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thesis entitled

The Effects of Maternal Dietary Zinc Deficiency
on the Growth and Immunocompetence
of Suckling A/J Mice

presented by

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has been accepted towards fulfillment
of the requirements for

Master of Science degree in Biochemistry

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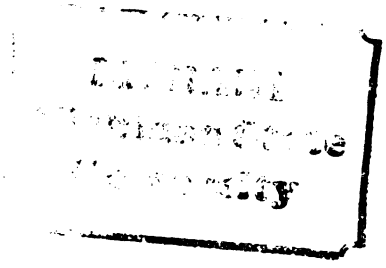
Date 3/23/82



THE EFFECTS OF MATERNAL DIETARY ZINC DEFICIENCY ON THE GROWTH AND IMMUNOCOMPETENCE OF SUCKLING A/J MICE

By

Kim Marie Hildebrandt

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the degree requirements
for the degree of

MASTER OF SCIENCE

Department of Biochemistry

1982

ABSTRACT

THE EFFECTS OF MATERNAL DIETARY ZINC DEFICIENCY ON THE GROWTH AND IMMUNOCOMPETENCE OF SUCKLING A/J MICE

By

Kim Marie Hildebrandt

The purpose of this research was to characterize the effects of marginal maternal dietary zinc deficiency during lactation on neonatal growth and immune function. Specific goals were to (a) define the zinc requirement for normal neonatal growth and mitogenic responsiveness, (b) characterize the consequences of maternal zinc deprivation on neonatal growth and antibody responsiveness to T-cell-independent (TI) and T-cell-dependent (TD) antigens and (c) determine whether or not zinc deficiency caused irreversible neonatal immune impairment. Results indicated that (a) 1.6 μg Zn/g maternal diet caused poor neonatal growth and altered mitogenic responsiveness while 5.8 μg Zn/g was sufficient, (b) maternal zinc deficiency caused reduced neonatal growth, TI antibody avidities, and antibody responsiveness against both TI and TD antigens and (c) zinc deficiency did not irreversibly impair postnatal immunocompetence and restoration could be effected by zinc supplementation. These data imply that normal growth and immunological ontogenesis in the suckling neonate was altered by maternal dietary zinc deficiency.

To My Morning Star

ACKNOWLEDGMENTS

I would like to express my sincere appreciation to Dr. Pamela Fraker for her guidance and support throughout my graduate career. Thanks are also due to the members of my guidance committee, Drs. Ronald Patterson, William Smith and William Wells and also to Dr. Loran Bieber who served as acting committee chairman. I am especially grateful to Dr. Richard Luecke for the advice and encouragement that he has provided throughout.

Special thanks to Paula Jardieu, Craig Zwickl and Don Williams for their technical assistance, moral support and friendship.

Finally, heartfelt thanks to George, Glenda, Ingrid, Linda, Sherry and Vickie, but especially to Julie and my dear family for the motivating encouragement, patient understanding and cherished memories.

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ABBREVIATIONS

ADCC	Antibody dependent cell-mediated cytotoxicity
AE	Acrodermatitis enteropathica
ConA	Concanavalin A
Dx	Dextran sulfate
FBS	Fetal bovine serum
^{125}I-Udr	^{125}I-Iododexoyuridine
LPS	Lipopolysaccharide
MBB	Modified barbital buffer
MEM	Minimum essential medium
NK	Natural killer cells
PBS	Phosphate buffered saline
PCM	Protein-calorie malnutrition
PFC	Plaque forming cells
PHA	Phytohemagglutin
PWM	Pokeweed mitogen
R(+)	Restricted
sIg	Surface immunoglobulin
SRBC	Sheep red blood cells
TD	T-cell-dependent
TI	T-cell-independent
TNBS	2,4,6-trinitrobenzene sulfonic acid

ABBREVIATIONS (Continued)

TNP	Trinitrophenyl
Zn(-)	Zinc deficient
Zn(+)	Zinc positive, control

INTRODUCTION

Zinc deficiency pervades human populations worldwide resulting mainly from inadequate dietary intake (1-3). Nutritional zinc deficiency is prevalent among underpriviledged nations subsisting primarily on cereal proteins containing the zinc-chelator phytate; the high incidence of protein-calorie malnutrition (PCM) in these developing countries appears to be the most common cause of zinc deficiency. The deficiency is generally severe among impoverished nations yet marginal cases have been reported within the United States among low socio-economic groups and during periods of relatively high zinc requirements such as rapid growth, pregnancy, and lactation (4-9).

A well documented instance of childhood zinc deficiency is that associated with the congenital disease acrodermatitis enteroptahica (AE) (10-15). This condition of zinc malabsorption is characterized by skin lesions, diarrhea, failure to thrive, frequently depressed immunoglobulin levels and impaired cell mediated immunity. Moynahan (16) first described the value of zinc supplementation in alleviating the clinical symptoms of AE while subsequent reports have shown that the immunodeficiencies of AE are also alleviated by zinc therapy (11,14,15).

Dietary zinc deficiency, rather than that associated with a disease state, is also prevalent among children. Since dietary zinc requirements are relatively high during periods of rapid growth, the

effects of insufficiency may be particularly severe during the perinatal and adolescent period (2). The occurrence of human nutritional zinc deficiency was first demonstrated by Prasad during a study of adolescent Egyptians (17). In a later Iranian study, zinc was postulated to be a limiting factor in nutrition of children, this most likely precipitated the observed growth retardation (2,18). Stimulation of growth was observed following zinc supplementation but full recovery was not effected. Inadequate zinc nutrition is not limited to the severely impoverished sectors of the world but may also be common among American preschool children from low income families (3). The infant may also be at risk of developing zinc deficiency which may result from maternal zinc depletion during gestation, increased neonatal requirements and/or inadequate neonatal intake (3,6,7,19,20). This risk may be especially great for the malnourished, preterm or small for gestational age infant (6,7,20).

The reported consequences of naturally occuring zinc deficiency are extremely deleterious to the neonate and adolescent; it is therefore not ethically justifiable to experimentally impose such a deficiency on a human subjects in order to study possible developmental aberrations. Several developmental studies have already been done with rodents and will be reviewed in the next section.

In rats, maternal zinc deprivation during gestation has been shown to have dramatic effects on embryonic and fetal development (21). Severe zinc deficiency during the first few days of pregnancy resulted in abnormal cleavage and blastulation of preimplantation eggs (22). If severe zinc deficiency was extended throughout gestation, these females lost rather than gained weight and less than 50% had

living young at term. When fetuses were carried to term, litter sizes were reduced, pup body weight was less than half normal and 98% of the neonates showed gross congenital malformations which affected nearly every organ and tissue; these problems included skeletal defects, hydroencephalus or hydranencephalus, small or missing eyes, hernias, and heart, lung and urogenital abnormalities (23). Even short-term deficiencies of zinc during gestation resulted in similar numbers and types of neonatal malformations; many of the young were stillborn and survival to weaning was poor (12-46% of normal) (24-26). Striking effects common to all periods of gestational zinc deficiency are the high incidence of stillbirths, fetal malformations and subsequent morbidity. Other investigators have also reported that low zinc intake during gestation results in a prolonged, difficult parturition and abnormal maternal behavior including failure to gather and nurse their young (27,28).

Indications that zinc was important to the development of the suckling neonate were first observed by Nishimura in mice (29) and later by Hurley in rats (30). In the former study newborn mice were removed from their mothers and reared by dams in later stages of lactation thus depriving the neonates of colostrum. These pups experienced growth retardation and rapidly developed physical anomalies including alopecia and cutaneous lesions. The lack of zinc was determined as the cause of these defects since protection from these symptoms could be effected by giving oral doses of zinc directly to the newborn pups. In Hurley's study of suckling rats (30), neonatal zinc deficiency was induced by depriving dams of dietary zinc throughout lactation. At weaning, maternal plasma zinc was reduced (less than 40%

of control) as was the amount of zinc per unit volume of milk (60% of control). On the basis of body weight, zinc deficient suckling pups could thus receive only half the amount of zinc received by control neonates. Mortality was high (60%) among pups suckling these severely deficient dams and surviving neonates weighed only half of the control pups. Besides growth retardation, pups displayed general alopecia, dermatitis, and reduced zinc content of plasma, carcass and organs.

Other studies of neonatal mice have investigated zinc deficiency imposed at parturition and extended throughout the fourth or eighth week postpartum (31-33). Growth retardation, alopecia, and developmental anomalies were severe and mortality was 90-100%. The effects of such profound maternal/neonatal zinc deficiency clearly precludes the study of postnatal animals.

The prevalence of zinc deficiency in children and the experimental evidence in rodents demonstrating its disruptive effects during the fetal/neonatal period suggests the need for further investigations of the interrelationship of zinc and development. The rapidly maturing suckling mouse has already been shown to be sensitive to the effects of severely suboptimal maternal zinc nutrition as evidenced by growth retardation, physical anomalies and high morbidity (31-33). Since the suckling neonate is also actively acquiring immunocompetence, one may anticipate severe immunological damage resulting from zinc deficiency imposed at this time. Previous work has established the importance of zinc in maintaining immunological function and will be reviewed in the following sections.

Studies from our laboratory explored the effects of dietary zinc deficiency on the antibody-mediated responses of young adult (5-6 week) A/J mice (34-36). Four to five weeks of subsequent zinc deprivation resulted in severe thymic atrophy; these mice displayed approximately 30% of the plaque forming cell (PFC) response to the T-cell-dependent antigen sheep red blood cells (SRBC) normally observed in mice fed the zinc adequate diet (34). Confirmatory results were reported by Fernandes (37) also using zinc deficient A/J mice. The suggestion that zinc deficiency interferes with T-cell helper function is supported by the observed thymic atrophy, reduction in T-cell-dependent IgG plaque formation, and the amelioration of SRBC responses by reconstitution with normal thymocytes (34-36). The effects of zinc deficiency and of the concomitant anorexia were distinct suggesting that zinc deficiency per se did result in a loss of immunity. Other investigators have suggested that dietary zinc intake is an important factor modulating cell-mediated immune responses. Fernandes (37) reported that zinc deficient adult mice had low natural killer (NK) cell activity, normal antibody-dependent cell mediated cytotoxicity (ADCC) and depressed T-killer cell activity against tumor cells. Chandra, in contrast, observed a significant increase in NK cell activity and a slight increase in ADCC (38). Recent work from our laboratory also suggests that zinc deficiency adversely effects the delayed type hypersensitivity reaction of the young adult mouse (39).

Zinc deficiency and antibody-mediated responses have also been studied in the neonatal mouse during the post-weaning period of immunological and physical development (40). In this study, 2 week old weanling mice were fed zinc-deficient diets for 1-2 weeks prior to

immunization with T-cell-independent (TI) or T-cell-dependent (TD) antigens. After only 7 days of dietary treatment, IgM responses to the TI antigen dextran were approximately 40% lower than controls. This reduction is dramatic in view of the relatively brief duration of the imposed zinc deficient feeding period. More striking effects were observed with the response against the TD antigen, sheep red blood cells. In this instance only 20% of the normal response was observed, while prolonging the deficiency to 11 days reduced the response to less than 10% of control. Accompanying the reduction in antibody responsiveness was the lowered neonatal body weight (50% of control) and thymus weight (10% of control). Despite these severe effects, the weanling mouse was able to completely restore body weight, thymus weight and TD antibody responsiveness after only four days of zinc-adequate feeding. This repair capacity was previously observed in the fully immunocompetent adult mouse (35); however, the onset of impairment and subsequent recovery occurred more slowly in the comparably affected adult mouse than in the neonatal mouse.

