
3	30.6 ± 3.6[a+]	38.8 ± 5.7[b,c]	71.6 ± 4.2[d]	67.7 ± 6.3
4	36.3 ± 6.5[a]	31.8 ± 3.2[b,c]	71.4 ± 3.1[d]	80.2 ± 5.0
6	31.0 ±13.4[a]	18.2 ± 4.2[b,c]	72.2 ± 5.0[d]	74.0 ± 5.2

Zinc determination (atomic absorption spectrophotometry) were made on plasma collected at 0900 hours from each experimental group. [+] Means ± SEM of 7 to 9 mice

[a] $p < .001$ or less as compared to zinc-adequate adrenalectomized level

[b] $p < .001$ or less as compared to zinc-adequate intact level

[c] NS as compared to zinc-deficient adrenalectomized level

[d] NS as compared to zinc-adequate intact level

adrenalectomized and zinc-deficient intact mice. Similarly, no difference in plasma zinc levels were found between zinc-adequate adrenalectomized and zinc-adequate intact mice. However, the results do show that the plasma zinc levels of adrenalectomized and intact zinc-deficient mice were significantly depressed (30-38 ug Zn/100 ml plasma) compared to adrenalectomized and intact mice fed zinc-adequate diet (70-100 ug Zn/100 ml plasma) throughout the experiment.

Determination of Antibody Mediated Response

To determine the effects of the elevated levels of adrenal cortical steroids on the immune function of the deficient mice, the antibody mediated response of the adrenalectomized and intact mice to SRBC was assessed by the Jerne plaque assay. The numbers of direct and indirect PFC/spleen produced in response to immunization with SRBC are shown in Figures 7 and 8, respectively.

At 3 and 4 weeks, both the direct and indirect responses of the adrenalectomized mice fed either adequate or zinc-deficient diet were higher than the antibody responses of the intact mice maintained on similar diets. Thus, for meaningful comparisons to be made between zinc-deficient adrenalectomized and zinc-deficient intact mice, their antibody responses should be expressed as a percentage of their respective controls. Indeed when compared in this manner, at 3 and 4 weeks, the direct responses of the adrenalectomized zinc-deficient mice and intact zinc-deficient mice were not statistically different. Moreover, the direct response of the zinc-deficient adrenalectomized and intact mice did not differ significantly from their respective controls. By week 6, a significant depression in the direct response was seen in both

Figure 7.

IgM PFC response of adrenalectomized and intact zinc-deficient and control A/J mice to SRBC. Each bar represents the mean ± SEM of 7 to 9 mice.

Statistical data as determined by Student's t-test.
3 + 4 weeks:
  Zn (-) adrenalectomized compared to Zn (+) adrenalectomized mice:NS
  Zn (-) intact compared to Zn (+) intact mice:NS
6 weeks:
  Zn (-) adrenalectomized compared to Zn (+) adrenalectomized mice:$p<.001$
  Zn (-) intact compared to Zn (+) intact mice:$p<.001$

