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thesis entitled

The Effects of a Marginal Deficiency
Of Zinc In Utero on Growth and
Immune Development in the Mouse: A Possible
Model for Normal Term Low Birth-Weight Infants

presented by

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has been accepted towards fulfillment
of the requirements for

M.S. degree in Biochemistry

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Date 11/15/89

THE EFFECTS OF A MARGINAL DEFICIENCY OF ZINC *IN UTERO* ON GROWTH AND IMMUNE DEVELOPMENT IN THE MOUSE: A POSSIBLE MODEL FOR NORMAL TERM LOW BIRTH-WEIGHT INFANTS

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A THESIS

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

Master of Science

Department of Biochemistry
1988

ABSTRACT

THE EFFECTS OF A MARGINAL DEFICIENCY
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MODEL FOR NORMAL TERM LOW BIRTH-WEIGHT INFANTS

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A marginal dietary deficiency of zinc during gestation in mice produced asymmetrically growth-retarded offspring with significant alterations in immunocompetence. A/J pregnant mice were fed a biotin-supplemented egg white diet containing 3.5 μg Zn/g diet from day 0-17 of gestation, after which they received zinc-sufficient diet. A transient deficiency of zinc was generated in the fetuses from day 15-18 which affected birth-weight, resulting in asymmetrically growth-retarded offspring. These neonates had normal survival rates, yet significant reductions in in vivo response to a T cell independent class I antigen which persisted at least until puberty. Phenotypic analysis demonstrated a reduction in T helper and B cells in the spleen of these neonates. No differences in cell-mediated immunity were evident as determined by mitogenic and MLC response. Therefore, a marginal deficiency of zinc in utero created impairments in growth and immune function which model closely the pathology of low birth-weight infants.

ACKNOWLEDGMENTS

I am deeply indepted to many people during my stay here at MSU, foremost amoung whom is Dr. Pamela J. Fraker. Her patience and understanding allowed me to develop both as a person and a scientist. I was challenged personally during the last three years, throughout which she supported me more than could ever be asked of just a boss or even collegue. I am proud to count her as one of my close friends. Thank you for all you've done.

I would also like to express my warmest thanks to all the members of the Fraker Lab who suffered through many hard times with me and shared in my joy: to Gerry Morford for his spiritual support and help, I have become a better Christian because of his efforts; to Dr. Louis King for all his help in so many different ways; and to Carmen Medina-Reyes, Joan Cook-Mills, Andrea Cress, Beth Garvy, Sue Leak, and Kara McCarty for things too numerous to mention. Additional thanks go to Lori Wallrath, Kevin Salyers, Randy Shoemaker, Dr. John Wang, and Dr. Dave McConnel for their help and support through the years. Extra extra special thanks goes to Lorri Morford, my good friend and companion in the mouse room, without whom I could not have done any of the experiments contained in this thesis.

I am also grateful for all of the help and support my family and friends have provided, I could not have done it without them; especially: my dad and grandparents, Kathy, Chris, and Mike Gibbons, Ron and Andrea Hartson, Don Motte, and all the members of the Hartson family. This thesis is dedicated to them and to my mother, Janice Elaine Gibbons, who in life taught me how to love and live, and in death taught me that life is too short not to. Thank you.

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Introduction

In the past century there has been a progressive decline in the infant mortality rate in the developed world, primarily due to advances in the prevention and treatment of infectious diseases (1). As the overall infant mortality rate has fallen, mortality of the low birth-weight (LBW) infant has increased in percentage to become the primary determinant of infant mortality. In the United States during the 1980's, LBW infants represented 7% of all births (2), and accounted for two thirds of all neonatal deaths (those occurring in the first 27 days postpartum) (1,3). These infants were 40 times more likely to die in the neonatal period than those of normal weight, 5 times more likely to die in the first year, and accounted for 20% of all postneonatal deaths (3). In underdeveloped countries with high population growth rates such as India and Bangladesh, the percentage of LBW infants among the total births is dramatically higher, being 30 and 50% respectively between 1979-83 (2).

Despite these grim statistics, little is known about the cause of LBW. One area which may help our understanding is in deficiencies of the trace metal zinc during gestation. Studies in animal models have firmly established that gestational deficiencies of zinc can indeed produce low birth-weight offspring (4). In addition, these studies have generated intriguing data which suggest that deficiencies of zinc during pregnancy can have an even more profound effect on the developing immune system (4,5). This correlates well with observations in humans indicating that low birth-weight infants are

much more likely to suffer recurrent infections and to die from common childhood diseases. Unfortunately, the studies to date have focused on rather severe deficiencies which produced overt signs of zinc deficiency in the pregnant dams and rather high offspring mortality. Therefore, even though growth-retarded offspring were produced with drastic reductions in immunocompetence, the relevance of this system as a model for low birth-weight infants must be questioned.

To better model the nutritional condition likely to be present in humans, especially in the U.S., a marginal level of zinc deficiency was developed in the mouse which produced near normal survival of the offspring, yet significant impairments in _in vivo_ B cell responses during gestation (6). The focus of this thesis was to further examine the immunological consequences of this marginal deficiency of zinc during _in utero_ development in the mouse. The first phase was to identify the precise timing of the deficiency of zinc actually incurred by the fetuses, as well as to better characterize the early state of immunocompetence in the neonatal animals (Chapter 1). Once the period of fetal zinc deficiency was determined, it was then possible to evaluate the state of the developing immune system gestationally in order to determine whether a specific block in ontogeny had occurred (Chapter 2).

To study the neonatal immunological manifestations of a marginal deficiency of zinc during pregnancy, it is necessary to be familiar with a broad range of disciplines such as nutrition, immunology, developmental biology, and biochemistry. To be complete, literature

reviews concerning general nutritional influences and other possible contributors to the incidence of low birth-weight infants have been included in this thesis, as well as the specific influences of zinc. Much of the human literature is conflicting, underscoring the importance of controlled animal studies to delineate the role of zinc gestationally in general development and more importantly, immune ontogeny. Reviews of the major areas of ontogeny and some other fetal-neonatal immunological topics have also been included in an appendix to further aid the reader in understanding the complexity of the system. It is hoped by the author that the role of zinc in intrauterine growth retardation and the acquisition of immunocompetence will become clearer, demonstrating the necessity of further research on this world-wide health problem.

Literature Review

Definitions of low birth-weight

Originally any human infant weighing 2500 grams (5.5 lbs) or less was considered to be low birth-weight (LBW). It was realized however, that premature, or more correctly preterm infants were not really of low birth-weight when their gestational age was taken into account, and therefore should not be considered as LBW infants. The definition was subsequently modified to include only those infants who were less than or equal to 2500 grams after 37 weeks of gestation. After further review, it became clear that the mechanism of determining birth-weight was much more complex, since children from different environments have different "normal" birth-weights. Therefore, it was recommended that diagnosis of low birth-weight be determined by comparison to birth-weight charts based on the local population into which the infant was born. If weight was below the tenth percentile, the infant should be considered LBW, or alternatively to be suffering from intrauterine growth retardation (IUGR) (7).

In practice however, LBW is still a catch-all term in the literature used for any infant below 2500 grams, regardless of gestational age. It is necessary then to examine further whether the subjects declared LBW are preterm (born prior to the 37 week of gestation) or full term but growth-retarded. This is very important since the pathology exhibited by these two groups is quite different. Preterm infants are "immature" in the development of many

of their organ systems including the immune system. They become deficient in several nutrients after birth due to immaturity of their digestive systems, and consequently have numerous pathological problems similar to a severely malnourished infant. The full term, or normal term, low birth-weight infant (NTLBW) is growth-retarded in some manner resulting in specific pathological abnormalities such as congenital malformations and respiratory diseases. One other term often used in the literature that is confusing is small-for-gestational age or SGA. This usually means NTLBW in practice, but by definition it could refer to preterm infants which are also growth-retarded.

Many professionals in the field of perinatology have recognized that there are two main types of IUGR: symmetric and asymmetric (7). The symmetric type is characterized by a decrease in linear growth believed to be initiated early in pregnancy. These infants rarely exhibit catch-up growth in childhood and adolescence. The asymmetric type of IUGR is by far the most common and least severe. It is characterized by normal body size, but decreases in body fat, muscle, and some visceral organs such as the liver, spleen, and thymus, giving the appearance of malnourishment. It is believed to have a late onset in pregnancy, and infants with this form show marked catch-up growth later in life.

Consideration of these forms is relevant to this thesis in that this represents one of the fundamental differences between the previous studies with animal models and the work presented here. As will be seen, the previous work generated offspring with a

symmetric form of IUGR, whereas this thesis will demonstrate that the marginal level of zinc utilized here resulted in offspring with an asymmetric IUGR, modelling more closely the most common form of IUGR in humans.

To summarize then: NTLBW infants are infants born after the normal 37-42 weeks gestation weighing 2500 grams (5.5 lbs) or less. The term small-for-gestational age is used loosely in the literature to describe both NTLBW and preterm infants even though these infants are different in most aspects. Additionally, there are two types of IUGR: symmetric and asymmetric, characterized by either decreases in body length and overall body size, or by normal length but decreases in total body mass, respectively. Each type has a different time of onset and long-term manifestation in the infant, and both forms can occur in either preterm or normal term infants. Therefore, in the determination of the low birth-weight infant, both birth-weight and body size must be considered, as well as comparison to the proper gestationally aged standard. In most studies this has not been the case. Consequently, predictions on the incidence of IUGR are in many cases subject to skepticism.

Correlates of low birth-weight

As indicated above, the incidence of low birth-weight (LBW) was approximately 7% of all births in the U.S. for 1979-83 (2). More recent statistics have indicated an upward trend suggesting the rate of LBW is now around 10% (unconfirmed). The problem becomes more severe if ethnicity of the mother is taken into account, with

the incidence of LBW being much greater in blacks. In the 1970's the LBW rate of blacks in the U.S. was 12.5% compared to 5.7% for caucasians (1). It has recently been reported that this rate has increased to close to 20% in blacks through the 1980's (unconfirmed).

Since LBW occurs more frequently in underdeveloped countries, many factors involved in their generation are likely to relate to the socioeconomic status of the mother. There have been several reports listing a large number of factors which are associated with an increased risk of having a LBW infant (7,1). These include income level, education, race, smoking, alcohol consumption, and others. One of the most controversial correlate to LBW is the age of the mother (reviewed in 8). It had been long accepted that women having their first child who are less than 18 or greater than 35 years old are at higher risk of having a LBW baby (1,7,9,10). For the adolescent it was believed that a competition for nutrients might exist between the potentially still growing mother and the rapidly growing fetus (11). Recently however, there have been more controlled studies indicating that differences in birth-weight were due not to age of the mother, but to other complicating factors such as race, education level, quality of diet, and the psychological state of the adolescent mother (12). It will be difficult to determine conclusively whether age of the mother is an independent factor since there are between 30 and 40 factors which may work in synergy. These factors make it extremely difficult to properly control human studies. Certainly teenage pregnancies contribute a larger than average share of LBW infants whether due to age or

other factors, representing a serious health problem which needs to be addressed in order to realistically reduce the incidence of LBW.

Placental malfunction

Whatever the indicator of intrauterine growth retardation (IUGR) might be, the impairment in growth implies a decrease in nutrient delivery or utilization during gestation. An area which has received increasing attention is the vascularization of the placenta and uterus. Any disruption of the flow of nutrients via the lifeline of the fetus could potentially lead to IUGR. There have been a plethora of reports suggesting that a reduction in the vascularization of the placenta is associated with IUGR (13-18, reviewed in 19).

The placenta is composed of fetal tissue derived from cells in the early blastocyte stage. These cells penetrate the wall of the uterus resulting in the interaction of fetal tissue and maternal blood vessels directly. The fetal trophoblast invades the uterine endometrium deeply, proceeding all the way to the spiral arteries of the uterus. There the trophoblast displaces the epithelial cells lining the maternal blood vessels, initiating a complex series of changes in the spiral arteries of the decidua and the underlining myometrium, ultimately resulting in the conversion of these arteries to uteroplacental arteries. The uteroplacental arteries are morphologically distinct in that they are devoid of muscular and elastic tissue, being replaced by fibrous tissue. The lumen of the vessel is dilated resulting in a large flow of blood into the

intervillous spaces of the placenta, to be collected by the fetus and returned to the maternal circulation via maternal veins which have undergone similar changes. The disruption of the integrity of the spiral arteries and their resultant conversion to uteroplacental arteries routinely have been referred to in the literature as "physiological changes" (20). If for some reason these physiological changes do not take place or are incomplete, blood flow into the intervillous space will be decreased, potentially resulting in fetal complications.

Two conditions which are known to contribute to IUGR are pre-eclampsia and pregnancy induced hypertension, both of which have been correlated to defects in the conversion of the spiral arteries to uteroplacental arteries (13-18). Khong et al. (18) demonstrated that mothers suffering from pre-eclampsia or hypertension may have incompletely changed spiral arteries, as well as a reduction in the number of physiological changes. In pre-eclampsia and some pregnancies resulting in small for gestational age (SGA) infants, the physiological changes were restricted to the decidual segments of the spiral artery. As previously mentioned, physiological change usually occurs over the entire length of the spiral artery, from the decidua to the myometrium. It's believed the trophoblast invades these two areas at different times during gestation (17). The first wave occurs in the decidua and is completed early in gestation (by 10 weeks in humans), while the second wave of invasion occurs at 16-18 weeks and is involved with the rest of the spiral artery in the myometrium. During pre-eclampsia, this second wave apparently

is impaired in some cases resulting in decreased flow of blood and possibly IUGR.

In addition to this incomplete conversion of the spiral artery, the complete absence of conversion was observed in some pre-eclamptic mothers and in pregnancies resulting in SGA infants (18). Nearly half of the arteries examined in these mothers were unaffected by the trophoblast and remained as they were in nonpregnant conditions. The authors were wisely reluctant to propose that a reduction in the number of uteroplacental arteries and/or incomplete physiological changes in some arteries will cause IUGR and pre-eclampsia. In their study instances of pre-eclampsia and SGA occurred in different cases. Also, there were cases of SGA without either defect present (i.e. normal physiological changes had occurred). Thus, in some pregnancies there appears to be a defect in the interaction of the invasive trophoblast with maternal tissues resulting in poorly developed placentation, decreased nutrient delivery to the fetus, and perhaps growth impairment. Additional evidence supporting this theory was the finding of intraluminal cytotrophoblast in the uteroplacental arteries in the third trimester of pregnancies with SGA and pre-eclampsia (18). This was considered to be highly unusual by the authors and was believed to have resulted from increased resistance to the movement of the trophoblast in the maternal tissue, or decreased mobility of the trophoblast, or both.

One problem inherent with these studies is that they were performed on tissue removed after caesarean (C) section. Often times the C section is indicated by some complicating factor during the

pregnancy and results in the forced birth of the infant. Therefore, there is a possibility that C section infants do not represent the product of a completely normal pregnancy. In any event, it seems likely that impaired placental transfer of blood and nutrients often results in pregnancies with a higher risk for IUGR than in normal pregnancies. The cause of the impaired spiral artery conversion however, remains an open question.

Whether deficiencies of zinc or other nutrients during gestation decrease the vascularization of the placental unit have not been examined. However, it seems unlikely that the growth retardation and immune problems associated with the offspring of marginally zinc deficient dams in this thesis would be due solely to a reduction in nutrient transfer to the fetus. As will be seen, there was a transient depression of zinc content in the fetuses which was quickly repaired with zinc replenishment in the diet of the dam. This suggests that placental transfer of nutrients was intact in this system.

General nutrition and intrauterine growth retardation

Since fetal growth is dependent in part on the amount of nutrients made available to the fetus, an attractive hypothesis for intrauterine growth retardation (IUGR) has been maternal undernutrition. Unfortunately no clear conclusions can be made from the human studies to date. It is impossible to completely assess the nutrient intake of a large number of subjects over the entire length of gestation. Thus, most studies have concentrated on

the effects of supplementation of various nutrients on fetal growth as measured predominantly by birth-weight (21-26). Studies in humans have been marred by difficulties in controlling for the other extrinsic factors which are believed to affect birth-weight (1), and with problems insuring adequate compliance with the supplementation regime.