Other investigators have studied the effects of prolonged zinc deprivation on neonatal immunocompetence during the combined suckling and post-weaning periods (31,33). Specifically, mitogenic and antibody responses were measured during the post-weaning period at 4 weeks of age. Because of the extended period of deficiency, growth retardation and hypoplasia of lymphoid organs were severe and morbidity exceeded 90%. The assessment of the surviving neonates indicated that splenic responsiveness to B and T cell mitogens were depressed as were antibody responses to SRBC. The time period of these studies encompassed a wide range of developmental transitions and incurred severe neonatal

mortality; in this thesis research, we attempted to avoid these problems by investigating marginal zinc deficiency during only the suckling period of development.

The aforementioned studies suggest that the human infant and neonatal animal may be particularly sensitive to the effects of dietary zinc deficiency both developmentally and immunologically. In particular the suckling neonate, which is rapidly acquiring the full complement of immunological capacities, may be highly susceptible to marked and possibly irreparable immunological damage. To date, no studies regarding the interrelationships of zinc and immune capacity have focused solely on this important developmental period. Thus the primary goals of this thesis were to further characterize this interrelationship by (1) defining the zinc requirement for normal neonatal growth and immunological function; (2) characterizing the consequences of maternal zinc deprivation on neonatal growth and immunological capacity as represented by responsiveness to TI and TD antigens; (3) determining whether or not zinc deficiency during the suckling period has lasting consequences to neonatal growth or immunological responsiveness.

CHAPTER 1

THE EFFECTS OF MATERNAL DIETARY ZINC ON GROWTH AND MITOGENIC RESPONSIVENESS IN SUCKLING MICE

Abstract

The purpose of this study was to determine the effects of varied levels of maternal dietary zinc on the growth and immunological development of the suckling A/J mouse. Beginning at 5 days postpartum, lactating dams were fed a biotin-fortified egg white diet containing zinc: 1.6 µg/g (low zinc), 3.6 µg/g (intermediate zinc), 5.8 µg/g (moderate zinc) and 30 µg/g (control). At 17 days of age, low zinc pups showed reduced body weight gain with splenic and thymic atrophy, intermediate zinc pups showed slight atrophy of spleen and thymus, and moderate zinc pups were unaffected. Splenic mitogenic responses of suckling neonates were also determined against Phytohemagglutin (PHA), Concanavalin A (ConA), Lipopolysaccharide (LPS), Pokeweed Mitogen (PWM) and dextran sulfate (Dx). Neonates from the low zinc group showed no detectable ConA response and only 50% of the control LPS and PWM responses; the intermediate zinc groups had reduced LPS responses while responses of the moderate zinc group were unaltered. It was concluded that with this diet the maternal dietary zinc requirement for normal neonatal growth to weaning and mitogenic responsiveness was not greater

than 5.8 μg Zn/g. However, 1.6 μg Zn/g resulted in neonatal zinc deficiency and growth retardation yet incurred no significant morbidity (10%). The low zinc groups displayed altered mitogenic responsiveness thus demonstrating both a qualitative and quantitative disruption in lymphocyte subpopulations.

Introduction

Earlier reports of the role of zinc deprivation indicated the importance of this trace element during the fetal-neonatal development of several species (41-45). In the rat, zinc deprivation during gestation is teratogenic (24) while postnatal deprivation has been shown to cause growth retardation and increased neonatal mortality (30). Maternal zinc deficiency imposed at parturition caused similar effects in weanling mice (46) with concomitant thymic atrophy and reduced splenic responses to mitogens in the pups at 4 weeks postpartum (31). Neonatal zinc/caloric deficiency beginning at 2 weeks of age also hindered growth and diminished antibody mediated responses of 3-4 week old mice to both T-cell-dependent (TD) and T-cell-independent (TI) antigens (40).

The effects of zinc deficiency have been more clearly defined in the fully immunocompetent young adult mouse and include atrophy of lymphoid organs, significant losses in body weight, reduced in vivo antibody and cell mediated responses (34,35,37) and altered in vitro mitogenic responses (47). The purpose of the present study, was to investigate the effects of varying levels of maternal dietary zinc on the rapidly developing yet functionally immature immune system (48-51) of the 17 day old suckling pup.

Because of the immaturity of neonatal in vivo responses, mitogenicity was selected as a probe to evaluate the immunological

potential of the neonate. Responses to several mitogens were measured in order to assess the possible effects of zinc deficiency on a broad range of lymphocyte subpopulations. Specifically, Phytohemagglutin (PHA) and Concanavalin A (ConA) are mitogens stimulating the Lyt $1^+,2^+,3^+$ and Lyt 1^+ subpopulations of T cells, respectively (52); Pokeweed Mitogen (PWM) responses require the interaction of B and T-helper cells (53); Lipopolysaccharide (LPS) stimulates B cells at intermediate stages of maturation and dextran sulfate (Dx) stimulates a more immature subpopulation of B cells (54-56). In the present study, equal numbers of cells were examined in culture from each dietary group; this allowed us to determine whether or not all subpopulations were equally affected.

Primary goals of this study were to define the maternal zinc requirement for normal neonatal growth and immunological development, to characterize the consequences of maternal zinc deprivation on neonatal growth and to assess the immunological development of these neonates by monitoring mitogenic responses of splenocytes. Results indicated that maternal zinc deficiency from day 5 to day 17 postpartum was sufficient to interfere with neonatal growth and immune development in the low zinc group (1.6 μg Zn/g) while incurring only 10% losses in neonatal life. Normal mitogenic responses and growth, however, could be maintained by pups in the moderate zinc group (5.8 μg Zn/g).

Materials and Methods

Mice and Diets

To obtain neonates for these experiments, it was necessary to develop a breeding protocol well suited to the inbred A/J mouse

(Jackson Laboratories, Bar Harbor, ME). The excitable temperament of this strain, low breeding rates, stress induced abortions, disruptive pheromone effects and cannibalism were all factors that had to be considered in these studies. Four week old male and female mice were housed separately in a temperature (24°) and light (12 hour dark/light) controlled room and given unlimited access to distilled water and stock laboratory diet (Mouse Breeder Blox, Allied Mills Inc., Chicago, IL). At 3-5 months of age and five days prior to breeding, nulliparous females were housed in pairs; males were subsequently introduced to these cages at 1600-1700 hours. For three consecutive days, at 0900 hours, females were checked for the presence of vaginal plugs. Since such detection is difficult in the mouse, confirmation was made by the visual determination of the estrous stage. This process did not reduce the success rate of breeding and avoided delay in remating unbred females. Pregnant females were housed in groups of three and, prior to the final trimester of gestation, were adjusted to a biotin-fortified egg white diet (Table 1) containing approximately 30 μg/g zinc. This gradual adjustment process minimized the agitation of pregnant females, eliminated the otherwise observed inanition and fetal resorption and promoted subsequent pup survival. Two to three days prior to parturition, visibly pregnant females were housed singly and provided with nesting material; cages were checked twice daily for new births. To prevent increased cannibalism, handling of pups and agitation of dams were minimized during the first week of life. Litters were not disturbed until the second day after birth; at this time, each litter could be successfully adjusted to five pups of average size, fostering pups as needed. From the fifth through the seventeenth day postpartum,

TABLE 1

Composition of Biotin-Fortified Egg White Diet

	g/kg
Glucose Monohydrate	585
Egg White Solids (spray dried)[a]	224
Corn Oil	100
Nonnutritive Fiber[a,b]	30
Salt Mix[b,c]	40
Vitamin Mix[d]	10
Ethoxyquin[e]	1

This diet is a modification of that previously described (57) with (a) increased protein and (b) supplemental zinc as zinc carbonate.

[a]Teklad Test Diets, Madison, Wisconsin.

[b]Wood cellulose-type fiber.

[c]Bernhart, F.W. and Tommarelli, R.M. (1966) J. Nutr. 89, 495, except that a U.S.P. grade of $CaHPO_4$ was used instead of a reagent grade; zinc carbonate was omitted.

[d]Composition similar to the AIN-76 mixture (1977) J. Nutr. 107, 1340, except that the biotin was increased to provide an additional 4 mg/kg diet.

[e]Santoquin, Monsanto Chemical Co., St. Louis, Missouri.

dams were given free access to acidified (4×10^{-5} M HCl) deionized (< 1 µg Zn/g) water and the biotin-fortified egg white diet containing one of the following levels of dietary zinc: 1.6 µg/g (low zinc), 3.6 µg/g (intermediate zinc), 5.8 µg/g (moderate zinc) and 30 µg/g (control). Within experimental limitations, this design minimized maternal stress and manipulation during the perinatal period and did not appreciably increase cannibalism or neonatal morbidity. In general, 50% of the bred females became pregnant, 90% of these delivered and 75% of the litters survived the first week postpartum. The overall success rate of breeding, delivery and neonatal survival was 10-25%; comparable results were obtained during studies with no dietary manipulations. Optimal results were achieved using 11-16 week old female mice weighing 19-22 grams and age matched, experienced males.

The introduction of extraneous zinc was minimized in all cases by washing feed jars and water bottles sequentially with detergent and 4N HCl followed by deionized water. Animals were housed on hardwood chip bedding (P.J. Murphy, Rochelle Park, NY; <1 µg Zn/g) in EDTA ((ethylenedinitrilo)-tetraacetic acid) washed cages constructed of polycarbonate and stainless steel.

Each of the four dietary groups consisted of 7-8 dams which bore their litters on the same day. The day of parturition was considered to be day 0 of neonatal life. Diet consumption and body weights of dams and pups were monitored every two to three days, between 0700 and 0900 hours.

Selection of Pups for Analysis

On the 17th day after birth and after 12 days of experimental feeding, five female and five male pups were chosen from each dietary group. Pups were randomly selected from the population falling within two standard deviations of the mean body weight for a given sex and dietary group.

Zinc Analysis

Flame atomic absorption spectrophotometry (Varian Techtron, A-175, Sunnyvale, CA) was used to determine the zinc content of the diet. Samples of known weight were added to preweighed flasks for wet-ashing in 20 ml 16M HNO_3 and 2.5 ml 11.6M $HClO_4$; the residue was diluted in a known weight of 1% HCl prior to analysis. Blanks consisting of the nitric-perchloric acid mixture were similarly prepared. Absorption values of all samples were obtained in triplicate at 214 nm against a commercially prepared zinc standard ($Zn(NO_3)_2$; Harleco, Philadelphia, PA) diluted in 1.0 N nitric acid (34).