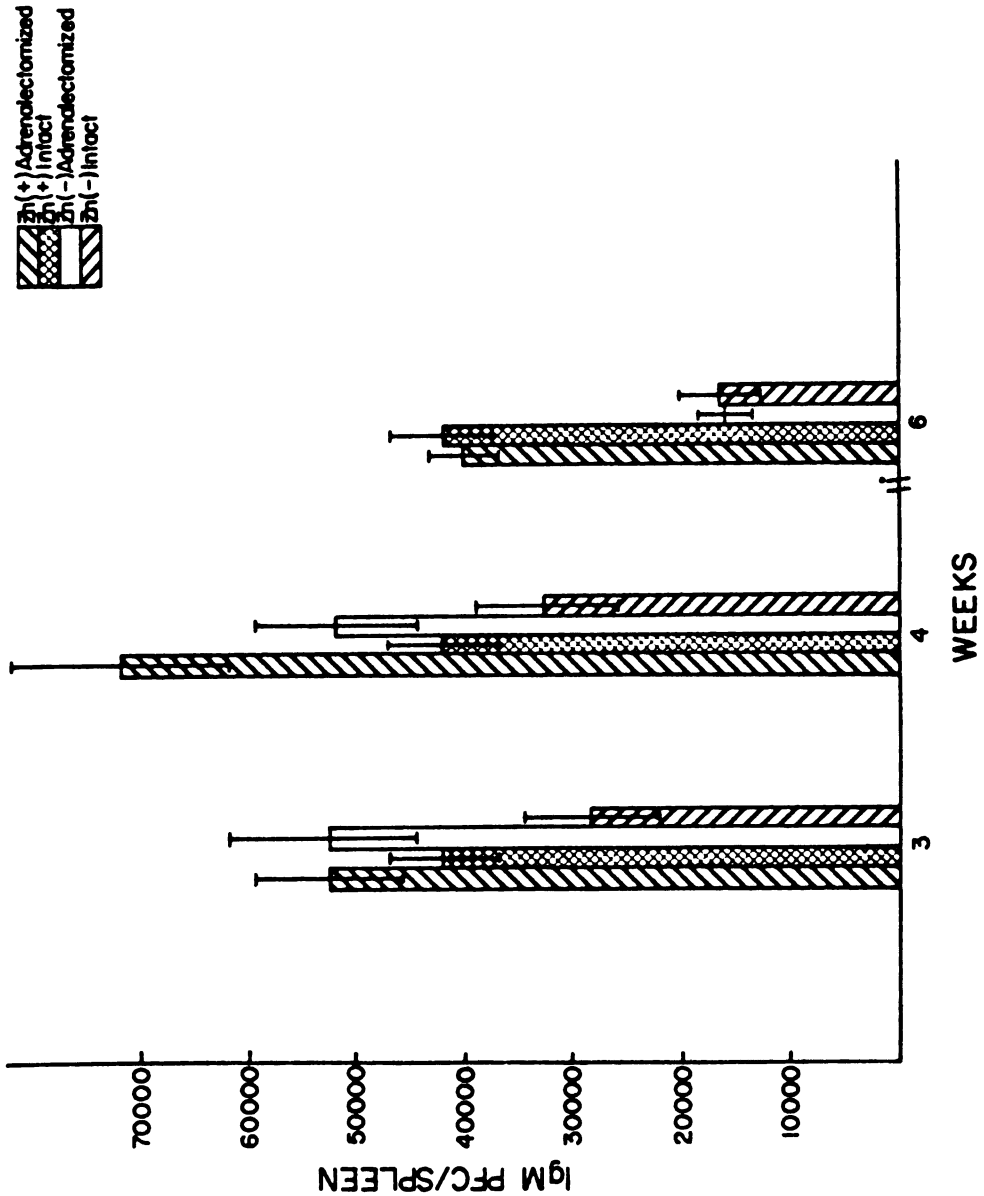
Zn(+)Adrenalectomized
Zn(+)Intact
Zn(-)Adrenalectomized
Zn(-)Intact
IgM PFC/SPLEEN
70000
60000
50000
40000
30000
20000
10000
3
4
6
WEEKS

Figure 7.

Figure 8.

IgG PFC response of adrenalectomized and intact zinc-deficient and control A/J mice to SRBC. Each bar represents mean ± SEM of 7 to 9 mice.

Statistical data as determine by Student's t-test.
3 weeks:
  Zn (-) adrenalectomized compared to Zn (+) adrenalectomized mice: $p<.01$
  Zn (-) intact compared to Zn (+) intact mice: $p<.01$
4 weeks:
  Zn (-) adrenalectomized compared to Zn (+) adrenalectomized mice: $p<.02$
  Zn (-) intact compared to Zn (-) intact mice: $p<.01$
6 weeks
  Zn (-) adrenalectomized compared to Zn (+) adrenalectomized mice: $p<.001$
  Zn (-) intact compared to Zn (-) intact mice: $p<.01$

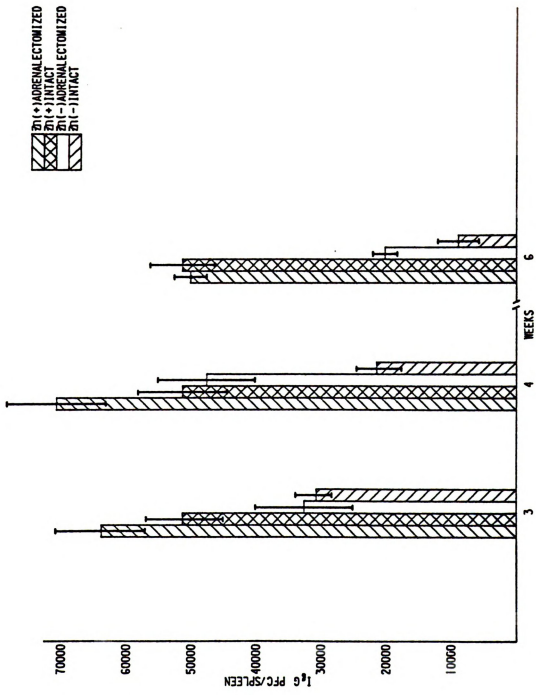
Zn(+)ADRENALECTOMIZED
Zn(+)INTACT
Zn(-)ADRENALECTOMIZED
Zn(-)INTACT
70000
60000
50000
40000
30000
20000
10000
IgG PFC/SPLEEN
3
4
6
WEEKS

Figure 8.

the adrenalectomized and intact deficient mice with their responses dropping to 37% and 40% of their respective control group.