In the 1970's most efforts were focused on examining the effects of protein supplementation in women considered to be at risk for undernutrition (reviewed in 22). It was believed at the time that deficiencies of protein were the primary cause of nutritionally derived IUGR, especially in women of low socioeconomic status. In a study of low income black women from New York City, protein supplementation had little effect on birth-weight when averaged for all of the subjects. Interestingly, there was an increase in the percentage of preterm infants born in the supplemented group, as well as an increase in neonatal mortality. Thus, for some of the women, the high protein (40 grams/day) supplement had an adverse effect on pregnancy outcome. Further analysis revealed that women in the supplementation group who had previously given birth to a low birth-weight (LBW) infant were most likely to be affected adversely by the increased protein. Women who were heavy smokers benefitted the most from the high protein supplement. Adverse effects of protein supplementation have been noted in other studies as well (28,29,30). Thus, some assessment of the existing nutritional status must be made to determine whether supplementation is in order. Further studies in other areas have shown that a more

balanced supplement of nutrients had a positive influence on birth-weight,resulting in increases of 200-300 grams (21-26). It is believed that increases of this magnitude are enough to have a positive effect on the number of LBW infants and a decrease in the neonatal mortality and morbidity rates.

The importance of general nutritional assessment of the pregnant mother is evident when critically reviewing the literature on adolescent pregnancies. As indicated above, there is no consensus on whether age is a definitive factor for the incidence of LBW (8). The main argument of adolescent pregnancies being at risk is a nutritional one: a competition may occur between the mother and fetus if the mother is still growing herself (11). In a study of 412 adolescent mothers age 12-15 years old, those classified as still growing based on comparison of their height with their mothers had significantly smaller infants than those deemed to have completed their growth (31). This is in contrast with others (12) who argued that menarche occurred after peak height velocity had been reached, and therefore the adolescent mother could not be in a high stage of growth. More accurate measurements of maternal growth and nutritional status are needed to reconcile these differences in observations. It is interesting to note that pregnant adolescents which gained the same amount of weight as older pregnant women during gestation were more likely to have LBW infants. Those adolescents who gained more than the control mothers had infants with normal birth-weights (10). Thus, for growing adolescent mothers

there seems to be a higher requirement for nutrients which may predispose them to incidence of IUGR.

Zinc in human intrauterine growth retardation

Aside from the general maintenance of proper nutrition during pregnancy, specific deficiencies of nutrients, particularly zinc, have received a great deal of attention in relation to low birthweight (LBW). Initially, studies in Egypt and Iran demonstrated that zinc deficiency resulted in impaired growth and development of children and adolescents. It was concluded that diets consisting mainly of plant products which were known to contain high amounts of the chelator phytic acid resulted in inadequate zinc intake and impairments in growth. These impairments were easily and quickly repairable with zinc supplementation, prompting nutritionists to try to determine the specific pathology(s) associated with zinc deficiency. There have been several reviews on general zinc deficiencies (32) and the immunological consequences (33) to which the reader is referred for further information.

Since postnatal deficiencies of zinc resulted in growth impairments, it was of interest to determine whether a deficiency of zinc during gestational growth would have the same impact. Severe deficiencies of zinc during pregnancy in animal models resulted in gross congenital malformations and fetal death (34,35). The disease acrodermatitis enteropathica has provided a natural model in which to study the effects of a severe deficiency of zinc during pregnancy in humans. This disease reduces the uptake of

zinc in the small intestine. Due to advances in treatment of acrodermatitis enteropathica, women with the disorder have succeeded in becoming pregnant. Occasionally the disease is exacerbated by the pregnancy, resulting in poor fetal outcome. Out of the seven pregnancies reported in (36), one abortion and two major congenital malformations occurred. This suggested that severe deficiencies of zinc during pregnancy in humans can also result in malformations.

Aside from the small number of cases of acrodermatitis enteropathica reported above, severe deficiencies of zinc during pregnancy are relatively rare in humans. In the U.S. marginal dietary deficiencies of zinc are likely to be more frequent than severe deficiencies. It has been shown that serum levels of zinc decline throughout gestation in normal pregnancies. Coupled with the observation that the average zinc intake in pregnant women in the U.S. is considerably less than the recommended daily allowance (37), it has been proposed that marginal deficiencies may exist quite commonly (37,38). It has been difficult to demonstrate however, that a marginal deficiency during gestation has a negative effect on fetal outcome in humans (see 39 for review).

There have been several studies reporting an association of low maternal zinc status and poor pregnancy outcome. Congenital malformations (40,41,42), anencephaly (43), and spina bifida (44) have all been documented to be associated with low maternal serum zinc during variable periods of gestation. These associations are not enough to determine causality and oftentimes are found in a

very small portion of the total population sampled. Marginal deficiencies of zinc have also been found to be associated with some cases of pregnancy-induced-hypertension and pre-eclampsia (45).

In an attempt to find a more sensitive measure of zinc status, some researchers have used maternal leukocyte zinc content as a predictor of fetal growth (48,49,50). In each of these studies, decreases in zinc content of the leukocytes were associated with intrauterine growth retardation (IUGR). However, caution must be used when interpreting these findings until the accuracy of leukocyte zinc assessment is validated (39). Others have shown this measure to be insensitive to changes in zinc status (51,52,53).

Paradoxically, increases in maternal plasma zinc levels have also correlated with incidence of IUGR (41,47,54,55). In each of these studies a negative or inverse correlation was found between zinc status and birth-weight. The authors postulated that altered zinc metabolism somehow affected placental development resulting in poor maternal-fetal transfer of nutrients.

There is a considerable amount of literature which indicates that there is no correlation between the incidence of IUGR and zinc status of the mother (56,57). In supplementation studies with zinc, very little to no effect on birth-weight has been shown (37,46,58,59), although reproductive performance was enhanced in a few of these studies (37,59,60). As indicated previously, supplementation studies are difficult to properly control and therefore are subject to skepticism. The efficacy of supplementation with zinc as general obstetric practice has been challenged recently

(61). A major concern is the effect of increased zinc on absorbtion of other metals, particularly copper (56). Just as high amounts of iron can impair zinc absorbtion (62), so too may zinc impair copper uptake.

To summarize, no clear picture exists for the effect of marginal deficiencies of zinc during pregnancy in humans. There have been many correlational studies which seem to indicate some association of maternal zinc status and IUGR, but more work needs to be done to elucidate the mechanism by which zinc affects pregnancy outcome. Fortunately, studies in animal models have produced more concrete results.

Zinc in animal studies

Extensive investigations have been performed on the rat demonstrating that a deficiency of zinc during pregnancy was teratogenic to the fetus (34,35,63-67). The type of defects produced in the fetuses were dependent on the timing of the deficiency during gestation. When a zinc deficient diet was fed to pregnant rats from conception to birth, 90% of the fetuses showed gross congenital malformations (34). When shorter periods of deficiency were used at different times during gestation, fewer numbers of offspring had malformations. The specific defects generated in the offspring were dependent on the developmental events at the time of the deficiency (34). For example, deprivation during the early stages of development resulted in significant malformations of the brain (34), while deficiencies during the last trimester resulted in

reductions of organ sizes, with little effect on the brain (68). It was concluded from these studies that even transitory periods of suboptimal zinc intake during gestation resulted in severe defects in the offspring not repairable with subsequent repletion of zinc.

Studies utilizing somewhat more moderate deficiency of zinc during pregnancy in the mouse resulted in decreases in litter sizes, significant growth retardation as evidenced by lower body weights and lengths at birth, and increased mortality in the offspring of zinc deprived dams (4). A so called moderate deficiency of zinc (MOD) was generated in outbred Swiss Webster mice by feeding a 5 ppm zinc diet from day 7 of gestation to birth, after which the mice were returned to control diet for the rest of the study. The authors felt that a deficiency during the critical stages of initial embryogenesis and implantation would result in malformations and fetal death as had occurred in previous studies with more severe deficiencies of zinc (34). Significant reductions in specific tissue sizes were also reported, with the spleen and thymus being most affected. Zinc repletion at parturition resulted in catch-up growth in these growth-retarded offspring by 6 to 8 weeks of age, including lymphoid organs returning to normal size. In contrast to the repair in tissue sizes and body weight, quite different results were obtained when immune function was determined in these offspring.

Severe alterations in immunocompetence as measured by antibody mediated responses and serum Ig levels were generated in the MOD offspring (5,69) (see below for explanation of immunological terminology). The offspring were assayed at 6 and 10 weeks of age,

as well as at 6 months to determine the persistence of the impairments. At 6 weeks of age, MOD offspring had severe reductions in serum levels of IgM, IgA, and IgG_{2a}. These persisted through 10 weeks of age, but were normal by 6 months except for IgM, which still was depressed as compared to control offspring. Since zinc deficiency has been shown to impair the development of the mammary glands (70), it was possible that alterations in immunocompetence were the result of impaired transport of nutrients in the dams' milk postnatally as opposed to the gestational deprivation of zinc directly affecting the fetuses. To control for this, cross-fostering (CF) of the MOD offspring to control dams was done. In the MOD offspring CF to control dams at birth, reductions in IgM were slightly less than in non-CF MOD offspring at 6 and 10 weeks, while levels of IgA and IgG_{2a} remained depressed. Antibody mediated responses, as determined by immunization with the T cell dependent antigen sheep red blood cells and assaying for plaque forming cells, were significantly lower in MOD offspring whether cross-fostered or not at all time points tested. The authors concluded that a moderate deficiency of zinc during gestation was largely responsible for the persistent impairments in immune response.

These studies were a good first step in the identification of possible roles of zinc during development. However, they were not very representative of the likely condition existing in humans in the U.S. Notable problems include the lack of weight gain in the dams during the first 17 days of gestation, the drastic reduction of food consumption, and the rather dramatic elevation in offspring

mortality at birth (near 50%). In addition, the surviving offspring suffered from a symmetric form of growth retardation, the least common form of intrauterine growth retardation in humans (7, see above). The value of these studies as a model for LBW in this country must therefore be questioned.

In an effort to better model human marginal deficiencies of zinc, subsequent studies from this group have focused on the rhesus monkey (71,72,73,93). A so called marginally zinc deficient diet was fed to pregnant females from conception through lactation, and to the offspring for an additional 6 months after lactation (through one year of age). Stillbirths, abortions, and delivery complications were more frequent in the deficient and pair fed females (74). Offspring of deficient females were more likely to be of low birth-weight, particularly male offspring. The increased sensitivity of male offspring has been reported elsewhere (75), and is believed to be related to their generally greater growth rates and differences in their body composition (i.e. males tend to be more lean). As in the murine study, low birth-weight was observed in conjunction with decreased length of the neonate and thereby represented the symmetrical form of intrauterine growth retardation. Behavioral abnormalities such as decreased sociability and exploration were also noted in the offspring in this study, as well as in a similar study with rhesus monkeys utilizing a severe deficiency in the diet during the last trimester of pregnancy (76). Interestingly, serum zinc status of the mothers was inversely correlated with birth-

weight in the zinc deficient group. This corroborates observations in some human studies (41,47,54,55).

Immunologically, the marginal deprivation of zinc during gestation resulted in reduced responsiveness to T cell mitogens but normal serum Ig profiles in the pregnant females (71). As stated earlier, the offspring were kept on the marginal diet through the first 12 months of postnatal life. Moderate reductions in their responsiveness to T cell mitogens concanavalin A (Con A), phytohemagglutinin (PHA-P), and pokeweed mitogen (PWM) were evident throughout the first year in the offspring. Serum IgM levels were differentially affected through the first year in these zinc deficient offspring, with increased levels of IgM at 3 and 5 months of age, and decreased levels at 7, 9, and 12 months off age as compared to control offspring levels. Serum IgA and IgG levels were unaffected in the deficient offspring.

Due to the dietary protocol used in the primate studies, with the offspring of zinc deficient females receiving the marginally zinc deficient diet after birth, it is difficult to interpret these immunological findings. It is clear however that the level of zinc fed to the females during gestation was still too severe, since obvious signs of zinc deficiency such as dermatitis and anorexia were present during gestation (71). An additional problem with this model is that fundamental immunological assays are not available to thoroughly evaluate immunocompetence and immune ontogeny. To precisely identify the role zinc plays in growth and especially immune development during gestation, it was necessary to develop an

animal model which was easier to work with, for which a number of assays were available to evaluate immune ontogeny, yet still was comparable to humans immunologically. The mouse has proven to be a reliable immunological model for humans and was therefore chosen to develop a truly marginal model of zinc deficiency during gestation.

The initial goal was to define a level of zinc in the diet which produced modest weight loss in the dams, near normal survival of the offspring, yet a significant impairment in their immune function. It was established that a marginal deficiency of zinc (MZD) during pregnancy in the mouse could indeed be generated which satisfied each of these criteria (6). Immunological defects in B cell development were demonstrated in day 18 fetuses by assessing the ability of fetal liver cells to produce antibodies to a specific antigen. This was accomplished by adoptively transferring day 18 fetal liver cells to irradiated adult mice and assaying for IgM plaque response to the T cell independent antigen trinitrophenyl-lipopolysaccharide. Recipients of fetal liver cells from MZD fetuses were able to produce only half of the plaques present in recipients of cells from control fetuses. This suggested a defect in B cell ontogeny, however the neonatal consequences of this defect remained unaddressed, as well as the effect marginal zinc deficiency may have had on other areas of the immune system. This thesis presents the subsequent characterization of this marginal system. The early neonatal immunological responses were assessed in addition to further evaluating the possible defect in the developing B cell. Before proceeding though, it is necessary to briefly review some of the

more practical aspects of immunological research during both pre- and postnatal development. Each of these concepts will be directly related to the subsequent studies presented in this thesis. More extensive reviews of immune ontogeny are presented in the appendix for additional information.

Murine Fetal/Neonatal Immunology

The major site of lymphopoiesis during gestation is the fetal liver (77). T cell precursors migrate to the thymus at around day 11 of gestation in the mouse (78), while B cells remain in the liver until around birth (the mouse has a 19-20 day gestational period). B cell development proceeds in discrete stages often times identifiable by the acquisition of specific surface molecules. The immature B cell acquires the surface molecule B220 at around day 12 of gestation, followed shortly thereafter by the production of cytoplasmic μ chains. The μ chain is the heavy chain component of IgM, the first immunoglobulin expressed by all B cells. Surface IgM, which must be acquired prior to antibody production, does not appear on the B cell until day 17. Therefore, evaluation of antibody production in the fetal liver can not be done prior to this time. At some time around birth, the B cell migrates out of the fetal liver to the bone marrow, the ultimate site of lymphopoiesis in the adult, and the spleen. Relatively mature B cells can be found in the spleen at around birth, with a steady increase in these cells occurring over the subsequent weeks (79).

Even though mature B cells can be found in the spleen shortly after birth, evaluation of their function is complicated by a state of immune suppression in the neonate. This immune suppression, which occurs in both mice and humans, is relatively nonspecific and lasts in the neonatal mouse until approximately four weeks of age (80). The mediator(s) of this have been reported to be of both a cellular and soluble nature. The practical ramifications of this are that T cell dependent antibody production cannot be assessed until close to puberty. However, there are antigens which act in a T cell independent manner which can be utilized to test for B cell function shortly after birth. For further information on both B and T cell ontogeny, as well as other fetal/neonatal immunological topics, the reader is referred to the appendix.

Chapter 1. The effects of a marginal deficiency of zinc *in utero* on growth and antibody mediated responses

Abstract

An improved model for studying the immunodeficiencies associated with normal term low birth-weight infants has been generated in the mouse by a marginal deficiency of zinc during gestation. A/J mice received diet containing 3.5 ug Zn/g diet (MZD), or 30 ug Zn/g diet ad libitum (ZA) or in restricted amounts (RES) from day 0-17 of gestation. MZD litter sizes were 68% of ZA litters, yet these offspring had normal survival rates. MZD dams and fetuses had transient reductions of zinc which returned to normal by birth after zinc replenishment on day 17. MZD fetuses had normal body lengths but significantly lower birth-weights (74% of ZA) indicating an asymmetric form intrauterine growth retardation. At day 13 and at puberty, MZD offspring exhibited a 40% reduction in response to a T-independent antigen. Therefore, a marginal deficiency of zinc *in utero* resulted in low birth-weight offspring with immunodeficiencies not readily repairable by zinc repletion.