Mitogens and Cell Culture Reagents

Eagles minimum essential medium (MEM), and Pokeweek Mitogen (PWM) were obtained from Gibco Laboratories, Grand Island, NY. Non-essential and essential amino acid mixtures were obtained from Microbiological Associates, Walkersville, MD. Fetal bovine serum (FBS) was obtained from Microbiological Associates Bioproducts, Walkersville, MD. Bacto Phytohemagglutin P (PHA) and Lipopolysaccharide W (LPS, E. coli 055:B5) were obtained from Difco Laboratories, Detroit, MI. Concanavalin A (ConA) and dextran sulfate (Dx, MW 500,000) were purchased from Sigma, St. Louis, MO.

Preparation of Cell Culture Reagents

The methods for cell culture are essentially those described by Mishell and Dutton (58). Briefly, the culture medium was prepared with Eagles MEM supplemented to 1% in L-glutamine (200 mM), 1% in nonessential amino acids (10 mM), 1% in sodium pyruvate (10 mM), 100 units/ml in penicillin and 100 µg/ml in streptomycin, 0.22% wt/vol in sodium bicarbonate, 5×10^{-5} M in 2-mercaptoethanol and 5% in FBS. Cultures were fed daily with 10 µl of the nutritional mixture previously described (58).

Preparation of Cell Suspensions for In Vitro Cultures

Neonatal spleens were removed aseptically and pressed through stainless steel screens (100 gauge) to form single cell suspensions. Splenocytes were then washed in the supplemented culture medium and resuspended to a final density of 2.5×10^6 viable cells/ml as determined by the trypan blue exclusion method (59).

In Vitro Mitogenic Stimulation

The neonatal groups were assayed in conjunction with eleven adult virgin female A/J mice (11-12 weeks old) reared on stock laboratory diet; the latter served as an internal control testing optimal culture conditions.

Cultures were prepared in round-bottom microtiter test plates (Gibco Laboratories, Grand Island, NY) according to the method of Mishell and Dutton (58) using 100 µl aliquots of 2.5×10^5 viable spleen cells. Mitogens were assayed in triplicate at the following final concentrations: PHA (100, 50, 25 µg/ml); ConA (10.0, 5.0, 2.5 µg/ml); LPS (100, 50, 25 µg/ml); PWM (100, 50 µl/ml) and Dx (75, 50, 25 µg/ml) (optimal doses, which were the same for all four dietary groups, are underlined).

Culture plates were incubated in a Streck Isolation Chamber (Streck Laboratories, Inc., Omaha, NE) at 37° in a humidified atmosphere of 10% CO_2, 7% O_2 and 83% N_2. After 56 hours of incubation, 10 µl of the nutritional mixture containing 0.1 µC ^{125}I-Udr (5 ^{125}I-Iododeoxyuridine; New England Nuclear, Boston, MA) was added to each well. Twelve hours later, cells were harvested onto glass microfiber filters (Whatman, Inc., Clifton, NJ) using a multiple sample harvester (Otto Hiller Co., Madison, WI). Samples were washed with trichloroacetic acid and the incorporation of isotope was measured using a Beckman gamma counter (Beckman Instruments, Palo Alto, CA; 64% counting efficiency). Lymphocyte recoveries and viabilities were comparable in each of the four dietary groups after the 68 hour culture period. Responses are reported only at optimum doses of mitogen.

Statistical Methods

All data were examined by two-way or one-way analysis of variance; treatment differences were determined by Tukey's test (60).

Results

Since there were no statistically significant differences between sexes ($P>0.05$) and no interaction ($P>0.05$) between sex and dietary treatments with respect to the neonatal variables monitored, male and female data were combined and presented only on the basis of treatment effects.

The feeding of the low zinc diet (1.6 μg/g) to the lactating dams caused the mean neonatal body weight to decrease significantly ($P<0.01$) from control group after 1 week of dietary treatment (Figure 1). This disparity between body weights of the low zinc group and those of the controls continued to increase throughout the experiment. At day 17 postpartum, deficient pups weighed an average of 5.2 grams (70% of the control pups); pups from the intermediate (3.6 μg Zn/g) and moderate (5.8 μg Zn/g) zinc group, however, did not vary significantly from control ($P>0.01$).

Other detrimental effects of maternal zinc deficiency included the hypoplasia of neonatal lymphoid organs (Table 2). Splenic atrophy was observed in both the low and intermediate zinc groups; final spleen weight of the low zinc group (1.6 μg Zn/g) was 42% of control. Effects on lymphocyte numbers were more severe, the low zinc group having only 18% as many splenocytes as normal pups. Thymus weights of the low and intermediate zinc groups were also lower than the control pups with the low zinc group at 63% of control.

Maternal diet consumption in the low zinc group (1.6 μg Zn/g) was significantly lower ($P<0.05$) than control intake but statistical differences between body weights were not observed among the four dietary groups (Table 3). The outward appearance of the low zinc group, however, differed from other treatment groups. Dams' coats were somewhat scruffy with a slightly yellow caste; neonates were more severely affected and suffered extensive dermatitis and alopecia, characteristic evidences of zinc deficiency (46).

The effects of maternal zinc intake on the in vitro mitogenic responses of suckling neonates is shown in Figure 2. As discussed in

Figure 1.

The effects of maternal dietary zinc levels on the body weight of suckling neonates. Five days postpartum, dams were fed one of the experimental diets containing 1.6, 3.6, 5.8 or 30 μg Zn/g. Each point represents the mean ± SEM of at least ten neonates (five males and females). Values significantly different ($P<0.01$) for control (30 μg Zn/g) are denoted by asterisks (*).

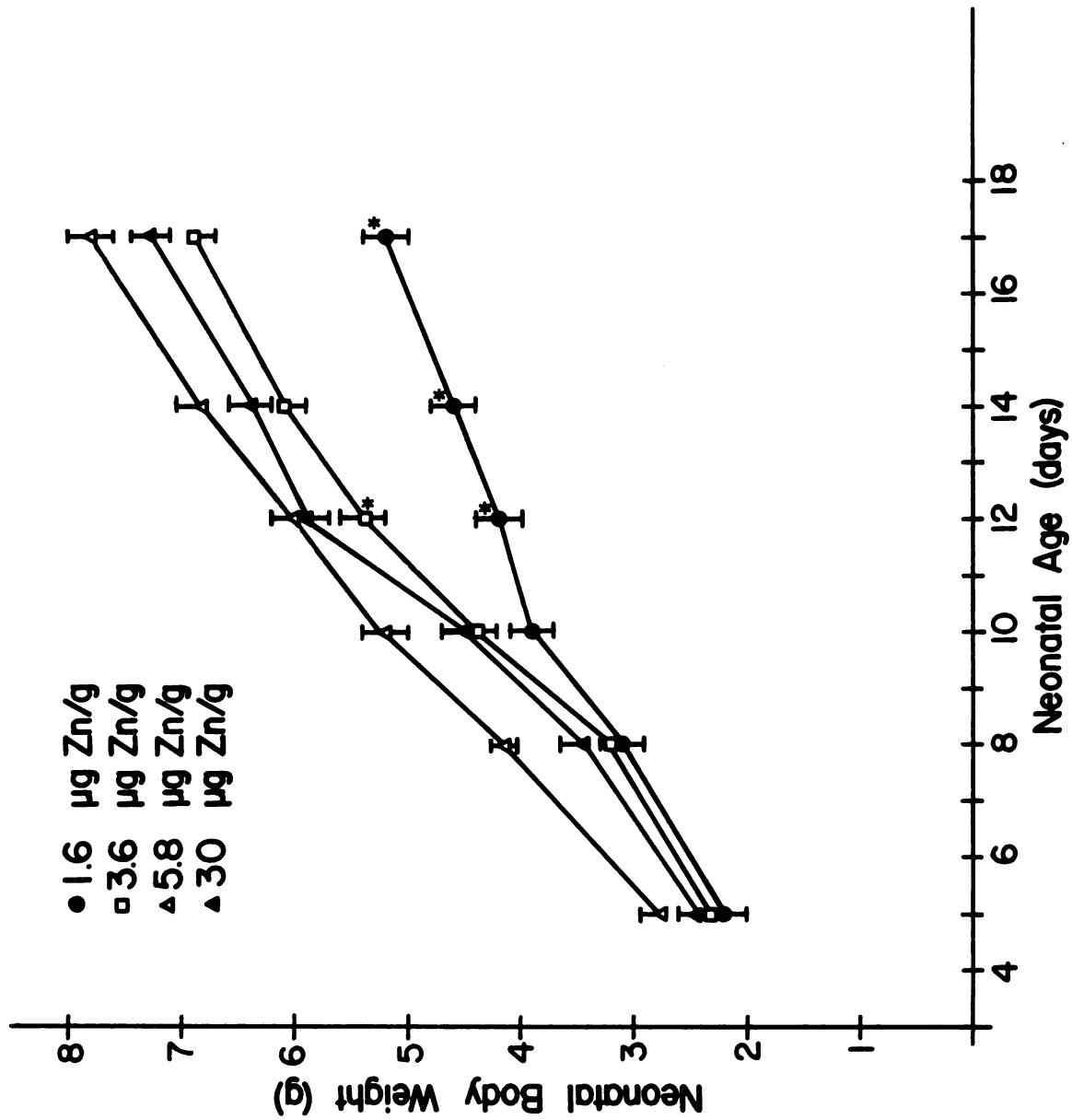

Neonatal Body Weight (g)
Neonatal Age (days)
● 1.6 µg Zn/g
□ 3.6 µg Zn/g
▲ 5.8 µg Zn/g
▲ 30 µg Zn/g

TABLE 2

The Effects of Maternal Dietary Zinc Levels on Neonatal Lymphoid Organ Weights and Number of Splenocytes[1]

Maternal Dietary Zinc (µg/g)	Lymphoid Organ Weights		Number of Splenocytes ($x10^{-6}$)
	Spleen (mg)	Thymus (mg)	
1.6	20.9 ± 1.8*	17.7 ± 1.3*	9.5 ± 3.7*
3.6	42.0 ± 1.8*	23.0 ± 1.3*	46.7 ± 3.4*
5.8	48.0 ± 1.8	29.2 ± 1.3	50.3 ± 3.7
30	49.8 ± 1.8	28.4 ± 1.3	54.3 ± 3.8

[1]Values represent the mean ± SEM of ten 17-day old pups (five males and five females) nursing dams maintained on diets beginning day 5 postpartum. Means significantly different ($P<0.05$) from the control are denoted by and asterisk (*).