In contrast, adrenalectomy appeared to delay, but not prevent, the loss of T-cell helper function. The indirect response of both intact and adrenalectomized zinc-deficient mice had dropped significantly by week 3, decreasing to 56% of adrenalectomized controls in the case of the adrenalectomized zinc-deficient mice and to 58% of intact controls in the case of the intact deficient mice. By week 4, in the presence of elevated levels of corticosterone, the response of the intact zinc-deficient mice had dropped to 41% of the intact control response. In contrast, the adrenalectomized zinc-deficient mice were able to maintain an indirect response approximately equal to their response of the previous week (60%). However, by week 6 the response of the adrenalectomized zinc-deficient mice had declined, reaching a value of 38% of adrenalectomized controls, while the response of the non-adrenalectomized mice had dropped further to approximately 20% of the intact control response.

Thymus Integrity

The thymus weight (Table 5) of the intact zinc-deficient mice decreased at 3, 4, and 6 weeks to 60%, 50%, and 20%, respectively, of intact control weights. In contrast, the thymus weight of the adrenalectomized zinc-deficient mice remained at 90% of the adrenalectomized control thymus weight throughout the experiment.

Since the thymuses of the adrenalectomized zinc-deficient mice did not undergo the large weight loss that was observed in the case of the nonadrenalectomized zinc-deficient mice, it was of interest to

examine these tissues histologically. At 3, 4, and 6 weeks, the thymuses from intact and adrenalectomized mice maintained on the zinc-adequate diet had a cortical:medullary ratio of 2.0, while the zinc-deficient intact mice had a cortical:medullary ratio of 1.0. This indicated preferential atrophy of the thymic cortex. [These values are in agreement with previously reported values (58).] Interestingly, the thymuses of the zinc-deficient adrenalectomized mice showed no such preferential involution of the cortex at 3 weeks. However, by 4 weeks and continuing through 6 weeks, the cortical:medullary thymic ratio of these mice was reduced to 1.0. This was the result of an apparent redistribution of lymphocytes which occurred in the absence of any thymic weight loss and paralleled the marginal increase in response seen at 4 weeks in the adrenalectomized zinc-deficient mice compared to intact zinc-deficient mice. There were no additional gross indications of abnormal pathology in thymuses from either group of deficient mice.

TABLE 5

Comparison of Thymic Weights and Ratios of Cortex to Medullary Area of Adrenalectomized and Intact Mice Maintained on Zinc-Deficient or Adequate Diets

Group	3 Weeks		4 Weeks		6 Weeks	
	Weight (mg)	C/M‡	Weight (mg)	C/M	Weight (mg)	C/M
Zinc-Deficient Adrenalectomized	31.2 ± 4.0[+a,d]	2.0 ± 0.2[*]	32.4 ± 3.7	1.1 ± 0.1	25.9 ± 1.5[a,c]	1.0 ± 0.1
Zinc-Deficient Intact	15.5 ± 4.0[b]	1.3 ± 0.1	15.5 ± 1.5[c]	1.2 ± 0.03	6.1 ± 1.3[c]	0.7 ± 0.05
Zinc-Adequate Adrenalectomized	34.1 ± 5.0	2.2 ± 0.1	36.6 ± 2.8	2.0 ± 0.2	27.3 ± 1.2	1.8 ± 0.1
Zinc-Adequate Intact	25.0 ± 4.0	2.0 ± 0.1	30.0 ± 1.2	2.1 ± 0.2	29.5 ± 3.7	1.9 ± 0.2

On the day of each Jerne assay, thymus weights were determined and tissues were prepared for histological examination (for method see text). [+] mean ± SEM of 7 to 9 mice, [*] mean ± SEM of 5 mice

[a] NS as compared to zinc-adequate adrenalectomized mice

[b] $p < .001$ as compared to zinc-adequate intact mice

[c] $p < .001$ or less as compared to zinc-adequate intact mice

[d] $p < .001$ or less as compared to zinc-deficient intact mice

‡ cortical/medullary area thymic ratio

Discussion

The results of the studies previously outlined in this thesis indicated that elevated levels of corticosterone were produced in the final stages of zinc deficiency. Since the mouse is a corticosterone sensitive species (37), if adrenal hormones played a role of the observed immune dysfunction, the effects of elevated glucocorticoids should be readily detected using these animals.