Key words Marginal zinc deficiency, gestation, low birth-weight, zinc kinetics, antibody mediated responses, B cells

Introduction

A marginal nutritional environment during prenatal development is thought to be responsible for the generation of a portion of normal term low birth-weight infants (NTLBW) (10,81). These children frequently have recurrent infections and a higher rate of mortality to ordinary childhood diseases (82), indications of a compromised immune system. Since zinc has been shown to be important for growth (83) and immune function (33), a marginal deficiency of zinc during pregnancy may be a contributing factor to the incidence of NTLBW infants. Suboptimal intake of zinc is possible among pregnant women in the U.S since their requirement for zinc may be higher, and their daily intake has been shown to be considerably less than two thirds the recommended daily allowance in some cases (37,38). Although several studies have indicated an association between maternal zinc status and pregnancy outcome (40,44), it remains controversial whether deficiencies of zinc during pregnancy in humans result in low birth-weight infants (39). Nevertheless, deficiencies of zinc in animal models such as the mouse (4,6) and rhesus monkey (74) have resulted in growth impaired offspring.

It was of interest to determine whether a marginal deficiency of zinc in utero could generate in an animal model immunodeficiencies which were similar to those found in the NTLBW infant. The mouse was chosen as a model due to its immunological similarity to humankind. Previous studies indicated that zinc deficiency during pregnancy resulted in smaller litter sizes, decreased postnatal survival, growth retardation, and decreases in offspring spleen and

thymus size in mice (4). Alterations in serum IgM, IgG_{2a}, and IgA levels were observed at six weeks of age despite zinc repletion beginning at birth (5,69). Reductions in antibody mediated responses to a T cell dependent antigen were evident in deficient offspring, as well as in their second and third filial generations. Even though the offspring of these deficient dams showed no sign of the congenital malformations often encountered in studies with severe zinc deprivation during pregnancy (34), the level of dietary zinc used was sufficiently suboptimal to cause nearly 50% mortality in the offspring at birth. Additionally, depressions in serum zinc status and weight of the surviving neonates were evident for at least 10 days after birth (4). It should be noted that immune impairments have also been demonstrated in the F_1 and F_2 offspring of calorically restricted rats during gestation as well (84).

Recently, a marginal level of dietary zinc for pregnant mice has been defined which produced modest decreases in dam weight gain and litter size, yet provided for near normal survival of the offspring (6). To test immune function in these offspring, day 18 fetal liver cells were adoptively transferred to normal irradiated hosts and challenged with a T cell independent antigen. Recipients which received liver cells from fetuses of marginally deficient dams produced only 50% as many antibody producing cells per spleen as those receiving control liver cells (6). This strongly suggested that B cell development was altered by the marginal deficiency of zinc during pregnancy.

This paper further characterizes the nutritional aspects of a marginal deficiency of zinc during pregnancy on the kinetics of zinc replenishment, weight gains, and the development of antibody mediated responses in the offspring postpartum. It is shown that indeed a marginal deficiency of zinc during development can generate NTLBW offspring in the mouse with significant and persistent impairments in immune responses. This system may then serve as a more useful model for determining possible immunodeficiencies encountered by NTLBW human infants.

Methods

Breeding Two weeks prior to breeding, 11-14 week old nulliparous A/J female mice (Jackson Lab, Bar Harbor, ME) weighing 18-25 g were housed five per cage and adjusted to a biotin-fortified egg white diet containing 30 ug Zn/g diet, the composition of which is shown in Table 1. Five days prior to breeding, females were housed two per cage to promote synchronization of their estrous cycles (app. 5-7 days)(85). Males were introduced at 1800 hrs. For three consecutive days at 0900 hrs. the females were checked for the presence of vaginal plugs, the presence of which was considered to mark day 0 of a 19-20 day gestational period.

Diet and Housing At day 0 of gestation, the dams were divided into three dietary groups: a control group receiving diet containing 30 ug Zn/g diet ad libitum (ZA), a marginally deficient group receiving diet containing 3.5 ug Zn/g diet (MZD), and a restricted control

group receiving 30 ug Zn/g diet restricted in amount to the daily intake of the marginally deficient dams (RES). All groups received 30 ug Zn/g diet ad libitum on day 17 of gestation, continuing throughout the rest of the study. The mice were allowed free access to acidified water (<1 ug/ml Zn). Feed jars and water bottles were acid washed to remove zinc. Polycarbonate cages and stainless steel tops were rinsed in EDTA. The dams were housed in pairs with one dam being pregnant per cage. Hardwood chip bedding was replaced weekly.

Sample selection All mice (dams, fetuses, and pups) were selected randomly. At least two fetuses from each litter were used for zinc analysis. For postnatal studies, complete litters were used when possible, with 2-12 litters used per assay. The number of pups and fetuses varied from 4-73, depending on the assay.

Zinc analysis Flame atomic absorption spectrophotometry (AA-175, Varian Techtron, Sunnyvale CA) was used to determine the zinc content of the diet, serum, and fetus. Solid samples were wet ashed in a nitric perchloric acid solution then diluted 1:5 in 1% HCL; serum samples were directly diluted 1:5 with 1% HCL prior to analysis. Absorption values were obtained at 213.7 nm using $Zn(NO_3)_2$ (Harleco, Phila., PA) diluted in 1% HCL as a standard.

Antibody mediated responses Pups were immunized intraperitoneally day 10 and 26 postpartum with the T cell independent antigen (TI)

Trinitrophenyl-lipopolysaccharide (TNP-LPS), using 1 ug dissolved in 25 ul of sterile PBS. TNP-LPS was prepared from picrylsulfonic acid (Sigma) and LPS (Sigma, Westphal prep.) according to the method of Walker et al (86). Spleens were harvested 3 days later by passage through a stainless steel mesh (100 gauge). Subsequent to washing the lymphocytes were counted and resuspended in 5 ml of Hank's balanced salt solution (HBSS) (pH 7.3) containing 100 U/ml of penicillin and 100 ug/ml of streptomycin. A 0.1 ml aliquot of splenocytes was mixed with 0.1 ml of TNP coupled sheep red blood cells (prepared by the method of Kettman and Dutton (87) modified by Mishell (88), in 1 ml of 0.6% agarose (SeaKem LE grade,FMC Bioproducts, Rockland,ME) solubilized in minimum essential media (MEM) (with HBSS, without glutamine and $NaHCO_3$, Whittaker MA Bioproducts), supplemented with 1mM Na-pyruvate, 4mM L-glutamine, and 26mM Tris, pH 7.3. The mixture was then overlaid onto an agarose-MEM feeder layer in 60 mm petri dishes. The plates were incubated for 2 hours at 37°C, after which 0.5 ml of nonhemolytic guinea pig complement (Cedarlane) diluted 1:10 was added for an additional hour to develop the plaques. Plaques were counted and expressed as plaque forming cells (PFC) per spleen and PFC's per 10^6 viable splenocytes. All samples were plated in duplicate. Six to thirteen pups were assayed per dietary group.

The affinity of the antibodies produced was determined by the addition of 0.1 ml of either $1x10^{-4}$M, $1x10^{-5}$M, or $1x10^{-6}$M TNP-lysine (TNP-lys) (prepared by the method of Okuyama and Satake (89) at the time of plating. TNP-lys competitively inhibits plaque formation

by occupying binding sites of the antibodies thus reducing the number of interactions between the antibodies and the TNP coupled sheep red blood cells. On day 29, $1x10^{-4}$M TNP-lys was substituted for $0.5x10^{-5}$M TNP-lys.

Statistics Dunnett's multiple comparison tests were performed after a one way analysis of variance. Statistical significance was indicated when $p<0.05$. Unequal sample sizes were adjusted for by a hierarchical between groups analysis (Crunch statistical package, Crunch Software Corporation, Oakland, CA.).

Results

Effects of diet on dams, fetuses, and pups The effects of a marginally zinc deficient diet on maternal weight gains during gestation were modest (Fig. 1). Only slight differences in weight were seen between MZD dams and ZA dams through day 14. By day 17 of gestation the body weights of MZD dams were 88% while the RES dams were 93% respectively the weight of the ZA dams. There was no difference in the amount of diet consumed between the dietary groups through day 17. The marginal diet nevertheless reduced litter sizes, with MZD dams producing litters averaging 3.7 pups while ZA dams had 5.5 pups per litter (Table 2). The number of surviving offspring at three weeks of age were similar for MZD and ZA groups. The RES dams litter sizes were intermediate but not statistically different from ZA dams.

At day 14-18 of gestation and at birth, dams from each group were bled and one femur and all of the fetuses were removed for zinc analysis. MZD dam serum zinc levels were markedly lower during this time when compared to controls, having only 34% the zinc content of the sera of the ZA dams through day 17 of gestation (Fig. 2A). However, MZD dam serum zinc status returned to near normal levels after only one day of zinc replenishment, having 92% of ZA sera levels on day 18. Zinc levels were normal at birth for the MZD dams. RES serum zinc levels were nearly identical to ZA dams for all of the time points examined. Femur zinc levels were similar between the three diet groups (data not shown).

The whole body zinc content of the MZD fetuses closely followed the serum zinc levels of their dams (Fig. 2B). Fetuses from the three dietary groups had similar zinc content on day 14 of gestation. However the zinc content of the MZD fetuses remained the same until day 17 while zinc levels in both the ZA and RES fetuses increased significantly. As a result, by day 17 the whole body zinc content of the MZD fetuses was 68% of ZA and RES fetuses. Yet after only one day of restoration of zinc, the fetuses from MZD dams were nearly equivalent (82%) to ZA fetuses. Interestingly, no significant differences in fetal weight were evident among the groups at any of the gestational time points (Table 3).

At birth the zinc status was completely restored in the MZD neonates and dams. However, this restoration of zinc did not prevent impairments in gestational growth as indicated by the decrease in body weight in the MZD offspring at birth (Table 3). The gestation-

al age at which parturition occurred varied between 19 and 20 days for all groups. This was of significance since birth on day 20 of gestation provided one more day of zinc repletion for these animals. As a result, when parturition occurred on the 20th day of gestation, no differences in fetal weight were evident between the three groups. For births occurring on the 19th day of gestation though, neonates from MZD dams had significant reductions in body weight, being approximately 75% as heavy as both RES and ZA offspring (Table 3). The crown to rump body length was measured in the newborns to further assess the type of growth impairment resulting from the marginally zinc deficient environment in utero (7). No differences were seen between three groups (data not shown) indicating an asymmetric type of intrauterine growth retardation (IUGR) (7). Neonatal weights and zinc status were similar for the three groups throughout the rest of the study.

Antibody Mediated Responses To assess the manifestations of the marginally deficient environment in utero on immune development, antibody mediated responses were tested in vivo. Because of the state of immunosuppression of T-cell-mediated function maintained postpartum in both the murine neonate and human infant (80), TNP-LPS (a so called T cell independent antigen (TI)) was used. Experiments with this antigen predominantly test the interaction of B cells and macrophages in the production of specific antibodies. At day 13 postpartum, there was a nearly 40% reduction in the number of IgM PFC's/spleen for the pups from MZD dams when compared to

pups from RES or ZA dams (Fig. 3). There was also a 40% reduction in PFC's/10^6 viable splenocytes for MZD neonates which indicates proportionally fewer responding cells. Table 4 shows liver, thymus, and body weights, splenocyte numbers and serum zinc levels on the day of the assay. Each of these were similar for pups of all dietary groups, yet when challenged immunologically, MZD pups were significantly compromised. It should be noted that the higher serum zinc levels of the neonates were in accordance with the findings of others (67).

The pattern of immune impairments seen in the earlier time point were also evident at day 29. Pups from MZD dams once again had a 40% reduction in PFC's/spleen compared to pups from RES and ZA dams (Fig. 4). The PFC's/10^6 viable splenocytes were also reduced when compared to RES offspring, but not significantly different from ZA levels. It should be noted that mice are weaned at day 21, being sexually mature by day 42 (85). Thus the immunodepression observed at day 29 represents a prolonged state of immunodeficiency.

Antibody Affinity Using the competitive inhibitor trinitrophenyl-lysine (TNP-lys) in the Jerne plaque assay, the relative affinity of the antibodies produced by MZD offspring was determined to be unaltered at day 13 (Fig. 5). Because the response was completely inhibited by $1x10^{-4}$M TNP-lys, it is possible that intermediate concentrations of inhibitor would have demonstrated some small differences in affinity. To address this, $0.5x10^{-5}$M TNP-lys was substituted for $1x10^{-4}$M TNP-lys in the subsequent day 29 assay.

Again, no differences were observed between the three groups (Fig. 5). Since the responses were nearly identical for both time points as indicated by slopes, it is presumed that no significant differences in the affinity of the antibodies were generated by the dietary intervention.

Table 1

Composition of diets

Ingredient	Amount
	g/kg
Glucose monohydrate	609
Egg white solids (sprayed dried)	200
Corn oil	100
Fiber[a]	30
Salt mix	40
Vitamin mix	10
Ethoxyquin[b]	1

[a] Cellulose-type fiber.

AIN-76 mineral mix without zinc carbonate, supplemented with ap - propriate amount of zinc from zinc carbonate to give final conc. of 30 or 3.5 μg Zn/g diet.

Composition similar to AIN-76 mixture except that the biotin level was increased to provide an additional 2 mg/kg of diet.

[b] Santoquin, Monsanto Chemical Co., St. Louis, MO. All other in - gredients purchased from Teklad Test Diets, Madison, WI. except Corn oil (Mich. State Univ. Food Services).

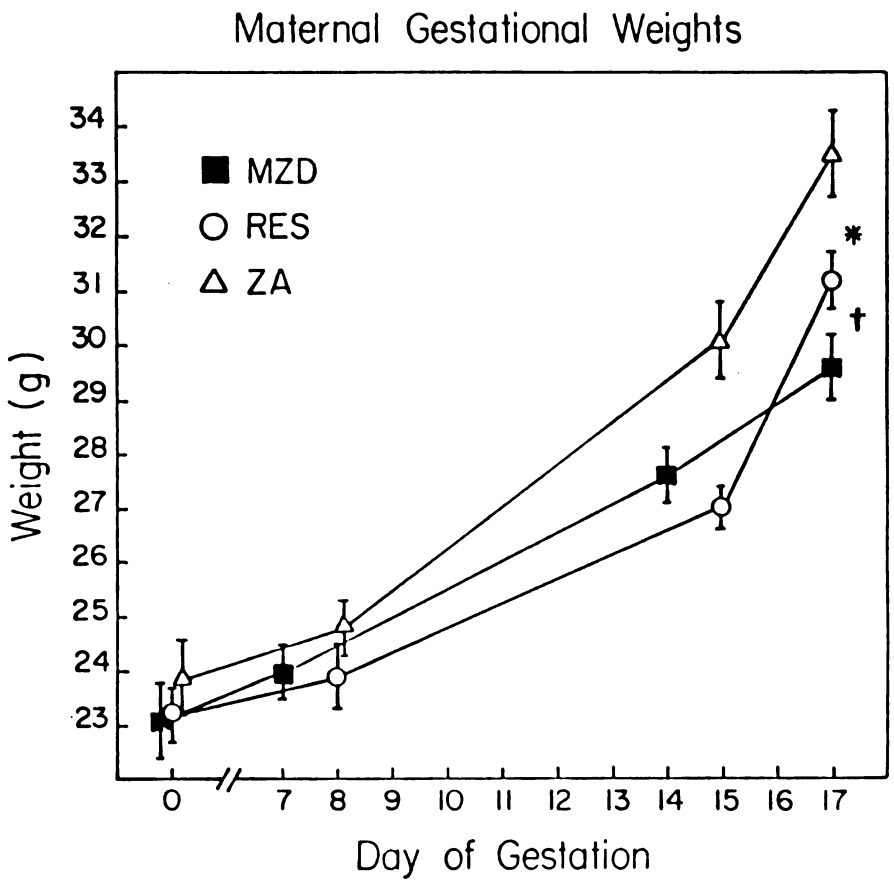

Figure 1. Effect of diet on maternal weights through gestation. Mean ± SEM of the weight of pregnant dams through day 17 of gestation. * - statistically different than ZA, $p<0.05$. † - statistically different than RES and ZA, $p<0.05$.