TABLE 3

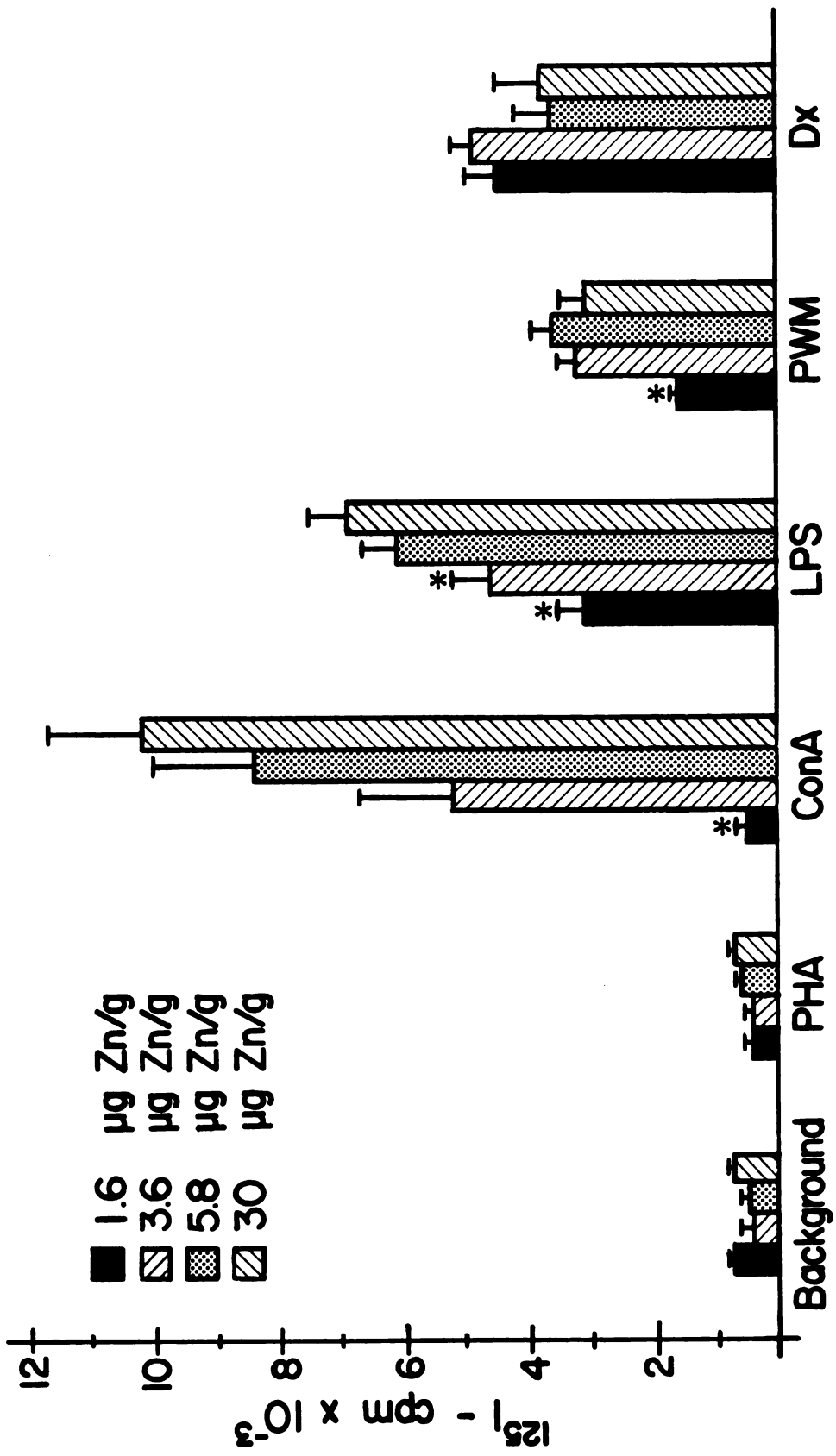
The Effect of Varying Amounts of Dietary Zinc on Maternal Body Weight and Diet Consumption from Day 5 to Day 17 of Lactation[1]

Maternal Dietary Zinc (µg/g)	Body Weights		Total Diet Consumed Per Dam (g)
	Initial (g)	Final (g)	
1.6	24.6 ± 1.2	22.8 ± 1.2	81.8 ± 4.5*
3.6	24.2 ± 1.2	26.1 ± 1.2	91.6 ± 4.5
5.8	24.9 ± 1.0	27.9 ± 1.0	104.0 ± 3.9
30	25.2 ± 1.2	25.7 ± 1.2	105.0 ± 4.5

[1]Values represent the mean ± SEM of 4-6 dams. Means significantly different ($P<0.05$) from control are denoted by an asterisk (*).

Figure 2.

Effects of maternal dietary zinc levels on the optimal mitogenic responses of 17 day old A/J neonates. Twelve days of maternal dietary feeding (1.6, 3.6, 5.8 or 30 μg Zn/g) preceeded the assessment of mitogenic responses. Responses were measured by incorporation of ^{125}I-Udr into DNA of splenocytes prepared from suckling neonates. Each neonatal bar represents the mean ± SEM of ten pups (five males and females). Values significantly different ($P<0.05$) from neonatal control are denoted by asterisks (*).


^{125}I - cpm x 10^{-3}
12
10
8
6
4
2
1.6 µg Zn/g
3.6 µg Zn/g
5.8 µg Zn/g
30 µg Zn/g
Background
PHA
ConA
LPS
PWM
Dx
*

Materials and Methods, these responses were tested over a range of doses for each mitogen and the optimum mitogenic dose was the same for all dietary groups (Figure 2). At 17 days of life, the control neonates gave substantial responses to ConA, LPS, PWM and Dx, but no PHA response was detectable. In comparison to neonatal controls, the moderate zinc group (5.8 μg Zn/g) responded to all mitogens with similar intensity. Splenocytes from the intermediate zinc group (3.6 μg Zn/g) exhibited reduced proliferation to LPS while other responses appeared normal. The low zinc group (1.6 μg Zn/g), however, gave no detectable ConA response and had a 50% reduction in the LPS and PWM responses. Neonatal Dx responses were not significantly altered by maternal zinc deprivation. The possibility that reduced responses in zinc deprived groups were due to increased fragility or death of lymphocytes during cell culture was eliminated since lymphocyte recoveries and viabilities were similar in all four dietary groups following culture.

Discussion

Previous studies in our laboratory have shown that zinc deficiency in weanling and young adult mice caused reduction in body weight, pronounced thymic atrophy and marked depression of antibody mediated responses to both TI and TD antigens (34,35,40). The focus of the present study was three-fold: (a) to establish a minimal level of dietary zinc required for normal neonatal growth and mitogenic responsiveness to weanling age, (b) to characterize the effects of varying maternal dietary zinc levels on neonatal growth and (c) to

detect possible changes in neonatal lymphocyte subpopulations resulting from varied maternal dietary zinc levels using mitogenicity as a probe.

Levels of zinc chosen for these studies were based on the information known for the young, growing female A/J mouse using a biotin-fortified egg white diet (57) and included a low zinc diet (1.6 μg Zn/g), an intermediate zinc diet (3.6 μg Zn/g), a moderate zinc diet (5.8 μg Zn/g) and a diet more than adequate in zinc (30 μg Zn/g). Based on the paramters measured in this study, 5.8 μg Zn/g maternal dietary zinc appeared to be sufficient to maintain the normal appearance and growth of suckling pups. The intermediate level was somewhat suboptimal in this regard but the low zinc level produced general dermatitis and alopecia, markedly reduced spleen and thymus weights, and reduced the overall growth rate of the pups while incurring only 10% morbidity.

In contrast to neonatal mice, dams from all dietary groups maintained comparable body weights after the 12 days of dietary zinc deficiency (Table 3). Dams from the low zinc group developed only slightly scruffy coats while their pups developed marked dermatitis and alopecia. This suggests that the dam appears to be unable to protect her offspring from the effects of zinc deficiency and that postnatal development may be particularly sensitive to changes in zinc nutrition (61). There is no evidence that the mammalian fetus can accumulate reserves of zinc, indicating that any restriction of neonatal zinc supply during the suckling period could have immediate consequences (61). Evidence for the absence of fetal reserves is given by rat studies in which the severe deprivation of maternal dietary zinc from day 0-28 postpartum and the concomitant decline in the production and

zinc content of dams' milk were sufficient to reduce neonatal growth rate and survival (90% morbidity) (30,61). In the present experiments, we acknowledge the fact that production, zinc content and nutritive value of murine milk may also be reduced following consumption of diets insufficient in zinc. However, the focus of this work was to monitor the effects of insufficient maternal zinc intake on neonatal immunological responsiveness rather than to quantitatively characterize changes in dams' milk.

Mitogenic responsiveness, under normal dietary treatment, generally varies with the mitogen, age, lymphoid organ and strain of mouse. The ability of murine splenocytes to respond to ConA is detectable at birth while responses to another T-cell mitogen, PHA, can not be detected until 3 weeks of age (48); neither response reaches adult levels until 4-8 weeks (48,62). Responses to B-cell mitogens Dx and LPS are acquired sequentially by fetal liver cells suggesting that Dx stimulates an immature population of B-cells while LPS activates B-cells in an intermediate stage of differentiation (54). Adult LPS responses have been reported with splenocytes from 1 week old neonates (62). Splenic PWM responses, which require interactions between B and T-helper cells, are significant in the 3 day old mouse and increase with age (53,63).

Mitogenic responses of the control A/J neonates in the present study concur with those described in the literature. Substantial responses to ConA, LPS, PWM and Dx were present at 17 days of life but the immature neonate could not respond to PHA (Figure 2). The experimental pups most severely effected by maternal zinc deprivation (1.6 μg/g) differed from the control by the absence of a ConA response and the reduced LPS and PWM responses; the intermediate zinc group (3.6

μg Zn/g) had only a depressed LPS response and the moderate zinc group (5.8 μg Zn/g) was unaffected. These results showed that reducing maternal dietary zinc intake created a loss of neonatal responsiveness to in vitro mitogenic stimulation which varied with the subpopulation of lymphocytes being studied. The loss of T-cell responsiveness was marked in the low zinc group (1.6 μg Zn/g) as evidenced by the complete absence of a ConA response. This reduction of T-cell function may have been, in part, responsible for the depressed PWM responses; normally PWM responses reflect the interaction of B and T-helper cells. The responsiveness of more mature B cells was impaired as evidenced by the half normal LPS response. The fully normal dextran response suggests that the immature population of B cells appears to be unaffected by the previously imposed dietary zinc deficiency. Zinc, however, is present in the in vitro system employed and may allow some lymphocyte recovery during the four day culture period. Despite this, neonatal mitogenic responses of the low zinc group were markedly altered indicating that maternal dietary zinc deficiency (1.6 μg/g) did affect both B and T cell subpopulations of the suckling neonate.

In summary, this work suggests that a maternal dietary zinc requirement for normal neonatal development is no greater than 5.8 μg Zn/g and that 1.6 μg Zn/g results in neonatal zinc deficiency and growth retardation. Dietary levels of 5.8 μg Zn/g were sufficient to maintain normal neonatal mitogenic responsiveness but reducing maternal dietary zinc intake to 1.6 μg/g altered the pattern of neonatal mitogenic responses thus suggesting a disruption in lymphocyte subpopulations and immunological impairment. Further in vivo studies will aid in delineating the specificity, extent and permanence of damage to the neonatal immune system.