A variety of approaches designed to quantitate the degree of immune impairment resulting from glucocorticoid elevation were attempted without success. It became apparent that adrenalectomy was the most acceptable method available. Using this means, it was possible to examine the effects of zinc deficiency on immunity in the absence of high levels of adrenal steroids.

However, a number of considerations were necessary in implementing this method of study. A role for adrenal hormones in regulation of serum zinc concentration has been proposed. Literature reports of studies designed to test this hypothesis, however, have been contradictory. Thus, ACTH or adrenal steroids have been shown to either decrease (59), increase (60), or have no effect on serum zinc levels (61). Further, adrenalectomy has been found both to decrease (62) or increase (63) serum zinc levels depending on the investigation. As a consequence of these observations, serum zinc levels of all mice were measured throughout the experiment to assess any possible variation in zinc levels due to adrenalectomy. The results indicate there was no significant difference in serum zinc levels between adrenalectomized and

nonadrenalectomized mice in either the zinc-deficient or zinc-adequate group and as such was not a consideration.

It has also been shown that adrenalectomy increases the immune responsiveness of mice (64). This may reflect the loss of glucocorticoid suppression of T-cell growth factor (TCGF), a mediator of T-cell proliferation (65). Removal of the steroid hormone, which has been shown to inhibit TCGF, would enhance the proliferation of T-cells and, thus, might account for the enhanced immune responsiveness of the adrenalectomized mice. However, by expressing the response of the adrenalectomized and intact zinc-deficient mice on a percentage control basis, meaningful comparisons are possible.

Thus to examine the role of adrenal steroids in the immune impairment observed in the zinc-deficient mice, the direct and indirect Jerne response was measured in zinc-deficient adrenalectomized and nonadrenalectomized mice. Adrenalectomy appeared to have little effect on the B-cell responsiveness of these mice since the direct responses of both the adrenalectomized and nonadrenalectomized zinc-deficient mice were comparable at the three time periods measured. The results were somewhat different in the case of the indirect or T-cell mediated response. The initial 50% drop in the indirect Jerne response was seen in the intact and adrenalectomized zinc-deficient mice prior to corticosterone elevation and is due to other factors associated with the absence of zinc. However, a second reduction in immune capacity (20%) was seen by week 4 in zinc-deficient mice and occurred in the presence of elevated levels of glucocorticoids. This reduction was not seen in the case of the adrenalectomized zinc-deficient mice and suggests a role

for corticosterone in this phase of the deficiency. This finding is also supported by results from the previous experiment which showed an additional 25% drop in IgG response in zinc-deficient mice following the appearance of elevated levels of corticosterone in the serum of these mice. We have not examined the cellular basis for this decline in immune function. Although previous studies have demonstrated the presence of suppressor cell activity following corticosteroid administration (66), this issue is unresolved since other studies have demonstrated an abrogation of suppression by in vivo administration of hydrocortisone (67).

In any case, the absence of corticosteroids appears to provide only a modest amount of short term protection against the effects of zinc deficiency on humoral immunity since by week 6 a second large decline in indirect response was observed in the adrenalectomized zinc-deficient mice. This drop paralleled the earlier drop in responsiveness of the intact zinc-deficient mice and thus was not prevented by adrenalectomy. Therefore, despite the temporary increase in immune responsiveness of the adrenalectomized zinc-deficient mice compared to intact zinc-deficient mice, it is clear that the adrenalectomized zinc-deficient mice ultimately experienced a significant decline in T-cell response. Since this decline occurred in the absence of elevated steroid levels in these mice, it would argue that the detrimental effects of zinc deficiency on the immune response are due in large part to the absence of zinc per se and not to secondary stress related events.