Table 2

Effects of diets on pregnancy performance

Diet group (n)	Pregnant dams completing parturition	Litter size (mean±SEM)	Surviving pups (3 weeks)
MZD (9)	73%	3.6 ± 0.5*	74%
RES (13)	68%	4.2 ± 0.5	91%
ZA (6)	57%	5.3 ± 0.4	76%

* Statistically different from ZA, $p<.05$ by Dunnett's

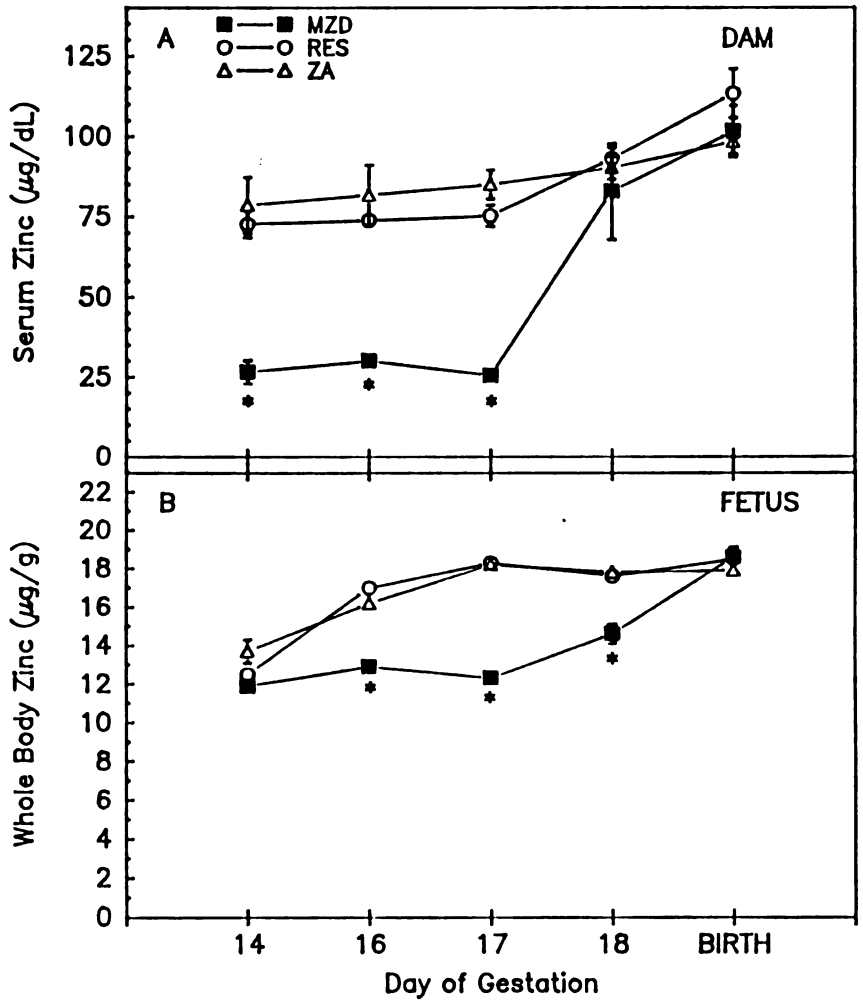

Figure 2. Zinc analysis of dam sera (A) and fetus whole body content (B) during the final trimester of pregnancy. * - statistically different than RES and ZA, $p<0.05$.

Table 3

Weights of fetuses through gestation

Day of gestation	MZD	RES	ZA
14	0.14 ± .02 (34)*	0.14 ± .02 (27)	0.15 ± .02 (33)
16	0.39 ± .06 (65)	0.37 ± .05 (65)	0.39 ± .04 (46)
17	0.55 ± .10 (73)	0.54 ± .08 (64)	0.59 ± .10 (65)
18	0.78 ± .11 (20)	0.80 ± .11 (26)	0.85 ± .08 (24)
birth(19)	0.87 ± .18 (35)	1.10 ± .10 (28)	1.18 ± .06 (5)
birth(20)	1.21 ± .14 (55)	1.22 ± .11 (35)	1.23 ± .10 (10)

* Mean ± SD. Number in brackets is total number of fetuses.
births occurring on day 19 "birth(19)" or 20 "birth(20)" of gestation
significantly different from ZA and RES, $p<.05$ by Dunnett's

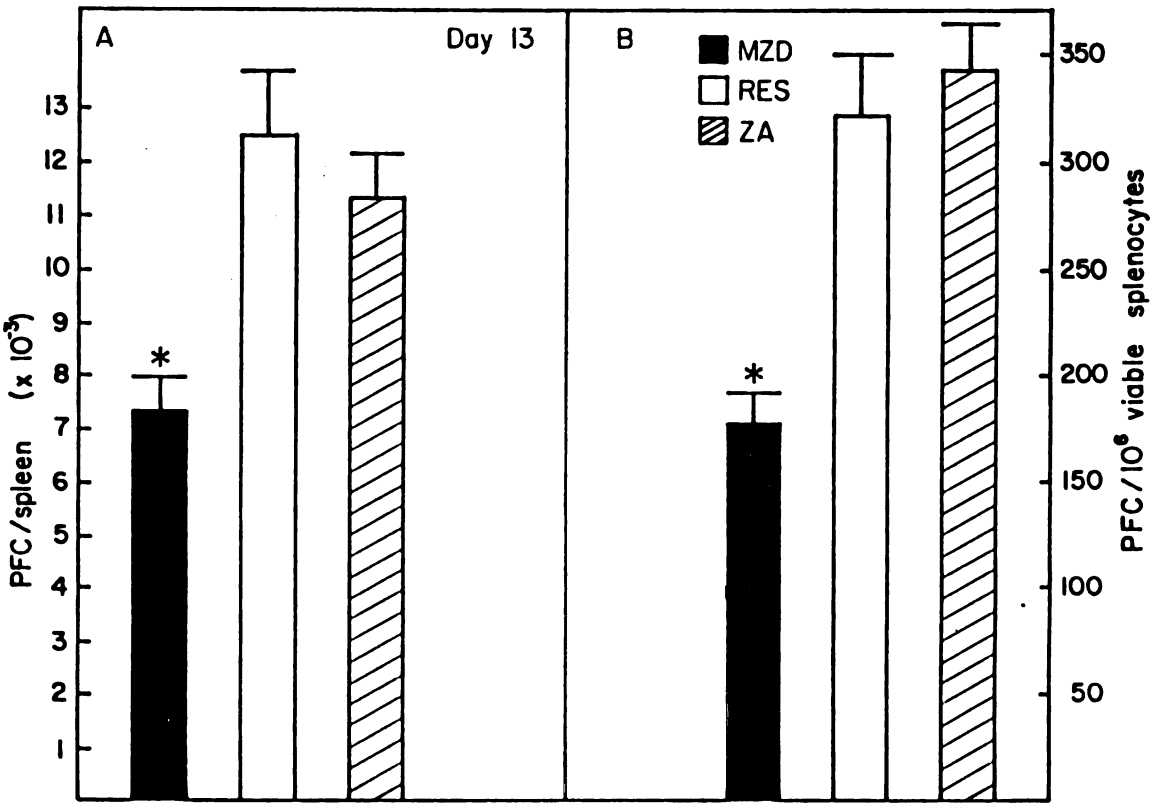

Figure 3. Results of Jerne plaque assay on 13 day old offspring. Panel (A) represents the number of PFC's per spleen. Panel (B) is PFC's per 10^6 lymphocytes. Bars represent mean ± SEM plaque forming cells of 7-11 pups. * - statistically different than RES and ZA, $p<0.05$.

Table 4

Organ weights, splenocyte numbers, and serum zinc content of 13 day old pups

Diet Group		Body	Thymus	Liver	Splenocytes	Serum Zinc
	(n)	(g)	(mg)	(mg)	(x10-6)	(µg/dl)
MZD	(7)	6.6 ± .2*	24 ± 2	261 ± 13	42.3 ± 3.5	153 ± 9
RES	(10)	6.2 ± .2	23 ± 2	213 ± 7	39.5 ± 2.5	149 ± 11
ZA	(11)	5.7 ± .2	21 ± 1	204 ± 7	31.1 ± 3.0	171 ± 12

* Mean ± SEM. Number in bracket is number of 13 day old pups per measure.

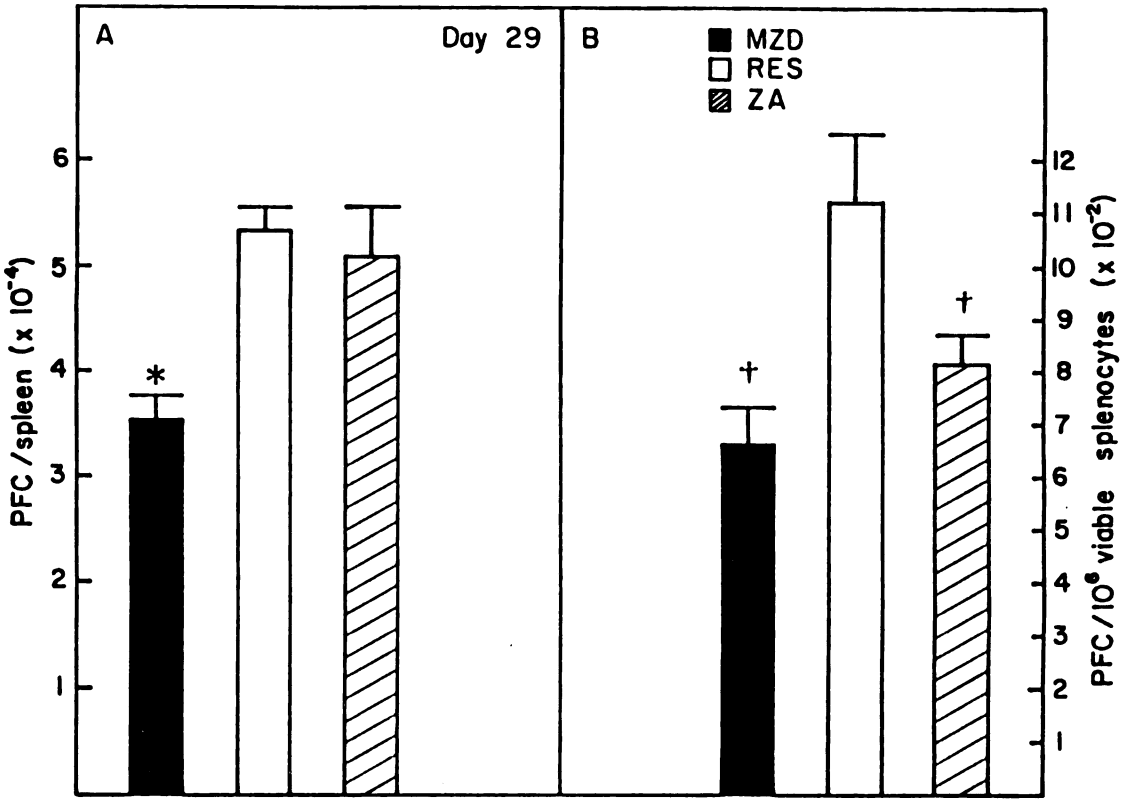

Figure 4. Results of Jerne plaque assay on 29 day old offspring. Panel (A) is PFC's per spleen. Panel (B) is PFC's per 10^6 splenocytes. Bars represent mean ± SEM plaque forming cells of 10-12 pups. * - statistically different than RES and ZA, $p<0.05$. † - statistically different from RES, $p<0.05$.

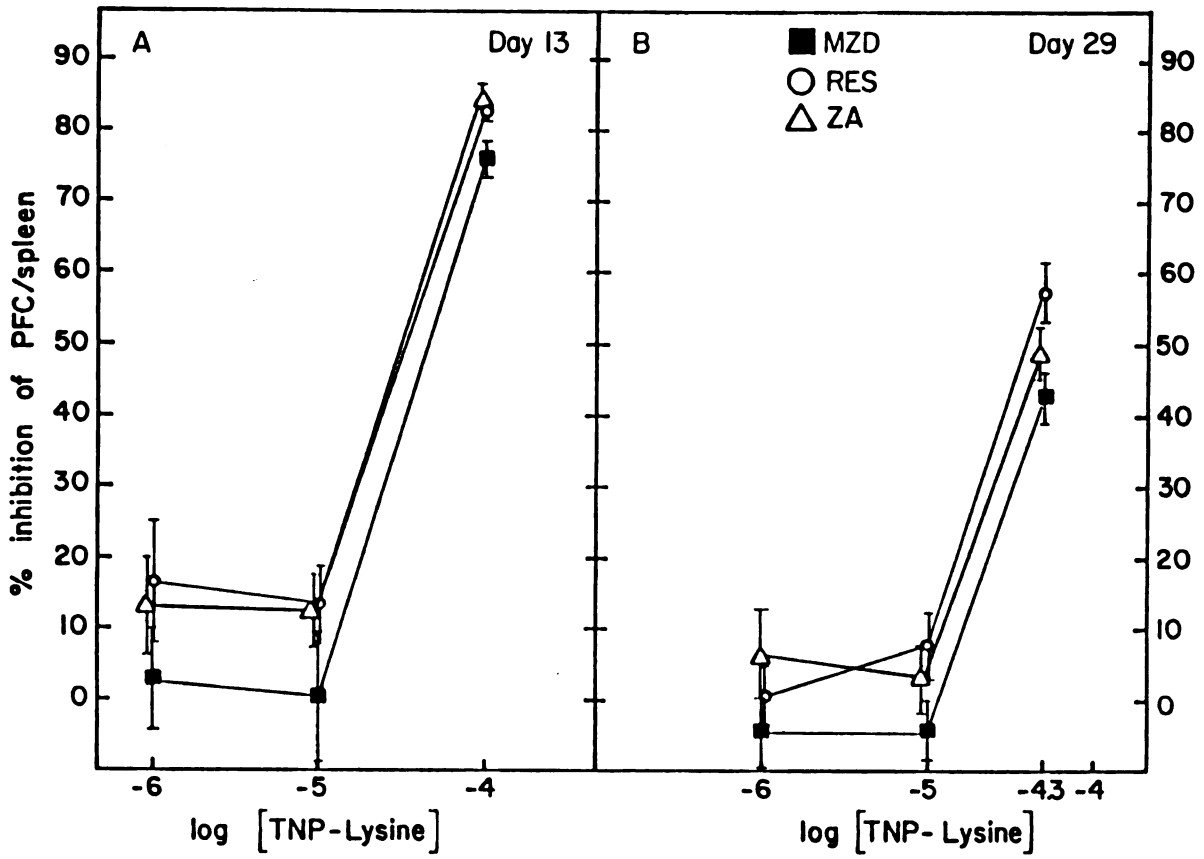

Figure 5. Relative affinity of antibodies produced in the Jerne plaque assays as determined by inhibition with TNP-lysine. Panel (A) shows the data from the day 13 plaque assay, while (B) contains data from day 29. Values are mean ± SEM.

Discussion

The purpose of this study was to further investigate the role of a marginal deficiency of zinc during pregnancy on neonatal immunocompetence. To generate a model which would more closely simulate the problems of the NTLBW infant, dams were fed a diet previously shown to be marginal in zinc (6) from day 0 to 17 of a 19-20 day gestational cycle, after which they received a diet adequate in zinc for the remainder of the study. This protocol was developed in light of the studies reported by Apgar, who found that zinc deficiency in the latter stages of gestation greatly interfered with parturition (35).

Day 14 of gestation was chosen to be the first time point for evaluating zinc status since it represented the beginning of the last trimester of pregnancy, with 90% of the total growth of the fetus occurring during subsequent days. The zinc status of the MZD dams as determined by serum zinc was significantly reduced, being only 34% of ZA dams. This degree of serum zinc depletion had previously been seen only in severely zinc deficient adult mice consuming a diet with less than 1 ug Zn/g diet (90). However, the MZD dams exhibited none of the visual characteristics normally associated with these severely zinc deficient adults (alopecia, parakeratosis, drastic weight lose etc...). Thus, despite markedly lower serum zinc, the dams appeared to be unaffected by the marginal diet. Interestingly, serum zinc levels of nonpregnant female mice fed the marginally deficient diet over the same time period (for 17 days) were the same as control levels (data not shown). Thus, apparently

the increased demand of zinc during pregnancy accounted for the drop in serum zinc levels.