CHAPTER 2

MATERNAL DIETARY ZINC DEFICIENCY AND THE EFFECTS OF NEONATAL ANTIBODY RESPONSIVENESS

Abstract

The effects of maternal dietary zinc deficiency on the immunological competency of the suckling A/J mouse were studied. Lactating dams were fed biotin-fortified egg white diet containing zinc: (1) 1.6 µg/g, ad libitum (zinc deficient), (2) 30 µg/g, ad libitum (control) and (3) 30 µg/g, daily intake restricted to that consumed by the deficient mouse (restricted). At 17-19 days postpartum, the suckling neonates were killed and antibody responses and avidities were determined against the T-cell-independent (TI) antigens, TNP-LPS (trinitrophenyl-Lipopolysaccharide) and TNP-Ficoll (trinitrophenyl-aminoethylcarbamylmethyl^{80}-Ficoll); responses were also determined against the T-cell-dependent (TD) antigen SRBC (sheep red blood cells) both before and after a two week repair period of zinc positive refeeding. Prior to zinc repletion, the zinc deficient and restricted pups displayed a similar reduction in body and lymphoid organ weights and in TI and TD antibody responses. In contrast, TI antibody avidities were reduced only in the zinc deficient group. Two weeks of zinc supplementation fully restored SRBC responses in the deficient and restricted

pups and body weights returned to near normal. This data indicated both qualitative and quantitative alterations in lymphocyte populations of the zinc deficient neonate and demonstrated that zinc deficiency during the suckling period did not irreversibly impair TD antibody responsiveness.

Introduction

Results from the previous study (Chapter 1) have established that the growth and immunocompetence of suckling mice were adversely effected by reduced maternal dietary zinc intake (1.6 μg/g). Further work, however, was necessary to establish the extent and nature of neonatal immunological impairment.

It is generally accepted that immunological maturity can be defined as the ability to elicit antibody responses of magnitude and affinity comparable to those of the normal adult. These responses are sequentially acquired with responses against T-cell-independent (TI) antigens appearing before those against T-cell-dependent (TD) antigens; during the first weeks of life TI antibody responses may approach 60-80% of adult values while the magnitude of TD responses remains depressed until 4-6 weeks of life (51,64-67). TI antigens can be divided into two classes as defined by the population of cells stimulated and by the time at which antibody responsiveness appears during ontogeny: (1) TI-1 antigens (trinitrophenyl-Lipopolysaccharide (TNP-LPS), TNP-Brucella abortus) elicit antibody responses from newborn splenocytes and (2) TI-2 antigens (TNP-Ficoll, TNP-dextran) stimulate antibody formation in 10-14 day old splenocytes; the magnitude of both responses increases with age (64). The TI antigen response pattern has been examined further in the immune defective CBA/N mouse strain (56). This strain bears an X-linked defect which leads to a delay in B cell

maturation whereby young adult male mice, although responsive to the TI-1 antigen (TNP-LPS), are incapable of responding to the T1-2 antigen (TNP-Ficoll); this response pattern is analagous to that acquired by the neonatal mouse. Apart from being able to use these TI antigens to detect differences in the antibody responsive state of B cells, one can also use the TNP-conjugated antigens to measure antibody avidities. This can give further information regarding the degree of neonatal B cell development since immature B cell populations generally produce antibodies of (a) restricted heterogeneity of affinity and (b) low average affinity (68). TD responses by definition are indicative of both the responsive state of B cells and the efficacy of T-helper cell function. As with TI antigens, sequential acquisition of responses to various TD antigens has also been reported (66,69). The onset of the in vivo responsiveness to the TD antigen SRBC (sheep red blood cells) has been extensively studied in the neonatal mouse (70-74); this response appeared sometime during the first 2 weeks of life and became adult-like after 4 weeks of age (70,74). By measuring TI and TD antibody responses, a general evaluation of neonatal immunological maturity can be obtained. Previous studies from our laboratory have indicated that mice made zinc-deficient during post-weaning or adult life were able to fully recover body weight and antibody mediated responsiveness to SRBC within two weeks of dietary zinc repletion (35,40). It was not known whether zinc deficiency imposed during the suckling period would allow similar restoration in the developing neonate or if these pups would be permanently impaired.

In the previous study of zinc deficient suckling mice (Chapter 1), delayed growth and altered mitogenic responsiveness were observed. The

zinc deficient neonate, physically immature for its age, may have also experienced a disruption in normal ontogenesis of the antibody mediated responsiveness and possibly sustained permanent immunological damage. The present study was designed to further test these hypotheses: (1) to discern the maturity of the antigenic responsive state of B cells relatively independent of the functional maturity of T cells, we measured antibody responses and avidities in the zinc deficient suckling neonate against antigens from the two TI classes (TNP-LPS, TNP-Ficoll); (2) to characterize neonatal TD antibody responsiveness and possible repair capacity, zinc deficient neonates were immunized with SRBC both before and after a two week period of zinc supplementation. The results reported herein demonstrated that zinc deficiency imposed during lactation depressed neonatal TI antibody avidity and reduced both TI and TD antibody responsiveness; subsequent zinc supplementation, however, allowed the complete restoration of TD antibody responses.

Materials and Methods

Mice and Diets

Adult female A/J mice were housed and bred as described in Chapter 1. Beginning at 5 days postpartum, dams were fed the biotin-fortified egg white diet containing varied amounts of zinc: (1) a zinc deficient diet (1.6 μg Zn/g), ad libitum [Zn(-), deficient], (2) a zinc adequate diet (30 μg Zn/g), ad libitum [Zn(+), control] and (3) a zinc adequate diet (30 μg Zn/g) with daily intake restricted to the amount consumed

by the zinc deficient groups on the previous day [R(+), restricted)]. Since the previous study indicated that the diet consumption of dams from the low zinc group (1.6 μg/g) was significantly lower than that of controls, the restricted dietary group was presently included to serve as a control for any effects due to inanition. Dietary groups were composed of at least 4 dams which bore their litters on the same day ± 1; the day of parturition was considered to be day 0 of neonatal life. Diet consumption and body weights were recorded between 0800 and 1000 hours. The zinc content of the diets was determined as described in Chapter 1.

Experiment 1. In this study, maturation of neonatal B cells was investigated. At 17 days of age, neonatal antibody responses and avidities were measured against the T-cell-independent (TI) antigens trinitrophenyl-Lipopolysaccharide (TNP-LPS; TI-1) and trinitrophenyl-Ficoll (TNP-Ficoll; TI-2).

Experiment 2. Neonatal T-cell-dependent (TD) antibody responses to sheep red blood cells were measured at 19 days of age following 14 days of maternal dietary zinc deficiency (day 5-19 postpartum); this also served as a baseline for repair studies (0 days repair). To test the immunological repair capacity of the suckling neonate, Zn(-) dams were returned to the Zn(+) diet; pups were subsequently weaned to this diet at 23 days postpartum and divided into groups of 4-5 according to sex. A two-week period of maternal/neonatal Zn(+) refeeding was allowed before immunization of the neonates (14 days repair).

Antigens and Immunizations

TNP-LPS (trinitrophenyl-Lipopolysaccharide, lot 415-459-3907; prepared by trichloroacetic acid extraction) and TNP-Ficoll (trinitro-

phenyl-aminoethylcarbamylmethyl^{80}-Ficoll; Cat. F-1300) were obtained from Biosearch, San Rafael, CA. Both antigens were dissolved in sterile phosphate buffered saline (PBS) and filtered (0.22 μM). Using a 27 1/2 gauge needle, neonates (12-13 days old) were injected i.p. with a 100 μl volume containing 1 μg TNP-LPS or 5 μg TNP-Ficoll; antibody responses were measured four days later. Slightly suboptimal doses of these antigens were used to enhance differences in avidities. Sheep red blood cells (SRBC, Gibco Diagnostics, Grand Island, NY) were washed and suspended in sterile PBS. Neonates (14 or 30 days old) were injected i.p. with 5 x 10^7 SRBC; antibody responses were measured five days later. In all experiments, at least 10 neonates were selected randomly from the population falling within two standard deviations of the mean body weight for a given sex and dietary group.

Haptenation of Sheep Red Blood Cells and L-Lysine

Trinitrophenylated sheep red blood cells (TNP-SRBC), for use as indicator cells in the Jerne Plaque assay, were made essentially by the method of Kettman and Dutton (75) as described by Mishell (76). SRBC were prepared for haptenation by washing in PBS containing 1% wt/vol dextrose. One ml of packed SRBC was added dropwise to 7 ml of 0.28 M Cacodylate buffer, pH 6.9, containing 130 mg picryl sulfonic acid (2,4,6-trinitrobenzene sulfonic acid (TNBS), Sigma, St. Louis, MO) and gently tumbled for 30 minutes at room temperature. Cells were centrifuged and washed sequentially with modified barbital buffer (MBB) (77), 6.3% wt/vol glycyl-glycine prepared in MBB and finally with MBB. Washed TNP-SRBC were resuspended in MBB for use at a concentration of 2 x 10^9 cells/ml. Throughout the haptenation and handling of cells, care was taken to avoid photodecomposition.

TNP-L-lysine was prepared according to the method of Okuyama and Satake (78) for use as an inhibitor of plaque formation. Briefly, 1.09 g L-lysine, 1.2 g sodium bicarbonate and 1.2 g TNBS were combined with 60 ml water and allowed to react for 2 hours at 25°. The pH was adjusted to 1.0 with 1 N HCl and the precipitate collected by centrifugation. The prepared TNP-L-lysine was recrystallized from hot methanol and spectrophotometrically tested for purity (= 12,500 at 340 nm).

Detection of Antibody-Producing Cells

A modification of the Jerne Plaque assay (79) as previously described in detail (34) was used to determine the number of direct (IgM) and indirect (IgG) plaque forming cells (PFC) in the neonatal spleen. Briefly, spleens were removed and individually pressed through stainless steel screens (100 gauge) to form single cell suspensions; splenocytes were washed and resuspended to a 1 ml volume. The medium used to maintain lymphocytes was Hanks salt solution (pH 7.4) containing penicillin (100 units/ml) and streptomycin (100 µg/ml). One-tenth ml of the splenocyte suspension (or dilution thereof) and 0.1 ml of the SRBC-indicator cells (2×10^8 TNP-SRBC or SRBC) were combined with 0.7 ml of 0.6% agarose-medium maintained at 53° and were poured onto a 60 mm petri-dish containing a 5 ml base of 1.2% agarose-medium. After a 2 hour incubation of anti-TNP plates in a 37° humidified culture chamber, 0.5 ml of non-hemolytic guinea pig complement was added to develop the direct (IgM) plaques. Anti-SRBC direct plates were similarly treated after 1.5 hour incubation; indirect (IgG) plaques were developed by the addition of 0.5 ml rabbit anti-mouse IgG 30 minutes prior to complement.

Each animal was assayed in duplicate and corrections were made for the background responses of unimmunized mice. Also, the number of viable lymphocytes per spleen was determined using the trypan blue exclusion method (59); this allowed the expression of data as both the average PFC per spleen and per 10^6 lymphocytes.