The effects of adrenalectomy are puzzling in the case of the thymic atrophy observed during zinc deficiency. Examination of the thymuses of zinc-deficient intact mice revealed that significant atrophy had occurred by week 3, reaching a value of 20% of control weight by the

end of the experiment. In contrast, the thymuses of the adrenalectomized zinc-deficient mice underwent no weight loss, remaining at 94% of controls by week 6. Further, histological examination of these thymuses revealed interesting differences. At 3 weeks, the thymuses of the adrenalectomized zinc-deficient mice had a cortical:medullary ratio of 2:1. This ratio was seen at all time intervals for the intact mice and appears typical for thymuses of young adult mice (58). In contrast, the ratio for the atrophying thymus from the intact zinc-deficient mice had dropped to 1:1 at this time, indicating cortical involution. One week later, the ratio for the adrenalectomized zinc-deficient mice had also dropped to 1:1, but this occurred in the absence of any thymic weight loss. Thus, this phenomena may be a result of a redistribution of thymocytes from the cortex to the medulla and might be responsible for the 20% elevation in indirect response of the adrenalectomized mice at a time when the response of the nonadrenalectomized mice was continuing to decline.

Since the adrenalectomized zinc-deficient mice had normal levels of plasma corticosterone, it was, therefore, surprising that the thymuses of these mice underwent preferential atrophy of the thymic cortex and indicated that other factors associated with zinc deficiency may be responsible for involution of this region. The question remaining, is why these mice with a thymus equal in weight to controls, and thus an enlarged medullary region, have an impaired T-cell helper function.

In summary, the elevated levels of adrenal steroids produced during the latter stages in zinc deficiency appear to play only a minor role in the loss of immunity resulting from zinc deficiency.

SUMMARY AND CONCLUSIONS

A salient feature of zinc deficiency is the increased vulnerability to disease which accompanies the deficiency. While our laboratory had characterized the preferential involution of lymphatic tissue and subsequent reduction in T-cell helper function, which is responsible for the immune dysfunction, the actual mechanism(s) responsible for these effects remained undefined.

The primary goal of this research was to attempt to determine the mechanism responsible for the immune impairment. The suggestion that nutritional deficiencies might constitute a stress on the animal coupled with the known immunosuppressive action of the stress hormones, lead to the investigation of the role of glucocorticoids in zinc deficiency.

Data from the first experiment revealed that significantly elevated levels of corticosterone were present in zinc-deficient mice and suggested that adrenal steroids might contribute substantially to the observed decline in immune function in these mice. However, the second series of experiments conducted with adrenalectomized zinc-deficient mice failed to support this hypothesis since only about 20% of the drop in T-cell function was prevented by this treatment. It appears then that the major detrimental effect of zinc deficiency on lymphocyte function is not a secondary consequence of elevated steroid levels. Thus, the suggestion that the thymic atrophy and T-cell dysfunction common to many nutritional deficiencies results in large part by

activation of the stress axis seems doubtful. Moreover, these data serve to further demonstrate the crucial role of zinc to lymphocyte integrity.

The question remaining then is, What mechanism is responsible for the immune impairment which occurs during zinc deficiency? Since these studies seem to demonstrate the importance of zinc to lymphocyte integrity, to answer this question it will probably be necessary to investigate the fundamental role(s) of zinc in lymphocyte function. A number of investigations would serve to support this approach. Zinc has been shown to be critical to normal nucleic acid synthesis function (68, 69). DNA and RNA polymerase (70) as well as terminal deoxynucleotidyl transferase (71) have all been shown to be zinc metalloenzymes. In the eukaryote, Euglena gracilis, Vallee (72) has shown that zinc-deficient cells contain nearly twice the amount of mRNA as zinc-adequate cells. Moreover, there is a significant difference in the base composition of the mRNA fractions of the zinc-deficient and zinc-adequate cells. Thus, the way in which zinc can regulate, modify, and influence transcription may be essential to the understanding of the deficiency.

The manner in which zinc stimulates resting cells to enter S phase and transform into blast cells also needs further study. Hart (73) and Skinner (74) have shown that zinc compounds can induce lymphocyte cultures to increase RNA and DNA synthesis. In addition, zinc removal by EDTA has been shown to inhibit PHA transformation of lymphocytes in vitro (69). Further, Newburne (75) has recently shown that lymphocytes from zinc-deficient mice have a depressed response to the T-cell mitogens, PHA and Con A.

Since the histological data suggests that immature T-cells are affected preferentially by zinc deficiency, it would also be of interest to investigate the stage(s) of lymphopoesis which is affected: i.e., proliferation and migration from the bone marrow or maturation and differentiation in the thymus or spleen. Good (76) found a decreased level of thymosin in zinc-deficient mice. Due to the severe thymic atrophy produced by zinc deficiency, this data is difficult to interpret. No definitive studies to date have been performed to answer this question.

Thus, the experiments detailed in this thesis constitute only the beginning of a series of experiments to determine the mechanism of immunosuppression resulting from zinc deficiency. Moreover, they emphasize the need for continuing investigation into the crucial role of zinc in lymphocyte function.

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