Since this extremely low level of serum zinc was maintained for at least four days (day 14-17) without any overt signs of zinc deficiency, it was unlikely the MZD dams became deficient much earlier than day 14. Dam femur zinc analysis indicated that tissue levels of zinc were unaffected by the diet. If tissue catabolism had occurred in the MZD dams, it was insufficient to boost zinc availability to the fetuses. This was supported by observations in zinc deficient pregnant rats which suggest that little zinc is made available to the fetus by soft tissue catabolism (e.g. bone and liver) (34). The majority of zinc released in severely deficient pregnant rats was from muscle catabolism (34). This was unlikely to have occurred in this study since weights were not dramatically lower in the MZD dams.

Regardless of the actual day the MZD dams first experienced reductions in serum zinc, the reduction of zinc in the MZD fetuses appears to have been transient, restricted to approximately day 15 to day 18 of gestation. The reduced zinc in MZD fetal tissues had no effect on gross anabolic processes up through day 18 as evidenced by fetal weights. Although the zinc levels were normal at birth for the MZD neonates, the prior reduction of zinc resulted in a significant lowering of birth-weight when born on day 19 of gestation, but had no effect on the MZD neonates born one day later. This would suggest that a narrow period of zinc deficiency was created that affected growth only between day 18 and 19 of gestation.

If this is true in humans, normal birth-weight may disguise transient nutritional deficiencies during gestation which may have affected other more sensitive developmental events (i.e. the immune system).

Decreased birth-weight in conjunction with normal body length suggests that the fetuses of MZD dams suffered from an asymmetric form of intrauterine growth retardation (IUGR) (7), simulating the most common form of IUGR in humans. Asymmetric IUGR is believed to result from a late onset of a nutritional deficiency in gestation and is quickly repaired after birth (7). Previous studies with gestational zinc deprivation resulted in a more symmetric form of IUGR (4). Decreases in body size and length were produced which persisted for up to six weeks of age.

Antibody mediated responses were evaluated as a means of determining possible neonatal immunological manifestations of the marginally zinc deficient environment in utero. Thirteen day old pups from MZD dams had drastic reductions in their ability to respond to TNP-LPS, indicating that some impairment of immune development was generated postnatally by the suboptimal zinc environment in utero, even though their thymus, liver, and body weights, splenocyte numbers, and zinc status were all normal. Only when challenged immunologically were any postnatal defects manifested. The same relative responses were evident in 29 day old offspring, indicating the impairment was persistent at least to puberty. It is of interest to note that only about 10 days of zinc adequate diet was required to repair the immune deficiencies created in suckling mice (91). Thus, the developing fetal immune system appears to be more

sensitive than either the adult or neonatal immune systems to deprivations of zinc.

It was of additional interest to determine whether there were any differences in the relative antibody affinity, possibly identifying whether the defect was in B cell differentiation and/or clonal selection. No differences in affinity were seen at either day 13 or day 29, suggesting that the types of clones responding were not significantly altered in MZD offspring. A possibility then may have been a reduced number of responding B cells. However, unlike zinc deficient adult mice in which the reduced antibody mediated responses were mainly due to an absolute reduction in the number of splenocytes (unpublished observations), the number of splenocytes in the MZD neonate was not altered at day 13. Analysis of the phenotypic distribution of B-lymphocytes in the offspring of marginally deficient dams will be needed to determine whether there have been any shifts in specific B cell populations or in their maturation.

This series of experiments in conjunction with the adoptive transfer studies previously reported (6) suggest possible defects in the development of B cells. The transient period of zinc deficiency in the fetus occurred during some critical stages of B cell ontogeny. For example, this period coincides with the expression of surface immunoglobulin (sIg) by pre-B cells in the fetal liver (77). Since zinc has been shown to be critical for normal cell cycling (92), perhaps some B cells were trapped at a sensitive stage of differentiation. The subsequent addition of zinc may have

allowed for continued cycling, but with delayed functional development. Defects in macrophage function and T cell-mediated suppression may also have occurred, but the experiments presented herein do not directly nor rigorously test for these functions.

This system is presented as a possible model for the NTLBW infant resulting from an asymmetric type of IUGR. These studies indicate that repair in weight of the offspring might not be sufficient to regenerate immune competence. The data also suggest that perhaps infants which were born with normal birth-weights yet were considered to be at risk for LBW during gestation, may still have an increased risk of immunodeficiencies through childhood. Research is progressing to identify the mechanism of immune impairments generated by a marginal deficiency of zinc during pregnancy. It is hoped that some indicators of the immune impairments seen in the mouse might easily be adapted for the clinical early detection of possible immunological deficiencies present in infants who were at risk for LBW.

Chapter 2. Evaluation of phenotypic distribution of lymphocytes and cell-mediated immunity

Abstract

A diet marginally deficient of zinc (MZD) fed to A/J pregnant mice from day 0-17 of gestation has previously been shown to result in growth impaired offspring with significant impairments in antibody mediated responses to a T cell independent antigen. This report further characterizes the immunocompetence of these offspring, demonstrating that both B and T helper cells were reduced in the spleen. To determine a functional correlation for the reduction of T helper cells, in vitro proliferative responses to Concanavalin A and phytohemagglutinin as well as a one way mixed lymphocyte culture were performed on 31 day old offspring. No differences were evident for any of these T cell specific responses suggesting in vitro proliferation was intact. A preliminary experiment with in vitro antibody responses was performed using fetal liver cells from day 18 to birth. The results suggest that a defect in trafficking of B cells out of the fetal liver occurs in MZD fetuses at birth, possible explaining the reduced number of B cells in the spleen.

Key words T cell, mitogens, marginal deficiency of zinc, gestation, fetal liver cells, FACS

Introduction

A marginal deficiency of zinc during prenatal development of the A/J mouse has previously been shown to produce offspring with an asymmetric like form of intrauterine growth retardation and severely depressed responses to antigenic challenge for at least until puberty (Chapt. 1). Zinc replenishment was able to quickly repair growth retardation but unable to overcome the significant impairment in immune response, suggesting that a long lasting defect was generated in the developing immune system. This system modelled quite closely the human condition of low birth-weight, and thereby provides a means to evaluate the possible immunodeficiencies associated with these infants.

The previous chapter focused on the immunological manifestations of the marginal deficiency of zinc in utero on B cells, completely ignoring any affects on T cell-mediated immunity. Studies utilizing non-human primates (rhesus monkey) indicated that proliferative responses to T cell specific mitogens were depressed in the offspring of zinc deficient dams for some time after birth when compared to control offspring (93). In humans, delayed type hypersensitivity reactions and proliferation to T cell specific mitogens have both been shown to be depressed in malnourished children including those suffering from intrauterine growth retardation (94). Thus, clinically there seems to exist some indications that T cell function may be altered.

To address whether any alterations in neonatal T cell responses resulted from the marginal zinc deprivation during pregnancy,

proliferative responses were evaluated to T cell specific mitogens, as well as a one way mixed lymphocyte culture (MLC). The mitogens used here are both lectins which predominantly activate T cells to proliferate. Activation results from the crosslinking of cell surface structures containing the carbohydrate bound by the lectin (95). The MLC is an in vitro assay which simulates the more realistic response of T cells to foreign (allogeneic) cells which differ in their major histocompatibility antigens (MHC). This is part of the response in tissue graft rejection. These assays are also easily adapted for use in human infants and thereby may provide a quick means of evaluating immune function in low birth-weight infants.

The generation of an effective immune response to most antigens requires the concerted action of a large number of immune cells. Additionally, immune cells must communicate with the surrounding vasculature to some extent in order to be delivered to the proper location in the organism. One mechanism developed to aid in these communications is the acquisition of specific cell surface molecules which regulate the effector functions as well as the localization of the immune cells. Many of these surface molecules delineate subpopulations of lymphocytes with specific effector functions (96). Examples of these are: surface immunoglobulin (sIg) found only on antibody producing B cells, Thy 1 found on T cells, Lyt 1 differentially expressed on all T cells as well as a small population of B cells, while Lyt 2 and L3T4 subdivide T cells into suppressor/cytotoxic and helper cells, respectively. Using these surface

markers, subpopulations of T and B lymphocytes were examined in the offspring of marginally deficient dams to assess whether any alterations in the relative proportions of these clones and subclones of lymphocytes had occurred.

Finally, if a critical stage of immune ontogeny was affected by the poor zinc environment in utero, examination of immune development coincident with the timing of the deficiency might reveal which stage was affected. It was previously reported that the fetuses of marginally zinc deficient (MZD) dams had significant reductions in zinc content between days 15-18 of gestation (Chapt. 2), and that B cell responses were altered in the fetal liver of offspring of MZD dams at day 18 of gestation (6). Therefore, a pilot experiment was done using B cell plaque response in the fetal liver to determine the state of immune development through this period of reduced zinc content.

Methods

Breeding The breeding protocol has been described in the previous chapter and will only briefly be outlined here. Ten week old A/J female mice were adjusted to a biotin fortified egg white diet containing 30 ug Zn/g diet two weeks prior to breeding. The males were introduced at 1800 hrs; female mice were then checked for three consecutive days at 900 hrs for the presence of a vaginal plug, which if found was considered to be day 0 of gestation. Pregnant mice were divided into two or three diet groups depending

on the experiment. One group was fed a diet containing 3.5 ug Zn/g diet (MZD) from day 0 to 17 of gestation, after which this group received 30 ug Zn/g diet for the rest of the study. The second group included in all experiments received 30 ug Zn/g diet restricted in amount to the daily intake of the MZD dams and hence were called restricted fed (RES). The third diet group used only in the in vitro fetal liver culture experiment in this report received 30 ug Zn/g diet ad libitum (ZA). The RES and ZA mice both received 30 ug Zn/g diet ad lib. from day 17 of gestation through the rest of the experiment. The ZA group was eliminated as a control for the phenotyping and cell-mediated experiments to conserve on the number dams distributed to each diet group.

Selection of dams and pups for analysis As previously described, all mice were selected randomly with one to four litters and four or more offspring used per assay. Sex of the neonates or fetuses was not considered in the distribution of samples for any of the experiments.

Monoclonal antibodies The following monoclonal antibodies were used to fluorescently label specific cell populations in the thymus and spleen: anti-Ig (goat anti-mouse IgG+M, FITC conjugated); anti-Thy 1.2 (rat IgG_{2b} anti-mouse Thy 1.2, FITC conjugated); anti-Lyt 1 (rat IgG_{2a} anti-mouse Lyt 1, FITC conjugated); anti-Lyt 2 (rat IgG_{2a} anti-mouse Lyt 2, biotin conjugated); and anti-L3T4 (rat IgG_{2b} antimouse L3T4, PE conjugated). A PE conjugated streptavidin

reagent was used in conjunction with the biotin conjugated anti-Lyt 2. All reagents were obtained from Becton Dickinson except anti-Ig, which was purchased from Tago, Inc. Appropriate amounts of each stock antibody were removed and centrifuged in an air fuge at 10,000xg for 15 min. at 4°C. Supernatants were then removed and diluted 1:40 in the buffer used to stain the cells. Avidin-PE was diluted 1:5 before use.

Cell suspensions for FACS analysis Thymus and spleen samples from 9 and 22 day old neonates from RES and MZD dams were removed and processed individually by passage through a 100 gauge stainless steel mesh screen. Harvesting was performed with Hank's balanced salt solution (HBSS) without phenol red, supplemented with 5% heat inactivated fetal calf serum (HIFCS) (MA Bioproducts) (pH 7.2 at room temp.). The cells were washed twice at room temp. by centrifugation at 400xg (1400 rpm) for 6 minutes, resuspended in 2-3 ml of harvest buffer, layered over 3 ml of a 1.083 g/ml histopaque gradient (Sigma), then centrifuged at 1550 rpm for 10 minutes with the brake off. The band which formed between the two layers contained the majority of the lymphocytes and was >90% free of red cells. This band was removed and immediately washed by centrifugation two times in harvest buffer at room temperature. After the final wash, the cells were resuspended in 1.0 ml of HBSS without phenol red (supplemented with 5% HIFCS and 0.15% sodium azide (pH 7.2 cold)) and kept on ice for the rest of the labelling procedure. The cells were counted to determine cell concentration; viability

was in most cases greater than 90% as assessed by the trypan blue dye exclusion method.

Staining with Fluorescent Antibodies The cells were then aliquoted out at $1x10^6$ cells/ml in less than 70ul of volume and kept on ice. The antibodies were added to the cells in 150ul amounts and incubated for 30 min on ice. The cell suspensions were washed twice with HBSS plus 5% HIFCS and 0.15% sodium azide (centrifuged for 5 min at 1400rpm at 6°C) and then resuspended in 1.0 ml of PBS with 2% paraformaldehyde to fix the cells for analysis. Samples requiring biotinylated antibodies (Lyt 2) were washed only one time after the initial addition of antibody. Then the second antibody was added for a 20 min. incubation, after which these samples were washed twice and fixed as above. Thymocytes labeled with anti-Ig were used as the control for nonspecific binding (<2%). AKR thymocytes which differed in their expression of Thy 1 (Thy 1.1 positive) were used for assessing nonspecific binding for anti-Thy 1.2. Autofluorescence (<3%) was used as a measure of fluorescent signal noise for FITC-Thy 1.2, FITC-Lyt 1, and PE-L3T4. Streptavidin-PE labeled splenocytes served as checks for nonspecific streptavidin binding to the cell surface (<2%). Labeled samples were kept in the dark on ice until FACS analysis.

FACS analysis The fluorescent samples were analyzed on an Ortho 50H Cytofluorograph with an Ortho 2150 computer data processor, using the 488 nm spectral line of an argon laser. Cell scatter

profiles were examined using low angle forward versus orthogonal light scatter. FITC was measured at 515-530 nm, while PE conjugated samples were measured using a 570 nm long pass filter. Fluorescent cytogram gating using forward scatter versus fluorescence was used to include the fluorescent cells in the normal splenic size range and eliminate debris, cell clumps, and off-scale fluorescence from data analysis. Greater than 95% of negative fluorescence was found in the nonfluorescent portion of the cytogram. Off scale fluorescence was less than 5% of the fluorescent population and contained cells clearly not lymphoid as determined by scatter profile. All data were corrected for background and off scale fluorescence of lymphoid cells.

Cell culture Spleens from 31 day old offspring of RES and MZD dams were aseptically removed and processed individually as previously described for the Jerne plaque assay. Briefly, spleens were removed with autoclaved utensils and placed in a sterile petri dish with 5 ml of sterile media. The medium used for harvesting and culturing was RPMI-1640 (MA Bioproducts) supplemented with: 0.01 M Hepes, pH 7.4; 0.6% $NaHCO_3$; 1.0 mM nonessential amino acids; 1 mM Na pyruvate; 100 units of penicillin; 100 ug/ml streptomycin; 50 ug/ml gentamicin; 2 mM L-glutamine (all from Gibco); $5x10^{-5}$ M 2-mercaptoethanol (Sigma, 1-2000 dilution of stock); and 5% FCS. Splenocytes were harvested in a hood by passage through an autoclaved, stainless steel, 100 gauge mesh screen. Suspensions were pipetted into sterile tubes, allowed to settle, transferred to

new sterile tubes, and washed twice by centrifugation (1400 rpm for 7 min). The cells were resuspended and counted on a hemocytometer in trypan blue to determine cell concentration and viability. Individual samples were resuspended at $2.5x10^6$ cells/ml with 100 µl of this distributed in 96-well flat-bottomed culture plates. The cultures were incubated in 7% CO_2 at 37°C (Forma Scientific water jacket heated).

Mitogenic assays Concanavalin A (Con A) (Sigma) was prepared by resuspension in RPMI media with all supplements except FCS (incomplete media), then filter-sterilized by passage through a 0.22 µm disposable filter. Dilutions were then made in incomplete media to give final concentrations in the wells of 1.0, 2.5, and 5.0 µg/ml. Phytohemagglutinin (PHA-P) (Sigma) was prepared in the same manner as Con A in final concentrations in the wells of 25, 50, and 100 µg/ml. The mitogens were added at the time of culture, three wells per mitogen concentration per sample. Each splenocyte suspension was also cultured without the addition of a mitogen to determine background proliferation. The plates were incubated for 48 hrs. before 1 µCi of methyl-[^{3}H]-thymidine (diluted in RPMI media alone) was added per well to radioactively label dividing cells. The cells were then frozen 18 hrs. later until subsequent harvesting. The samples were harvested with a multiple sample harvester (Otto Hiller Co., Madison, WI) which precipitated the DNA onto a filter by washing with cold trichloroacetic acid. The filter paper was then placed in vials containing 3 ml of scintillation

cocktail (6 g PPO and 75 g POPOP in one liter of toluene) and counted in a scintillation counter. The data were expressed as the mean cpm of the three wells minus background.