Assay of Anti-TNP Antibody Avidity

Antibody avidity was assayed using Anderson's method of plaque inhibition by free antigen (80) where plaques formed by low avidity antibody required higher concentrations of antigen for inhibition than high avidity plaques. Average avidities are represented by the reciprocal of the inhibitor concentration required for 50% inhibition of plaque formation (81). TNP-L-lysine, containing the antigenic TNP-hapten, served as the inhibitor of TNP-plaque formation by competing with TNP-SRBC for anti-TNP antibodies. The formation of antibody/TNP-L-lysine complexes thus prevented lysis of TNP-SRBC and subsequent plaque formation. The pattern of inhibition was assessed at four final concentrations of TNP-lysine: 10^{-4}M, 10^{-5}M, 0.5×10^5 M, 10^{-6} M. TNP-L-lysine was added in 0.1 ml volumes to the 0.7 ml agarose medium used in the Jerne Plaque assay described above.

Statistical Methods

Data were treated statistically by one-way or two-way analysis of variance; treatment differences were determined by Tukey's test (60).

Results

Since there were no statistically significant differences between sexes ($P>0.05$) and no interaction ($P>0.05$) between sex and dietary treatment with respect to the neonatal variables monitored, male and female data were combined and presented on the basis of treatment effects.

Marginal dietary zinc deficiency during lactation (days 5-17 postpartum, approx.) and the accompanying anorexia caused marked growth retardation among the Zn(-) and R(+) neonates (Table 4; Table 5, 0 Days Repair); at 17-19 days of age, the Zn(-) and R(+) pups weighed significantly less ($P<0.05$) than the controls. Maternal body weights seemed to be relatively unaffected by the zinc deficiency despite the significant decrease in food intake ($P<0.05$). In general, dams maintained 90% of their initial body weight while the zinc deficient neonate could maintain only 75% of the growth rate observed in control pups (Table 4; Table 5, 0 Days Repair). As previously observed (Chapter 1), dams from the deficient groups had somewhat scruffy coats while neonates were more severely affected and showed extensive dermatitis and alopecia. Besides the detrimental effects on neonatal growth, maternal zinc deficiency also caused reduction in the weight of the neonatal spleen (44% of the control) and thymus (63% of the control) (Table 5). Splenocyte numbers were also severely reduced, being approximately 40% of the controls (Table 4, Table 5).

Neonatal antibody responses to both TI antigens, TNP-LPS and TNP-Ficoll, are shown in Figure 3. On a PFC/spleen basis (Figure 3, Panel A), Zn(-) responses were approximately 47% of control while R(+)

TABLE 4

The Effects of Maternal Zinc Deficient Feeding (Day 5-17 Postpartum) on Diet Consumption, Body Weights and Number of Splenocytes[1]

Dietary Group	Total Diet Consumed Per Dam (g)	Dam Body Weight Initial (g)	Dam Body Weight Final (g)	Neonatal Body Weight Initial (g)	Neonatal Body Weight Final (g)	Number of Neonatal Splenocytes ($x10^{-6}$)
TNP-LPS Experiment:						
Zn(-)	68.1 ± 3.6^{a*}	22.1 ± 0.9	21.1 ± 0.7	2.1 ± 0.1	5.1 ± 0.1^{a}	16.2 ± 1.7^{a}
R(+)	---	22.4 ± 0.9	24.0 ± 0.7	2.1 ± 0.1	5.4 ± 0.1^{b}	18.6 ± 1.8^{b}
Zn(+)	86.3 ± 3.0^{a}	23.9 ± 0.8	22.0 ± 0.7	2.1 ± 0.1	6.8 ± 0.1^{ab}	41.9 ± 1.7^{ab}
TNP-Ficoll Experiment:						
Zn(-)	72.6 ± 1.1^{b}	23.8 ± 0.9	21.2 ± 0.5^{c}	2.4 ± 0.1	5.0 ± 0.1^{c}	17.2 ± 3.8^{c}
R(+)	---	23.7 ± 1.0	20.1 ± 0.6^{d}	2.5 1 0.1	5.1 ± 0.1^{d}	19.2 ± 3.9^{d}
Zn(+)	91.3 ± 4.5^{b}	24.2 ± 1.0	24.5 ± 0.6^{cd}	2.3 ± 0.1	6.2 ± 0.1^{cd}	42.1 ± 3.9^{cd}

[1]Values represent the mean ± SEM of at least 4 dams or 10 17-day old neonates (five males and females).

*Values which share common superscripts within columns are significantly different (P>0.05).

TABLE 5

The Effects of Refeeding Zinc Adequate Diet on Diet Consumption, Body and Lymphoid Organ Weights and Number of Splenocytes in Previously Zinc Deficient Mice[1]

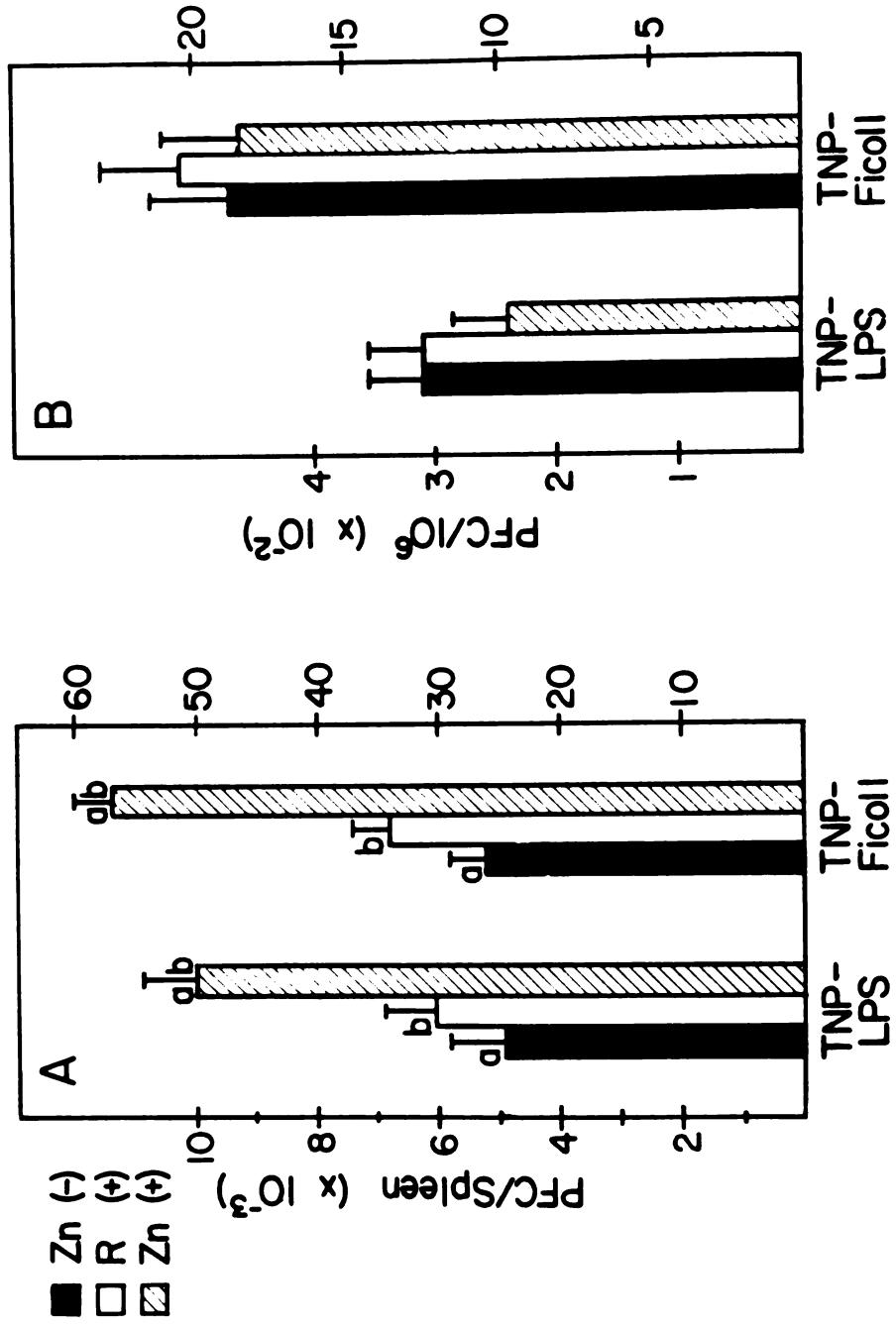
Dietary Group	Total Diet Consumption: Dam (g)	Total Diet Consumption: Neonate (g)	Dam Body Weight: Initial (g)	Dam Body Weight: Final (g)	Neonatal Body Weight: Initial (g)	Neonatal Body Weight: Final (g)	Neonatal Organ Weights: Spleen (mg)	Neonatal Organ Weights: Thymus (mg)	Number of Neonatal Splenocytes ($\times 10^{-6}$)
0 Days Repair*									
Zn(-)	81.3 ± 4.0^{a+}	---	21.4 ± 0.4	19.8 ± 0.7^{a}	2.2 ± 0.1	5.4 ± 0.1^{a}	16.0 ± 1.7^{a}	17.7 ± 1.3^{a}	20.5 ± 5.1^{a}
R(+)	---	---	22.2 ± 0.4	22.1 ± 0.6	2.1 ± 0.1	5.7 ± 0.1^{b}	22.6 ± 2.0^{b}	---	23.6 ± 6.6
Zn(+)	119.0 ± 3.7^{a}	---	22.9 ± 0.4	22.8 ± 0.6^{a}	2.2 ± 0.1	7.6 ± 0.1^{ab}	36.4 ± 1.9^{ab}	28.4 ± 1.3^{a}	47.6 ± 5.4^{a}
14 Days Repair**									
Zn(-)	125.0 ± 5.0^{b}	40.1 ± 2.6	22.3 ± 0.5	23.2 ± 0.6^{c}	2.1 ± 0.1	13.3 ± 0.2^{cd}	74.6 ± 4.3^{c}	35.5 ± 1.5	66.6 ± 6.4
R(+)	---	---	22.8 ± 0.6	20.4 ± 0.7^{cd}	2.2 ± 0.1	14.2 ± 0.2^{ce}	71.2 ± 4.3^{d}	36.8 ± 1.8	60.2 ± 6.2
Zn(+)	154.0 ± 5.5^{b}	45.1 ± 2.6	23.3 ± 0.7	23.9 ± 0.6^{d}	2.2 ± 0.1	16.0 ± 0.2^{de}	95.8 ± 4.0^{cd}	38.6 ± 1.6	62.2 ± 5.8

[1]Values represent the mean ± SEM of at least 5 dams or 10 pups.
[2]Neonatal diet consumption is reported for the post-weaning period.
[+]Means which share common superscripts are significantly different ($P<0.05$).

*In this experiment (baseline), dams were fed their respective diets from day 5-19 postpartum.
**In this experiment (repair), experimental dams were fed the Zn(-) diet from day 5-16 postpartum and then returned to the Zn(+) diet. All pups were weaned to this diet at 23 days postpartum. Two weeks of Zn(+) refeeding elapsed prior to neonatal immunization. Final values reported above were measured at day 35.

Figure 3.

Effect of maternal dietary zinc deficiency (day 5-17 postpartum) on neonatal IgM PFC response to TNP-LPS and TNP-Ficoll. The two panels show the PFC/spleen (A) and PFC/10^6 lymphocytes (B) of the 16-17 day old neonates immunized 4 days previously with 1 μg TNP-LPS or 5 μg TNP-Ficoll. Values represent the mean ± SEM of at least 10 (5 per sex). Within panels, bars significantly different ($P<0.05$) from one another share common superscripts.


A
B
PFC/Spleen (x 10⁻³)
PFC/10⁶ (x 10⁻²)
TNP-LPS
TNP-Ficoll
Zn (-)
R
Zn (+)
Zn (+)

responses were nearly 60% of control; despite these differences, the PFC/spleen responses of the Zn(-) and R(+) pups were not significantly different from each other ($P>0.05$). On the basis of PFC/10^6 lymphocytes (Figure 3, Panel B), all dietary groups responded similarly. This suggested that although the reduced PFC/spleen response of the Zn(-) pups was caused by zinc deficiency and the accompanying dietary restriction, the functional capacity to produce TI antibody was not affected by dietary treatment.

The inhibition curves for determining the avidities of neonatal TNP-LPS and TNP-Ficoll plaques are shown in Figure 4, Panels A and B, respectively. The apparent average avidities of the Zn(-), R(+), and Zn(+) responses to TNP-LPS were obtained, respectively, at 4.5×10^4, 1.7×10^5, and 2.0×10^5 while the corresponding responses to TNP-Ficoll were obtained at 3.6×10^4, 7.7×10^4 and 1.0×10^5. Close agreement of the Zn(+) and R(+) values were observed within both experiments while the Zn(-) values were approximately 3-4 fold lower. Since increases in avidity correlate with the maturation of B cells (68), this data suggests that the maturity of neonatal Zn(-) B cells was less than that of the R(+) or control pups.

Neonatal antibody responses to the TD antigen SRBC prior to zinc supplementation are shown in Figure 5 (0 Days Repair). PFC/spleen responses of the Zn(-) and R(+) groups were significantly lower ($P<0.05$) than controls (approximately 32% of control and 58% of control, respectively) yet were not significantly different from each other ($P>0.05$); similar responses were observed in all groups on a PFC/10^6 lymphocyte basis. As seen with the TI antibody responses,

Figure 4.

Effect of maternal dietary zinc deficiency (day 5-17 postpartum) on the inhibition of neonatal anti-TNP antibody by competing TNP-L-lysine. Percent inhibition of neonatal IgM plaque formation at 16-17 days of age plotted against the molar cncentration of inhibitor. Each point represents the mean ± SEM of at least 10 pups (5 per sex). Panel A: Inhibition of response to TNP-LPS. Panel B: Inhibition of response to TNP-Ficoll.

A
● Zn (-)
○ R (+)
▲ Zn (+)
% Inhibition
100
80
60
40
20
10⁻⁶
0.5 x 10⁻⁵
10⁻⁵
10⁻⁴
Concentration Inhibitor (M)

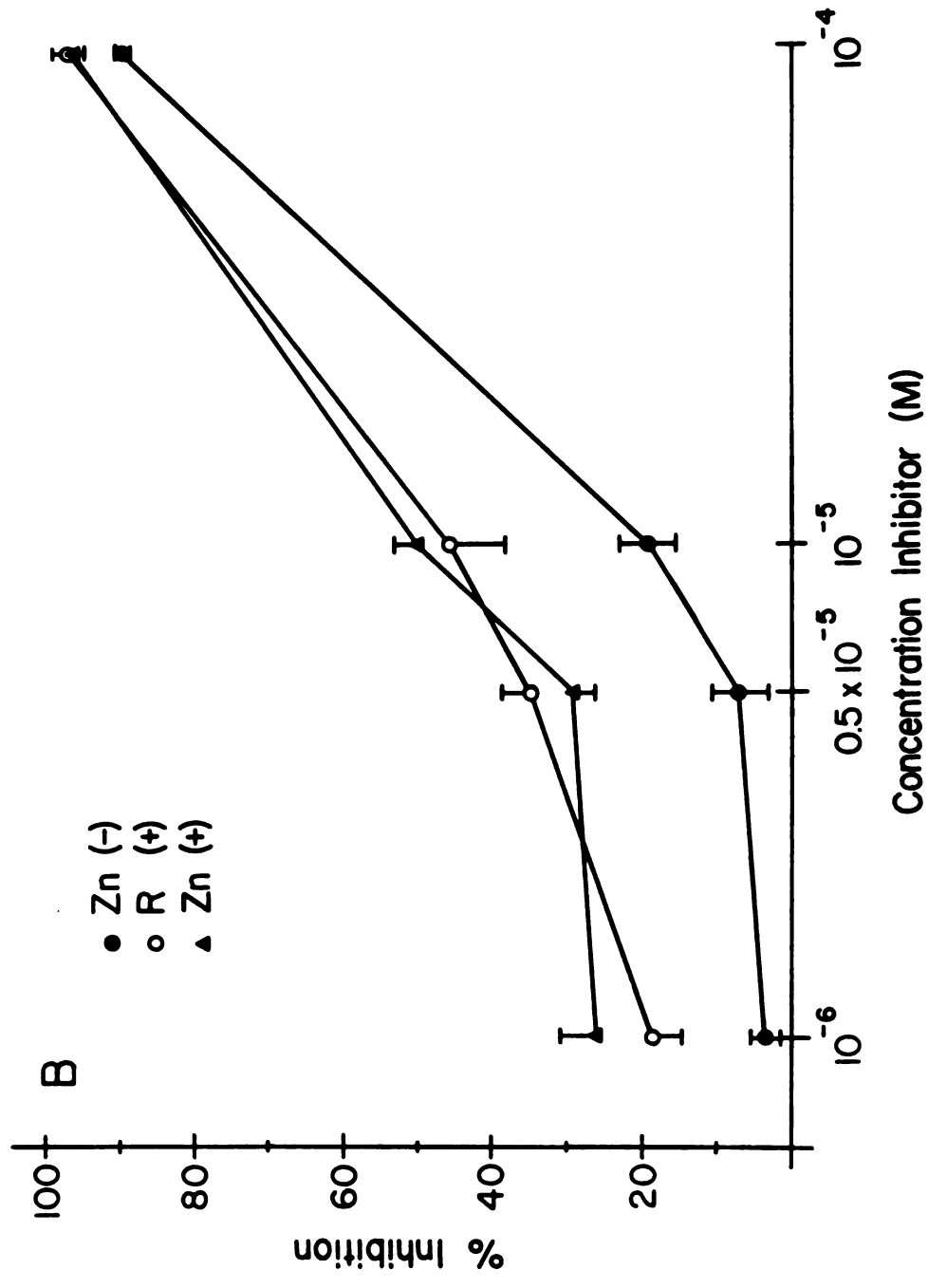
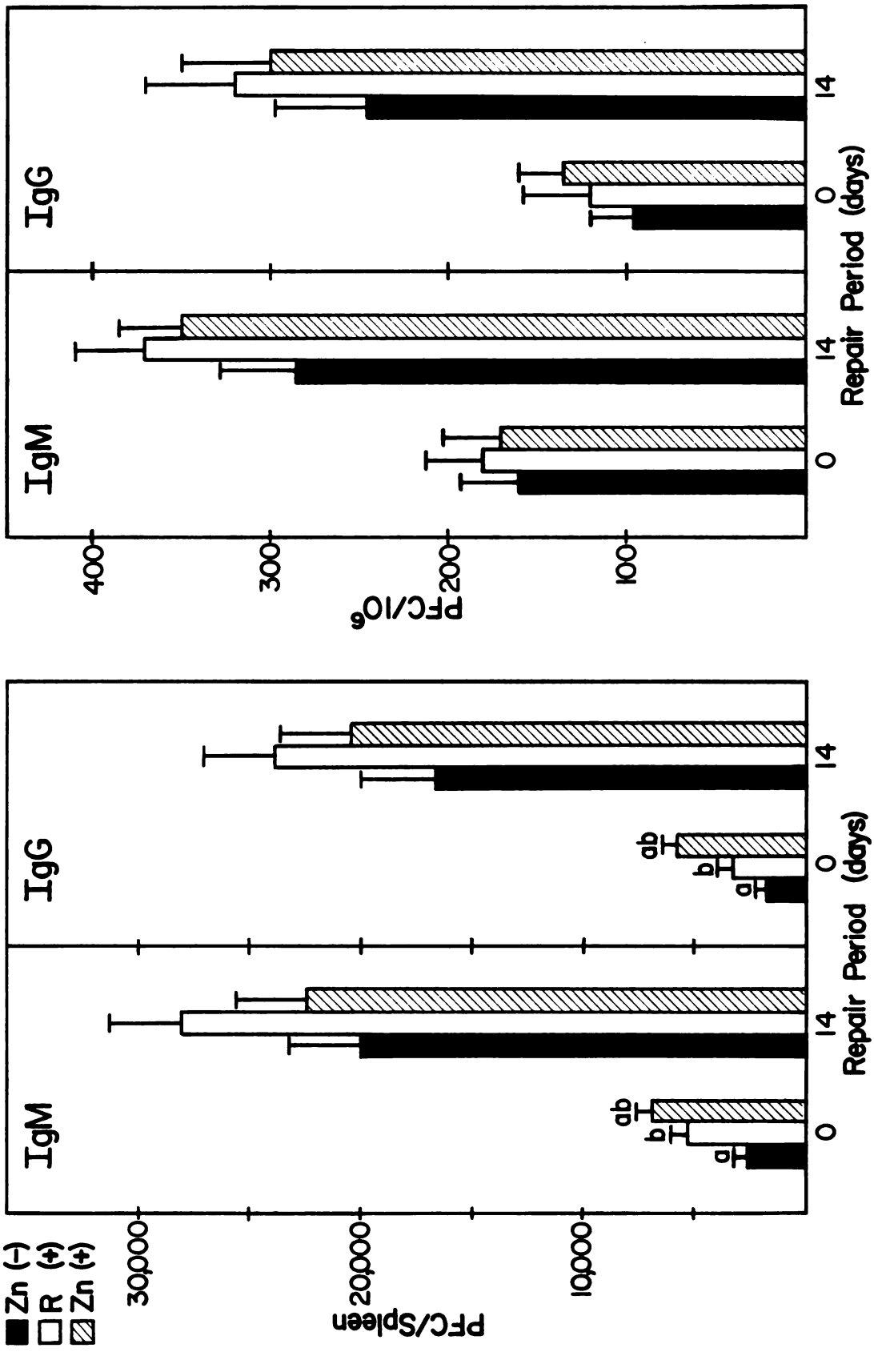

B
Zn (-)
R (+)
Zn (+)
% Inhibition
100
80
60
40
20
10^{-6}
0.5×10^{-5}
10^{-5}
10^{-4}
Concentration Inhibitor (M)

Figure 5.

Effects of dietary zinc supplementation on SRBC plaque-forming response of previously zinc deficient neonatal mice. Five days postpartum, dams were divided among three dietary groups: deficient, restricted and control. After 9-11 days of dietary treatment, neonates from each group were immunized with a 5×10^7 SRBC (0 days repair). The remaining groups were fed a zinc adequate diet for 14 days prior to immunization (14 days repair). Direct (IgM) and indirect (IgG) responses are shown as both PFC/spleen and PFC/10^6 lymphocytes. Each value represents the mean ± SEM of at least 10 pups. Bars within the same panel which share a common superscript vary significantly from one another ($P<0.05$).