Mixed lymphocyte culture Allogeneic C57BL/6 (H-2^b) mouse spleens were removed and processed exactly as described previously. The cells were pooled, resuspended at $7.5x10^6$ cells/ml and treated with 25 µg/ml of mitomycin C (Sigma) for 30 min., washed twice by centrifugation, and resuspended again at 2.5, 5.0, and $7.5x10^6$ cells/ml. One hundred microliters of these suspensions were added to the pup sample splenocytes ($2.5x10^6$ cells/ml) and cultured for four days. Pup splenocyte suspensions were cultured without C57BL/6 cells as a control. The cultures were radioactively labeled with methyl-[^{3}H]-thymidine 18 hr. after the four day incubation, after which they were frozen and harvested as above.

Fetal liver cultures In a pilot experiment, livers from MZD, RES, and ZA fetuses at day 16-18 of gestation and from pups born on day 19 of gestation were aseptically removed using autoclaved utensils and processed in a manner similar to the Jerne plaque assay described previously (Chapt. 1). Livers were harvested using PBS containing 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml of gentamicin, and 2% HIFCS. Four or five livers were pooled from each diet group per day, washed twice by centrifugation (1200-1400 rpm at room temp. for five min.), and resuspended in 5 ml of culture media (RPMI-1640 supplemented as above except containing 5%

FCS and 0.5% globulin free BSA (Sigma)). Cell density and viability was determined by counting nucleated cells on a hemocytometer in the presence of trypan blue dye. The density of the cell suspension was adjusted to $1x10^6$ cells/ml, with 0.5 ml plated in 24 well plates (16 mm wells). TNP-LPS was added (0 or 0.1 μg/ml final concentration in the well, six wells/conc.) and the cells cultured for 6 days at 37°C in an atmosphere consisting of 10% CO_2, 7% O_2, and 83% N_2. The cultures were then harvested by removal of the cells by pipette and rinsing the wells with buffer (Hank's balanced salt solution with 1% HIFCS). The cells were then centrifuged at 1200-1400 rpm to pellet the cells and plated along with TNP coupled sheep red blood cells in the Jerne plaque assay as previously described (Chapt. 1). Due to the high variability of the plaque response in these cultures, the plates containing the highest and lowest plaque numbers for that set were not included in the final calculation of plaque response. The remaining four plates were averaged and expressed as plaque forming cells/10^7 cells.

One day after the initial culture was set up the experiment was repeated using livers from pups born on day 20 of gestation from all diet groups. The procedure was exactly as above using all of the same reagents. The bone marrow of adult A/J female mice was removed and cultured along with the same concentrations of TNP-LPS as a positive control on both assay days. Plaque response from these cultures were used to normalize the data.

Statistics Statistical significance was determined for FACS data by Student's T test. Significance was inferred when $p<0.05$.

Results

Phenotypic distribution of lymphocytes in the spleen and thymus

Analysis of the thymus of 9 day old mice revealed no difference in percent positive cells for any of the markers tested (Table 5). As expected, >95% of cells in the thymus were positive for Thy 1.2 in both MZD and RES neonates. This was identical to the adult value obtained on the same day. Lyt 1 was also found to label 95% of the thymocytes at day 9 postpartum, slightly more than adult levels for this marker (90%). The day 9 postpartum results for Lyt 2 for both diet groups were somewhat reduced from experimental adult levels, 65-69% versus 80%. Both pup and adult percentages of Lyt 2^+ cells found for this assay were low in comparison to values previously obtained in this lab and elsewhere (85-90%) (97). L3T4 positive cells were 80% of the total cells in the thymus, again identical to adult levels. Since all values were similar between the two groups and consistent with published results except Lyt 2, only Lyt 2 labelling was repeated at day 22 postpartum. In this assay the percent Lyt 2 positive cells was consistent with published adult values (85%), the 22 day old block fed control mice had identical percentages, and there were no differences between the two diet groups.

Quite different results were obtained when the peripheral immune system was evaluated. As a measure of the periphery, the spleen

was chosen as the representative organ for analysis (Table 6). FACS analysis of Thy 1.2 positive cells in the spleen revealed that at 9 days postpartum there were very few positive cells. Even though the percent positive cells was only 1% of the total, a statistical difference ($p<.05$) was obtained between the diet groups, with RES neonates containing twice the number of positive cells as the MZD neonates. Lyt 1 analysis suggested that perhaps MZD neonates had slightly higher numbers of these cells then RES offspring at day 9, however this difference was not statistically different. Lyt 2 positive cells were undetectable in the spleen at day 9, consistent with published observations (79). L3T4 positive cells were lower in the MZD neonates than RES (4.3 versus 6.9%, respectively, $p<.05$), as were surface Ig positive cells (7.1 versus 9.5%, $p<.05$).

FACS analysis was repeated on splenocytes from 22 day old offspring utilizing Lyt 2, L3T4, and sIg markers. Lyt 2 positive cells were not significantly different between the two groups. Both numbers were still quite low when compared to adult levels for this marker in the spleen but consistent with accepted values. Both L3T4 and sIg positive cells in the spleen were significantly reduced ($p<.05$) in MZD as compared to RES offspring. Adult levels of sIg were evident in the RES mice, while L3T4 remained well below adult levels at this age.

Functional analysis of splenocytes In order to evaluate the functional capability of the peripheral T cells, mitogenic responses

to concanavalin A (Con A) and phytohemagglutinin (PHA-P) were tested in vitro on 31 day old mice. Stimulation with Con A (1.0, 2.5, and 5.0 ug/ml) resulted in marked proliferation, with maximal amounts of methyl-[^{3}H]-thymidine incorporation of >100,000 cpm produced by addition of 1.0 ug Con A/ml (Fig. 6). No differences were evident between the diet groups for any of the concentrations used. Adult positive controls responded with maximal incorporation of 200,000 cpm. Proliferation in response to PHA-P (25, 50, 100 ug/ml) was markedly lower than Con A response as expected, with maximal incorporation of 12,000 cpm occurring with 50 ug PHA-P/ml (Fig. 7). The responses on average were higher in the MZD offspring than RES, but these differences were not statistically significant. Adult positive control responses were higher with a maximal incorporation of 30,000 cpm, consistent with previous results in this lab (data not shown). Background proliferation was less than 5000 cpm.

In addition to mitogenic responses, a one way mixed lymphocyte culture (MLC) was examined concurrently, using mitomycin C-treated C57Bl/6 spleen cells as the stimulating cells (Fig. 8). Similar amounts of methyl-[^{3}H]-thymidine were incorporated between the diet groups with maximal responses (11,000 cpm) resulting from 7.5×10^5 C57Bl/6 cells/ml. Adult positive controls responded maximally with 80,000 cpm, again consistent with previously published values from this lab. Background again was less than 5000 cpm.

Fetal liver cultures Fetuses from day 17 and 18 of gestation, and from births which occurred on day 19 and 20 of gestation (B19 and B20, respectively) were sacrificed and their livers removed for culture (Fig. 9). Since the experiment with B20 liver cells was done one day after the other time points, the data were normalized prior to direct comparison of the time points. Adult positive bone marrow samples gave slightly different plaque responses on the two assay days. Thus, the percentage difference between the two controls (the first day was 75% of the second days assay) were used to normalize the liver data from the different experimental days.

In preliminary results in response to 0.1 μg/ml TNP-LPS, the fetuses from ZA dams had a greater plaque response than both RES and MZD fetuses for day 18 and B19 (Fig. 9). Maximal number of plaque forming cells were present in B19 fetuses for all groups. Interestingly, the MZD fetuses failed to show a significant decrease in plaque numbers one day later (B20) as had RES and ZA fetuses. The number of plaque forming cells from the MZD fetuses on B20 remained nearly the same as the previous days response, and was roughly 2 fold higher than ZA fetuses and 4 fold higher when compared to RES fetuses at this time point. Fetal liver cells from day 17 were unresponsive to TNP-LPS for all diet groups.

Table 5

Phenotypic distribution of lymphocytes in the thymus

		percent positive fluorescence			
Age (days)	Diet group	Thy 1.2	Lyt 1	Lyt 2	L3T4
9	MZD	97 ± 1	94 ± 2	69 ± 6	81 ± 5
	RES	95 ± 2	95 ± 1	65 ± 12	79 ± 4
22	MZD	ND	ND	83 ± 2	ND
	RES	ND	ND	85 ± 3	ND
Adult	control	97	90	80	79

Flow cytometric analysis of thymuses from 9 and 22 day old neonates. Data expressed as the mean ± SD of percent positively fluorescent cells using 5-9 samples per antibody. Adult positive control included for comparison. ND - not done.

Table 6

Phenotypic distribution of lymphocytes in the spleen

		percent positive fluorescence				
Age	Diet	Thy 1.2	Lyt 1	Lyt 2	L3T4	sIg
9	MZD	0.5 ± .2*	5.3 ± 1.5	0	4.3 ± 1.3*	7.1 ± .8*
	RES	1.1 ± .4	4.7 ± .7	0	6.9 ± 1.8	9.5 ± 2.2
22	MZD	ND	ND	3.3†	6.9 ± .6*	27.8 ± 1.9*
	RES	ND	ND	4.0†	8.3 ± .2	31.4 ± 2.6
Adult	control	22	30	8.0	17	30

Flow cytometric analysis of splenocytes from 9 and 22 day old neonates. Data expressed as the mean ± SD of percent positively fluorescent cells using 5-9 samples per antibody. Adult positive control included for comparison. * - statistically significant, $p<0.05$. ND - not done.
† - Value of 5 samples pooled for one reading.

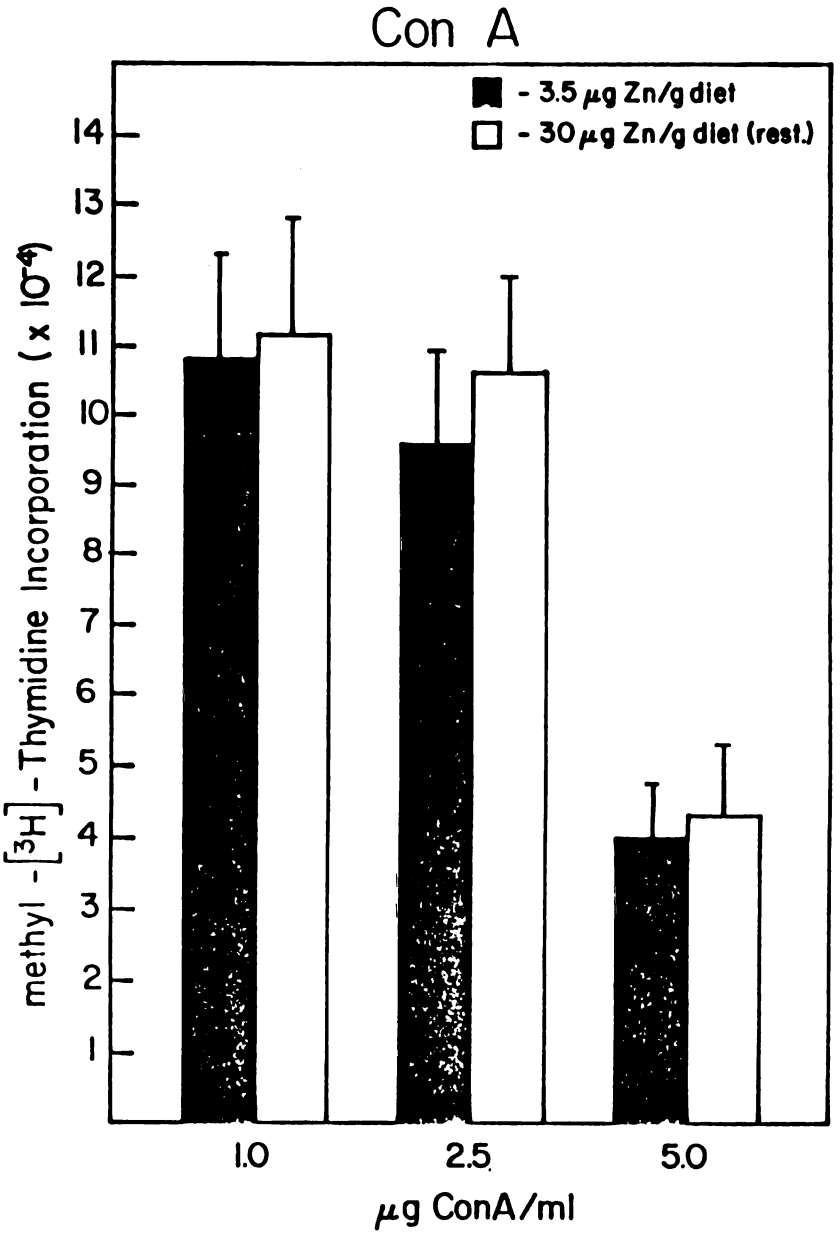

Figure 6. Proliferative response of splenocytes to Con A. Splenocytes from 31 day old offspring were cultured in the presence of either 5, 2.5, 1, or 0 μg Con A/ml for 48 hr., after which the cultures were labeled with methyl-[^{3}H]-thymidine and harvested 18 hr. later. The data is expressed as the mean ± SEM incorporation minus background incorporation.

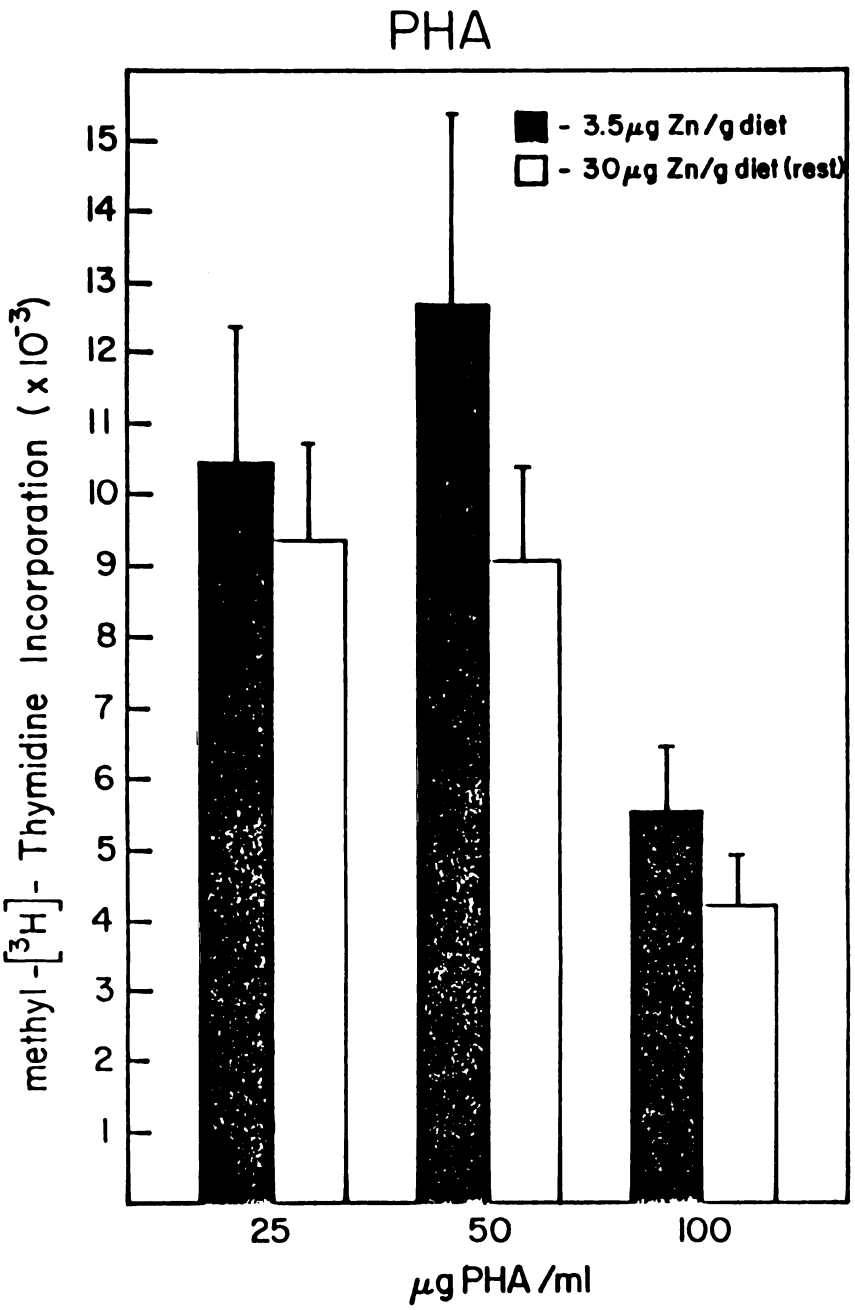

Figure 7. Proliferative response of splenocytes to PHA-P. Splenocytes from 31 day old offspring were cultured in the presence of either 100, 50, 25, or 0 μg PHA-P/ml for 48 hr., after which the cultures were labeled with methyl-[^{3}H]-thymidine and harvested 18 hr. later. The data is expressed as the mean ± SEM incorporation minus background incorporation.