Zn (-)
R (+)
Zn (+)
PFC/Spleen
30000
20000
10000
IgM
IgG
a
ab
b
0
14
Repair Period (days)
PFC/10^6
400
300
200
100

this suggested that although reduced PFC/spleen responses of the Zn(-) pups were caused by zinc deficiency and the accompanying anorexia, the ability to produce antibody was not altered by dietary treatment.

In the zinc repletion experiment (Table 5; Figure 5, 14 Days Repair), experimental dams were fed the Zn(-) diet for 11 days (day 5-16 postpartum) before returning to Zn(+) diet. A two week period of maternal/neonatal Zn(+) refeeding elapsed prior to neonatal immunization with the TD antigen SRBC. This repair period did allow considerable "catch-up" growth in the previously zinc deficient (83% of the controls) and companion restricted (89% of the controls) neonates; pups from the restricted group did show statistically greater weight gains than did those pups from the deficient group ($P<0.05$). Relative to the controls and prior to zinc supplementation, the Zn(-) neonates had depressed splenocyte numbers (43% of the control) and spleen and thymus weights (44% and 62% of the control, respectively); these differences were significant ($P<0.05$). Following two weeks of Zn(+) refeeding, thymus weights and splenocyte numbers returned to normal while spleen weights remained significantly lower ($P<0.05$) than controls (78% of normal). Similar effects were also seen in the R(+) group after companion Zn(-) mice were returned to the Zn(+) diet. Zn(-) pups differed from other groups, however, by the occurrence of marked alopecia; this effect was also reversed following two weeks of zinc supplementation.

Despite the depressed PFC/spleen SRBC responses of the Zn(-), (IgM, 37% of the controls, IgG, 28% of the controls) (Figure 5, 0 Days Repair), the mean IgM and IgG responses of the repairing Zn(-) pups (Figure 5, 14 Days Repair) equalled the responses of the R(+) and Zn(+)

pups on the basis of both PFC/spleen and PFC/10^6 lymphocytes ($P>0.05$) (in this experiment only female data was available). Results from the repair study indicated that the detrimental effects of maternal zinc deficiency on the TD antibody responsiveness of suckling female neonates could be reversed within 2 weeks of Zn(+) refeeding.

Discussion

The previous mitogenic study (Chapter 1) indicated that maternal zinc deficiency altered both B and T lymphocyte subpopulations in the neonate. Specific goals of the present experiments were to further investigate the effects of maternal zinc deficiency on the suckling neonate by (a) assessing the development of B cells by measuring antibody responses and avidities against the TI antigens, TNP-LPS and TNP-Ficoll, (b) characterizing B and T-helper cell maturity by measuring the TD antibody responses against SRBC and (c) evaluating neonatal potential for restoration of immunological impairment following zinc supplementation.

The effects of maternal dietary zinc deficiency (day 5-17 postpartum) demonstrated that the delayed growth observed in the zinc deficient neonate paralleled that in the restricted neonates. Although the impaired growth of the zinc deficient neonate appears to be caused by the accompanying inanition, the possibility remains that the restricted animals were partially zinc deficient. This concept is not unprecedented as human studies have demonstrated the interrelationship of malnutrition and zinc deficiency. Childhood malnutrition and clinical zinc deficiency share the common features of wasting, anorexia, and

stunting of growth; several reports suggest that children suffering from malnutrition are actually zinc deficient (82-86). This interrelationship of zinc deficiency and malnutrition suggests that anorexia in the present study may not have been solely responsible for the impaired growth of the Zn(-) neonates and that the contributions of zinc deficiency per se are important. In the study of zinc supplementation (repair), partial recovery of neonatal body weight was observed in both the zinc deficient and restricted neonates, but the effects were distinct: final body weights of the Zn(-) pups were significantly less than those of the R(+) pup ($P<0.05$). This further supports the premise that the effects of zinc deficiency, per se, were important factors in determining the acquisition and recovery of neonatal body weight.

It is generally established that the neonatal mouse is relatively immunoincompetent with regards to both humoral and cell-mediated reactions (51,63,70). Even though the lymphocytes present are approaching adult-like numbers and functionality, the expression of neonatal immune responses are depressed because of high levels of nonspecific immunosuppression (51,65,87); the presence of suppressor T cells thus limits the number of functional in vivo assays which can be utilized during the early neonatal period. Specifically, suppressor T cells can interfere with T-helper cell function (TD antibody responses) and can also negatively interfere with TI B cell function. Despite these problems, antibody responses to the TI antigens TNP-LPS and TNP-Ficoll and to the TD antigen SRBC are substantial in the suckling neonate and were thus chosen for the present study.

Antibody responses of the Zn(-) neonates against both TNP-LPS (TI-1) and TNP-Ficoll (TI-2) were similarly depressed from control values (47% control); R(+) pups displayed 60% of the normal PFC/spleen responses. This suggests that while the lowered TI responsiveness observed in the Zn(-) neonate may have been partially caused by the accompanying effects of inanition, zinc deficiency per se appears to have further reduced these responses. The depression of TI-1 and TI-2 responses implies impairment of both immature and relatively mature B cell subpopulations. This may reflect a disruption in the normal development of the repertoire of B cell clones. Further aberrations in neonatal B cell maturation resulting from zinc deficiency were evidenced by the depression of TI antibody avidities. This reduction suggested that B cells of the zinc deficient neonate were less mature than B cells of the restricted or control pups.

It has been established that the ability of the neonatal mouse to respond to TD antigens is generally acquired after the capacity to produce antibodies against TI antigens. These neonatal TD responses are generally low but can not be attributed to a quantitative deficiency in lymphocytes since the number of Thy-1 bearing T cells and immunoglobulin-bearing B cells are approaching adult levels by two weeks of age (88). Again, qualitative maturational changes may be required before adult-like responsiveness can be achieved. Specifically, adult-like SRBC antibody responses are not obtained until 4-8 weeks of age (51,70,74).

In the present study of SRBC responses (0 Days Repair), both the Zn(-) and R(+) neonates displayed reduced PFC/spleen responses. In the Zn(-) neonate, the IgM and IgG responses were 37% and 28% of

control, respectively, while the R(+) pups displayed 66% and 51% of the respective IgM and IgG responses. The effect of zinc deficiency seems to be more detrimental to neonatal TD antibody responsiveness than does the accompanying dietary restriction. Also, the reduction of IgG plaque formation was greater than that observed in IgM plaque formation suggesting that, against SRBC, T-helper cell function was more severely impaired by the previously imposed zinc deficiency than was B cell function; these results concur with those previously observed in the young adult and weaning mouse (35,40).

After a two week period of zinc supplementation, SRBC PFC responses of the formerly zinc deficient pups equalled those of the restricted and control pups. This loss and subsequent restoration of TD antibody mediated responses agrees with previous studies from our laboratory where mice made zinc deficient during post-weaning or adult life were able to fully recover antibody mediated responsiveness to SRBC within 2 weeks of dietary zinc repletion (35,40). Although the suckling neonates were able to recover TD antibody responsiveness, one can not be certain that other forms of immunological damage are equally reparable. Long-term immunological consequences of neonatal zinc deficiency have not been established.

In summary, this work suggests that maternal dietary zinc deficiency during lactation severely compromised neonatal growth, caused quantitative losses in neonatal lymphocyte numbers and qualitative impairment of lymphocyte functionality. Immunological impairment was evidenced by the reduced antibody responses to three antigens which, normally, are sequentially acquired during ontogeny. Specifically, antibody responses against TI-1 (TNP-LPS), TI-2 (TNP-Ficoll) and TD

(SRBC) antigens were all reduced in the Zn(-) neonate; in addition, TI antibody avidities were markedly reduced in the Zn(-) neonates. The impairment of neonatal TD responses resulting from zinc deficiency was not permanent and responsiveness could be restored within 2 weeks of dietary zinc supplementation. These results suggest a disruption in the normal maturation of B and T-helper cell function resulting from zinc deficiency; responsiveness, in part, is reparable upon zinc supplementation. However, the extent and long-term consequences of immunological damage incurred during neonatal zinc deficiency have not yet been characterized.

SUMMARY AND CONCLUSIONS

There is a considerable amount of evidence which indicated that conditions of zinc deficiency and/or malnutrition during the neonatal period are responsible for increased susceptibility to infection and disease, impaired growth and reduced immune capacity. The relationship between maternal dietary zinc deprivation and immunocompetence of the suckling neonate has not however been well established. Thus, the primary aim of this research was to characterize neonatal immune impairment resulting from maternal zinc deficiency during lactation.

Data from the first experiment established that 1.6 μg/g maternal dietary zinc was sufficient to induce a zinc deficient state in the neonate with reduced growth, a reduction in lymphoid organ weight and altered in vitro mitogenic responsiveness. The reduction in ConA, PWM and LPS mitogenic responses suggested that zinc deficiency may adversely effect both B and T cell subpopulations of the neonate.

Results of the second study established that the combined effects of maternal dietary zinc deficiency and the accompanying anorexia were responsible for reduced neonatal growth and the atrophy of lymphoid organs. Two weeks of subsequent zinc supplementation allowed the body weights of both the zinc deficient and restricted pups to return to near normal. Final body weights of the deficient neonates, however, were significantly lower than those of the restricted pups suggesting that the effects of zinc deficiency per se were important factors in determining the recovery of neonatal body weight.

Antibody responses against TNP-LPS (a TI-1 antigen), TNP-Ficoll (a TI-2 antigen) and SRBC (a TD antigen) are normally acquired in that sequence during ontogeny. In these studies, PFC/spleen responses against all three antigens were depressed in the Zn(-) neonate while the PFC/10^6 lymphocyte response did not differ from control. This implies that the reduced number of splenocytes in the Zn(-) neonate caused a proportional loss in overall responsiveness (PFC/spleen). However, a different subpopulation of lymphocytes is stimulated by each of the three antigens and the reduction of all three antibody responses may represent a disruption in the normal ontogenesis of humoral immunity.

In the zinc deficient neonate, antibody avidities against both TNP-LPS and TNP-Ficoll were markedly lower than those of the control or restricted mouse. Since increases in avidity correlate with increased maturity of B cells, this data suggests that zinc deficiency suspended the maturation of TI-responsive B cells in the neonate.

Results of the repair study indicated that the zinc deficient neonate is capable of a marked recovery of body weight and full restoration of TD antibody responsiveness following dietary zinc supplementation. This suggests that immunological impairment caused by zinc deficiency was reversible in the neonatal animal.

The results of this research indicated that maternal dietary zinc deficiency during lactation adversely affected the growth and immunocompetence of the suckling neonate. Both qualitative and quantitative changes in lymphocyte subpopulations were observed implying that normal immunological ontogenesis was altered by zinc deficiency. Neonatal immune function was not irreversibly impaired as evidenced by the

restoration of TD antibody responsiveness upon zinc supplementation. This research has demonstrated the importance of zinc to the suckling neonate and suggests the need for further study examining the role of zinc during immunological development.

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