Figure 8. Proliferative response of splenocytes to allogeneic mitomycin C treated splenocytes in a one way mixed lymphocyte culture. Splenocytes from 31 day old offspring were cultured in the presence of 7.5, 5, or 2.5×10^5 cells/ml, or without allogeneic cells for background response for 4 days prior to the addition of methyl-[^{3}H]-thymidine. The cells were labeled for 18 hr. before harvesting. The data is expressed as the mean ± SEM incorporation of label minus background incorporation.

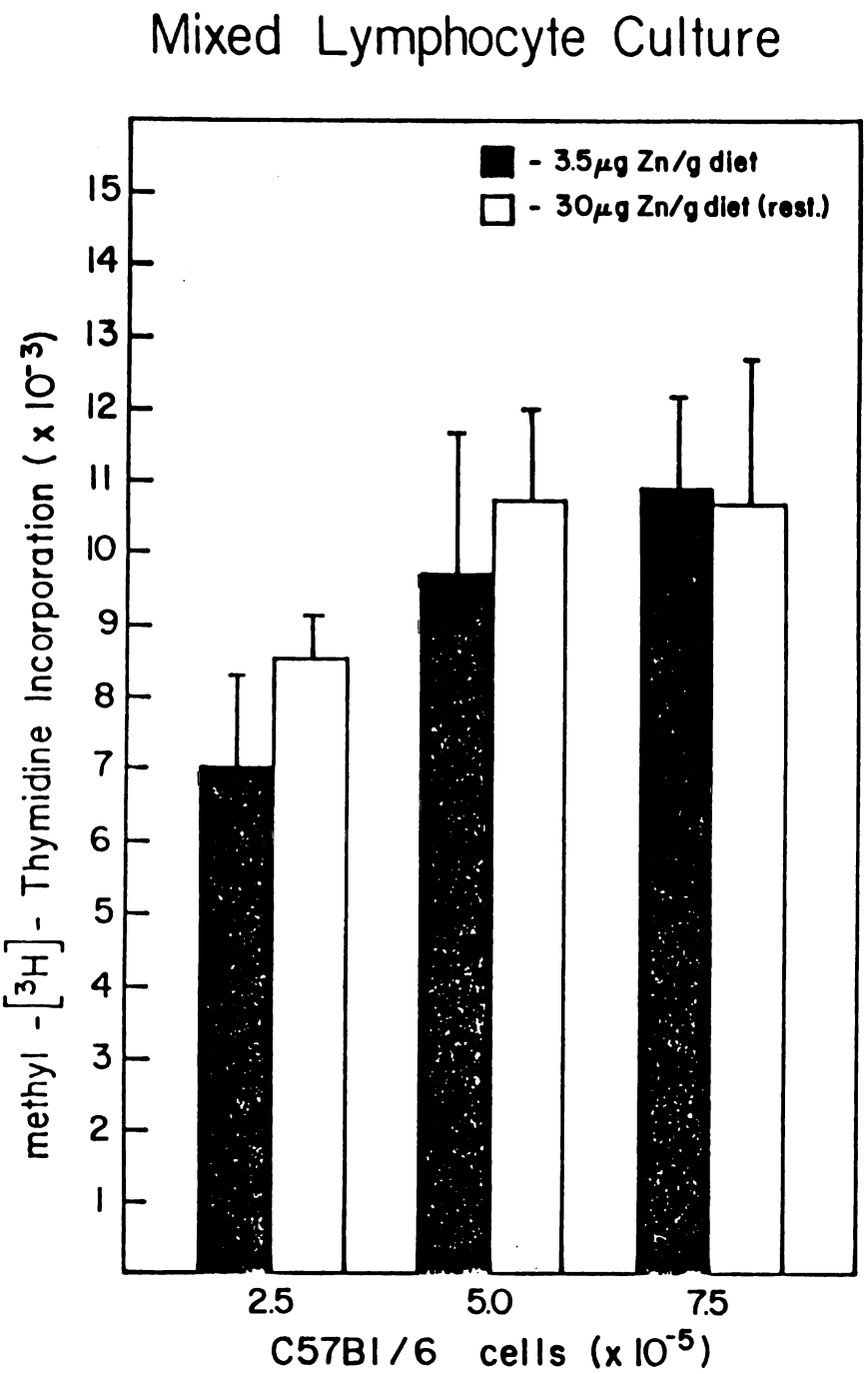

Figure 8. Proliferative response of splenocytes to allogeneic mitomycin C treated splenocytes in a one mixed lymphocyte culture

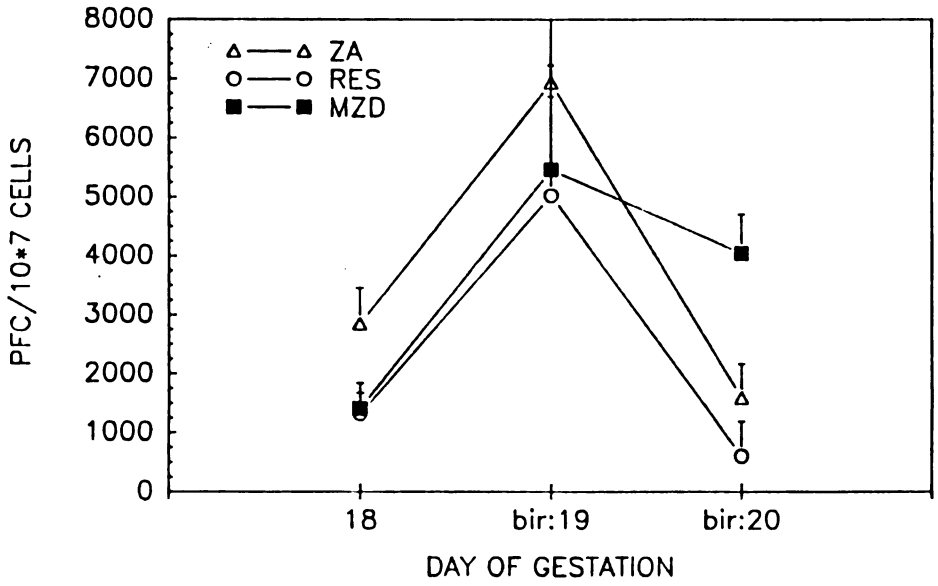

Figure 9. Results of fetal liver <u>in vitro</u> antibody mediated response to TNP-LPS. Fetal liver cells from day 17 and 18 of gestation, and livers from neonates born on day 19 and 20 of gestation were cultured in the presence of 0.1 μg TNP-LPS/ml for six days prior to analysis by the Jerne plaque assay. The data represents the mean ± SD of the four median wells out of six. Data from different assay days were normalized by comparison of adult positive bone marrow samples cultured at the same time.

Discussion

Neonates born of dams fed a marginally zinc deficient diet during gestation have previously been shown to be impaired in antibody mediated responses to T cell independent antigens at least until puberty, despite having a normal physical appearance and normal numbers of splenocytes (Chapt. 1). Since plaque forming cell response was reduced in the spleen of MZD neonates, one explanation may have simply been a reduction in the number of B cells present. To address this and the more general question of the status of lymphocyte subpopulations, the phenotypic distribution of both B and T cell populations in the spleen were assessed in neonates, as well as the percentage of T cell populations in the thymus.

Analysis of the thymus, the site of T cell development in mammals, revealed no differences in specific T cell populations as determined by the proportion of thymocytes positive for Thy 1.2, L3T4, and Lyt 1 and 2. In contrast to this, offspring of MZD dams had persistent reductions in splenic B cells and T helper cells. These reductions particularly would affect antibody responses dependent upon T cell help, as well as cytotoxic cell-mediated responses. The reductions in T cell independent responses previously demonstrated (Chapt. 1) were likely to have been due in part to this reduction of B cells in the spleens of these animals. However, it is unlikely that the 40% reduction in the number of plaque forming cells in the MZD neonatal spleen evident at both 13 and 29 days of age were accounted for solely by the 12-25% reduction in B cells. There are a number of other possible reasons for a defect in plaque

production such as: immaturity of the B cells activated by the TNP-LPS, a defect in antigen processing by macrophages or other accessory cells, a defect in B cell specific lymphokines or cytokines, or increased suppression mediated by T suppressor cells, many of which remain to be addressed.

Since alterations in the number of T cells were evident in MZD offspring, it was of interest to determine whether any defects could be demonstrated in T cell function. One method to evaluate T cells is to measure their proliferative response to T cell specific mitogens. Proliferation is dependent on the production of soluble growth factors such as IL-2 and IL-4 by T helper cells. Thus, these assays potentially could demonstrate a functional correlation with the reduced numbers of T helper cells seen at day 22. Mitogenic responses were tested in 31 day old offspring, only two days later than antibody-mediated responses were previously shown to be dramatically depressed (Chapt. 1). No differences were evident between MZD and RES offspring for either Con A or PHA, suggesting that *in vitro* T cell function was intact. As a further measure of T cell function, splenocytes were cultured in the presence of mitomycin C treated allogeneic cells in a one way MLC. This assay was somewhat more representative of an *in vivo* response, simulating the initial phases of a MHC class I restricted cytotoxic response. Again, no differences between the groups were seen, suggesting *in vitro* T cell function in the spleen was unaltered in the MZD offspring at 31 days of age.

There are at least two interpretations for this discrepancy as to the state of T helper cells: 1) the in vitro assays were too artificial to detect a small reduction in helper cells, or 2) the differences in T helper cell numbers seen at 22 days were repaired by the time of the assay (31 days of age). The latter seems most likely, even though antibody mediated responses were down at this age. It should be reemphasized that the evaluation of the number of antibody producing cells was done utilizing a type 1 T cell independent antigen, and therefore should not detect alterations in T helper cells. Two experiments are indicated to resolve this: 1) evaluation of the phenotypic distribution of splenocytes from older offspring to determine whether indeed T helper cells are still reduced, and 2) a repeat of the in vitro assays presented here at an earlier age. Unfortunately, the in vitro assays had been performed previously but the data were lost due to contamination.

To examine the kinetics of B cell development during the critical stages of ontogeny which occurred in parallel with reduced zinc in the fetuses, fetal liver cells from day 17 and 18 of gestation, and liver cells from neonates born on day 19 and 20 of gestation were tested in vitro for plaque forming cell production in response to TNP-LPS. Preliminary results suggest a defect in B cell migration out of the liver in MZD offspring. This normally occurs around birth, with B cells migrating out of the liver for colonization of the bone marrow and spleen (77,79).

This would suggest that perhaps during the transient period of reduced zinc in the fetus (between day 15-18 of gestation), there

was a block in the development of trafficking molecules on the surface of MZD fetal B cells which may have altered subsequent homing patterns to the periphery. This would account for the reduction of numbers of B cells present in the neonatal spleen as evidenced by flow cytometric analysis. Interestingly, no differences in T cell populations in the thymus were evident, yet there was a reduction in T helper cells in the spleen. The thymus is seeded by T stem cells sometime around day 11 of gestation (78), well before the fetuses of MZD dams experienced reductions of zinc. Thus perhaps both B and T cell populations in their respective origins may have been able to develop, however the lowering of zinc content may have impaired the acquisition of the necessary surface molecules to migrate out to the periphery. There is considerable evidence to support the notion that the expression of cell surface molecules is necessary for lymphocyte trafficking (98,99).

As indicated above, it is unlikely that the reduction of numbers of B cells seen in this study would have entirely accounted for the lowering of plaque forming cell numbers. One additional possibility may have been a reduction of amount of surface Ig on the B cells present in the spleen. It is well established that B cells from fetal and neonatal animals are extremely susceptible to tolerization, and that once sIg is removed from the surface of the immature B cell, reexpression does not take place (80). Perhaps a similar mechanism is involved in the acquisition of sIg and other trafficking molecules which is somehow dependent upon zinc. The transient period of lower zinc in the fetuses of MZD dams may have interrupted

the process of expression of these molecules sufficiently to reduce the numbers expressed, but not enough to abort further maturation of the cells upon zinc replenishment. This would have resulted in both a reduced number of B cells and a reduction in their function.

Focusing on B cells, the next step will be to examine both the liver and the bone marrow with the in vitro plaque assay during the latter stages of gestation and early in neonatal life to follow the migration of the B cells during development. This in conjunction with FACS analysis using the surface markers B220, IgM, and IgD should clearly identify whether indeed there is a blockage of B cell trafficking. Should this prove to be the case, it will be necessary to further delineate which surface molecule(s) is altered, then examine the synthetic process of its expression to elucidate the actual mechanism of action of zinc on this portion of ontogeny.

Summary and Conclusions

As indicated in the text, there is an increase in the incidence of low birth-weight (LBW) infants in the U.S. despite the recent advances in neonatal treatment. There may be several factors involved in the generation of LBW, yet they all have an implicit mechanism of action; namely, a decreased nutrient supply to the rapidly developing fetus. Chapter 1 demonstrates that dietary deficiencies of a single trace element, zinc, during gestation can indeed result in growth-retarded offspring in a murine model. The deficiency transmitted to the fetuses was shown to be transient, restricted to day 15-18 of gestation (the mouse has a 19-20 day gestational cycle). The lower zinc content of the marginally zinc deficient (MZD) fetuses impaired growth only at parturition when this occurred on day 19 of gestation, as evidenced by their lower birth-weights. When the fetuses were born on day 20 of gestation however, no differences in weight were evident. The extra day *in utero* provided enough nutrients to promote continued growth, most likely at an accelerated rate since these neonates from MZD dams were able to obtain the same birth-weight. There were no differences in body lengths at birth indicating an asymmetric form of intrauterine growth retardation. This type of growth retardation is the most common form in humans. Thus, the marginal deficiency of zinc *in utero* in the mouse generated offspring with similar physical characteristics to those of the normal term LBW infant. In previous studies with gestational deficiencies of zinc, the surviving offspring exhibited the more severe and less common

symmetrical form of intrauterine growth retardation, and therefore represented a model for a minority of human infants. Whether gestational deficiencies of zinc in humans result in LBW infants remains controversial. However, regardless of whether this is a human problem or not, this system also provides a good model for the determination of how a marginal level of zinc affects immune development.

When immune function was assessed in MZD offspring, significant impairments in antibody mediated responses to a T cell independent antigen were evident at least until puberty. Phenotypic analysis of the subpopulations of lymphocytes in the spleen suggested that this impairment was in part due to an overall reduction of B cells. There may be other contributing mechanisms to this impairment such as enhanced immunosuppression or defective accessory cell function, however no determination of these have been made to date.

Phenotypic analysis also revealed a significant reduction in the number of T helper cells present in the spleen. To determine whether a functional correlate to this was evident, proliferative responses to T cell specific mitogens were assessed in vitro, as well as evaluating the proliferative response to allogeneic cells in a one way MLC. No differences were seen between the MZD and control offspring suggesting in vitro T cell function was intact despite the reductions in helper cells. One problem with this assay was that it was performed on 31 day old offspring, while the latest phenotypic evaluation was done on 22 day old neonates. Thus, it is possible that mitogenic responses may be reduced in younger

offspring. This experiment as well as proliferative responses to B cell specific mitogens are particularly important since these are assays which are quite readily adapted for clinical use in humans. One of the long-term objectives is to develop a clinically useful assay to assess immunocompetence in LBW infants.

In a preliminary experiment to determine the actual mechanism of action of zinc in immune ontogeny, a possible defect in B cell trafficking out of the fetal liver was suggested. This was interesting since one interpretation for the reduced number of B cells in the spleen of MZD neonates was a decrease in B cells homing to the spleen. This perhaps is mediated by surface molecules on the B cells which determine their migratory path. Taken together, one theory for the mechanism of action of the marginal deficiency of zinc during pregnancy is a decrease in the acquisition of cell surface molecules during immune system development in gestation. Briefly, the reduction in zinc content of the MZD fetuses from day 15-18 of gestation coincides with the acquisition of surface Ig and perhaps other molecules involved in trafficking. A transient reduction of zinc during this time may have suspended the development of immature B cells, thereby reducing the number of surface molecules expressed. The replenishment of zinc to the B cells may have allowed continued cycling, but the stage at which one or more specific surface molecules were acquired was passed. Just as the immature B cell is unable to reexpress surface Ig once it has been lost, so too may these cells lose the ability to reexpress other surface molecules necessary for trafficking. Should this prove to be the case, aside

from the clinical significance these studies may have someday, this system may provide one mechanism to generate developmentally arrested B cells. These cells perhaps could be clonally expanded by long-term culturing techniques, which would provide large numbers of cells to address the fundamental biochemical role of zinc in the development of the immune system.

Appendix

Immunology of the fetus and newborn

As indicated in the text, to determine the defects resulting from a marginal deficiency of zinc in utero it is necessary to examine the ontogenic events of the immune system coincident with the timing of the deficiency. Presented in this appendix are reviews on B, T, and macrophage ontogeny. Somewhat related discussions on the state of immunosuppression in the neonate and the role the maternal immune system plays in boosting immunocompetence of the neonate. The focus of the review is on murine ontogeny, except for the last section on maternal contributions, which primarily addresses humans. Many immune ontological developments are similar between the two species, however direct comparisons should be made with caution. Woefully lacking from these reviews are the interactions of interleukins and other soluble factors in the ontogeny of the immune system. This is a complicated area and beyond the scope of this review. Suffice it to say that soluble factors play crucial roles in the maturation of the immune system. It should be clear from the discussions below that immune ontogeny is a complex series of events, each dependent upon certain developments in the preceding compartment. Interruptions of these events may block subsequent stages or result in functionally immature progeny.

B-cell Ontogeny

Ontogeny is defined as the course of development of an individual organism. The ontogeny of the immune system is initially dependent upon hematopoiesis: the production of erythroid, myeloid, and lymphoid (lymphopoiesis) cell types. The initial site of lymphopoiesis during embryogenesis is located in the yolk sac, with subsequent development in the fetal liver and spleen, and finally the bone marrow after birth (100). It is not known in mammals whether stem cells migrate from the yolk sac to the fetal liver, or if lymphopoiesis independently develops intraembryonically as is the case in birds. The primary site of B cell development embryonically in mammals is the fetal liver, with pre-B cells being identified at day 12 of gestation in the mouse (77,100). A large expansion of these pre-B cells occurs in the fetal liver between day 13 and 16 of gestation, with the subsequent rise in surface IgM (sIgM) positive cells at around day 17. Colonization of the bone marrow occurs near the end of gestation, as evidenced by the appearance of 14.8 or B220 positive cells (79). B220 is a large glycoprotein related to the T200 family of proteins expressed on T cells. The 14.8 antibody is believed to be a marker for B cell precursors prior to cytoplasmic μ expression, as well as being found on all B cell populations and a subpopulation of T cells. Cells positive for 14.8 have been reported in the fetal liver at day 11, consistent with its putative role as an early B cell marker (79).

The bifurcation of a subpopulation of early stem cells committed to the B cell lineage and their subsequent transition to the pre-B cell stage is poorly characterized in the fetus, but some advances have been made in the adult bone marrow. Whether the ontological developments which occur in the bone marrow are the same as in the fetal liver remains to be proven. Phenotypic and functional differences have been demonstrated between B cells from the two tissues which indicate that cells from the fetal liver are less mature (77,101,102). It seems reasonable to assume though that the mechanisms of ontogeny are the same, with the populations of pre-B cells in the fetal liver synchronized in a less mature state, whereas the bone marrow contains cells of all stages of development.

Park and Osmond defined three discrete pre-B cell populations in the adult bone marrow which differed in their size and expression of the nuclear enzyme terminal deoxynucleotidyl transferase (TdT), and the cell surface glycoprotein B220 (103). TdT is found predominantly in immature lymphoid cells and is believed to be involved in the generation of immunological diversity (104). All three populations: TdT^+, $B220^-$ cells of 9.5 μm diameter; TdT^+, $B220^+$ cells of 10 μm; and TdT^-, $B220^+$ cells of 11.5 μm diameter, lacked cytoplasmic μ chains and thereby represented pre-pre-B cells technically. It was proposed that this order was the actual order of maturation, subsequently giving rise to cells containing cytoplasmic μ chains. Pre-B cells have previously been reported to be highly heterogeneous in size, presumably due to their active state of proliferation (100). Landreth et al. demonstrated the order of

maturation of B cells in the bone marrow based on size, cytoplasmic μ (cμ) and surface μ (sμ) expression, and amount of tritiated thymidine incorporated (105). By autoradiography and staining with fluorescent antibodies to the μ chain, they showed for the first time that precursor cells which contained no μ chains predominantly fed into the pool of large $c\mu^+$, $s\mu^-$ cells pre B cells. These divided into small $c\mu^+$, $s\mu^-$ pre-B cells before expressing surface Ig giving rise to large $c\mu^+$, $s\mu^+$ virgin B cells. These ultimately gave rise to the small virgin B cell which left the bone marrow to colonize the periphery. The fate of these newly formed B cells in the peripheral lymphoid system is reviewed in (106) and will not be discussed here.

In experiments to determine the status of pre-B cell Ig gene rearrangements in mature bone marrow, Coffman identified another surface molecule which further subdivided pre-B cells (107). All of the cells selected were $B220^+$ pre-B cells as evidenced by the lack of surface Ig expression. The marker ThB stained the small $B220^+$ pre-B cells, which upon further analysis had almost complete rearrangement in the J locus of Ig heavy chain genes, and approximately half the light chain J locus rearrangement seen in mature B cells. Large pre-B cells were ThB^- and lacked any light chain J region rearrangements, but still had near complete heavy chain J rearrangements.

Since Ig heavy chain genes were nearly completely rearranged in large $B220^+$ cells, rearrangement at this locus must occur at very early stages of development, perhaps even prior to B220 expression.

This coincides well with Park and Osmond's findings that TdT$^+$ cells precede B220 surface expression. The disappearance of TdT in the nucleus while the pre-B cell is still in the large proliferative state suggests that TdT function is not a major contributing factor to light chain diversity. Light chain J locus DNA is not rearranged prior to the small pre-B cell stage as defined by ThB$^+$. These studies taken together map out a tentative pathway for B cell development in the adult bone marrow and presumably in the fetal liver. Some progenitor cell as yet to be defined gives rise to TdT$^+$ cells which rearrange their heavy chain genes prior to acquiring surface B220. Nuclear TdT expression is then lost, with the subsequent cytoplasmic expression of μ chains. This cell, which is a large pre-B cell, divides a few times at most (105) resulting in small daughter pre-B cells. These cells are ThB$^+$ and represent the first stage of B cell ontogeny with light chain gene rearrangement. Surface IgM expression occurs in the next stage of development resulting in the rise of the virgin B cell.

IgM is the first immunoglobulin to appear on the surface of murine B cells, whether from the bone marrow or the fetal liver. IgM$^+$ cells have been reported in the fetal liver, spleen and bone marrow at day 17 of gestation (79). In the liver, IgM expression peaks at birth declining thereafter. In the spleen there is a steady rise in IgM$^+$ cells with adult levels (approximately 35% of spleen cells) being reached at 5 weeks of age (108). Ia antigens are rapidly expressed on IgM$^+$ cells after birth, with nearly 95% being Ia$^+$ at 9 days old (80). In the adult bone marrow during

ontogeny, IgD and complement receptor expression (C3) apparently commence after migration to the spleen (106). A subset of IgM^+ cells in the spleen are positive for IgD within a few days after birth (80,108). As with IgM, IgD attains adult levels of expression at around 5 weeks, being coexpressed on the vast majority of mature peripheral IgM^+ B cells (109).

T cell ontogeny

As with B cell ontogeny, there are many similarities between fetal and adult T cell development, but direct correlation of specific events remains to be demonstrated. The site of T cell ontogeny for both is the thymus. The thymus microenvironment is formed in mid gestation; the framework epithelial components coming from the ectoderm and endoderm of the third pharyngeal cleft and pouch at around day 10 of gestation. By day 14 the fetal thymus has developed many of the structural characteristics of the adult thymus such as desmosomes and tonofilaments. Ia+ stromal cells of bone marrow origin first appear at this time in medullary regions, with the rest of the basic thymic architecture being mostly complete by day 17 of gestation. There are three main stromal cell types identified at this time which seem to play critical roles in thymic education and T cell development via direct cell-cell interactions (110,111). The first to be encountered by thymocytes in adults and during fetal ontogeny are $I\text{-}A^-$ macrophages. Interactions with macrophages occur in the cortex and enhance expression of L3T4, Lyt2, and the T cell receptor (TCR). This is believed to aid in

the selection of the T cell repertoire which is necessary prior to thymic education. Thymocytes then migrate to the subcapsular region of the cortex where there reside very large stromal cells termed "thymic nurse cells" or TNC's. These are so-called because up to 200 small T cells are contained within the cell membrane in intimate contact with the plasma membrane of the caveolae, the organelle containing the T cells (111,112). TNC's are MHC class I and II positive and believed to be involved in thymic education. After residence in the nurse cells, the thymocytes migrate into the medullary regions of the thymus and interact with bone marrow-derived dendritic cells which are MHC class I and II positive. These cells reside at the corticomedullary junction, as well as being somewhat dispersed throughout the medulla of the thymus, and appear as early as day 14 of gestation. Interactions with dendritic cells are believed to be necessary for T cell tolerance (111). Antigens found in the maternal circulation are apparently prevented from being processed by thymic dendritic cells of the fetus during gestation and therefore are not available to be tolerized (110).

Colonization of the thymus occurs at day 11 of gestation by a prothymocyte of undescribed phenotype believed to come from either the fetal liver or yolk sac (78). The predominate thymocyte subset at day 14 of gestation is a $Thy1^+$, Ly-1 low density +, L3T4 and Lyt-2 negative T cell which is believed to be the intrathymic stem cell of the adult thymus. This cell, located in the cortex, gives rise to nearly adult levels of $L3T4^+, Lyt2^-$; $L3T4^-, Lyt2^+$; and double positive T cells by day 19 of gestation after interaction with the

stromal cells listed above. TCR gene rearrangements occur in fetal thymocytes at day 14-15 for gamma and ß chains, and sometime after for α chains (78,113,114). Transcription follows shortly afterward. Gamma chain transcription is at maximal levels at day 15, declining slowly through the rest of gestation. Transcription drops off significantly after birth to the very low levels seen in adult thymocytes. The first appearance of complete α/ß TCR's occurs at day 17 of gestation.

The cell type(s) exported by the thymus to the peripheral immune system has not clearly been established (97). It was believed that medullary thymocytes which were phenotypically similar to adult T cells were the only exported T cell from the thymus. However, if the adult thymus exports approximately 10^6 cells per day, and the turnover rate of the total T cell pool is estimated at $< 10^7$ cells per day, it is mathematically impossible to supply the periphery solely with mature T cells (97). Some expansion of the T cell pool therefore must occur extra-thymically, suggesting the existence of a post-thymic precursor (97). Limiting dilution assays have demonstrated the presence of such a cell type in the fetal liver at day 17, in the spleen and marrow at birth, and in lymphoid tissues and marrow in adults (97). These cells phenotypically resembled immature cortical thymocytes (Lyt $1,2,3^+$, $L3T4^+$, $Thy1^+$) and were able to extensively repopulate T cell depleted hosts. Interestingly, a small population of immature cortical thymocytes express very high amounts of MEL-14, a surface molecule believed to be necessary for emigration of thymocytes to the periphery (78). It has yet to be

determined whether these cells represent the post-thymic progenitors which seem to maintain the relatively constant homeostatic levels of T cells in the peripheral immune system.

T cell populations in the spleen after birth increase gradually, reaching adult levels of Thy1, Lyt1, Lyt2, and L3T4 sometime after five weeks of age (108,115). The actual proportion of the different T cell populations seen in the adult are established by two weeks of age (115) though reduced in numbers. The Lyt1,2,3+ immature T cell population reported by Cantor and Boyse to be nearly 100% of splenic T cells early in postnatal life (96) has not been confirmed in subsequent studies using fluorescently labeled monoclonal antibody technology (108,115). However, a recent study with adult human blood has identified a population of immature T4,T8+ T cells (the human equivalent of Lyt1,2,3$^+$,L3T4$^+$ cells in the mouse) which constituted 3% of the total blood population (116). When activated with the T cell mitogen concanavalin A, this population expanded to 60% of the total blood T cells. Perhaps for some reason the mice in Cantor and Boyse's study were mitogenically stimulated resulting in a larger than normal Lyt1,2,3+ population when analyzed. In any event, the peripheral immune system early in postnatal life does not contain an unusually high percentage of immature T cells, indicating two things: 1) the ontological development of thymocytes is likely to be similar in fetal and adult animals, and 2) phenotypically the cells are available for normal immune responses in the neonate, albeit in greatly reduced number compared to adults. Functionally though, neonates have been shown to be relatively

unresponsive in a number of assays (80). The question then becomes, is the lack of response due to lack of numbers, or to some active suppression in the neonate?

Macrophage Ontogeny and the general state of immunosuppression in the neonate

Macrophages are descended from the pluripotential stem cell precursor common to all hematopoietic cells. Lymphoid and myeloid branches segregate early in hematopoiesis, with the myeloid branch ultimately giving rise to megakaryocytes, erythrocytes, and granulocytes. Macrophages are a part of the granulocytic lineage which include neutrophils, eosinophils and others. Macrophages have been reported in the fetal yolk sac at day 10, and in the fetal liver at day 12 of gestation. There is evidence which suggests however, that most macrophages do not become functionally active until sometime after birth in the mouse (reviewed in 80,117). Lu and Unanue have demonstrated that neonatal macrophages are deficient in antigen-presentation capacity mainly due to their lack of surface Ia molecules (117). Ia expression in adult macrophages is dependent in part on the presence of gamma-interferon (INF-gamma) which is released by T cells in response to antigen presentation. Basal levels of Ia bearing macrophages are generated in the absence of INF-gamma during normal ontogeny. These "mature" macrophages initiate a cascade of responses by presenting antigen to T cells activating them to secrete soluble factors such as INF-gamma, which then activates more macrophages to process antigen etc. Ia

expression in the neonate does occur in the presence of INF-gamma indicating that the ability to express Ia is intact. It was believed that lack of Ia expression was due to a general lack of T cells in the neonate. Yet when INF-gamma is injected into neonatal mice, Ia expression is not induced suggesting that the lack of macrophage function is the result of suppressive influences in the neonatal environment. Possible mediators of suppression are neonatal suppressor cells and inhibitory substances such as prostaglandin like molecules and alpha-fetoprotein (AFP). Suppressor cells were demonstrated when neonatal splenocytes were injected intraperitoneally into adult mice. These splenocytes inhibited Ia expression of resident peritoneal macrophages in response to injections of INF-gamma. Suppression was shown to have been mediated by a macrophage/monocyte precursor via prostaglandin release (118).

Murine AFP is a 74 kD glycoprotein present in high amounts in fetal serum, amniotic fluid, and for some time in neonatal serum (reviewed in 80). It represents the second most abundant protein in these fluids next to albumin, to which it has a fair homology, and is present in mg/ml quantities. High amounts are found in these fluids until shortly after birth, at which time the concentration of AFP drops dramatically to near insignificant levels at two weeks of age. In view of its curious normal expression in fetal and newborn serum, and pathological expression in some liver cancers, a role as an immunoregulatory protein was sought. AFP has been shown to inhibit Ia expression in adult macrophages without altering class I molecule or complement receptor expression (117).

This suppression of Ia expression occurred without activating prostaglandin release.

In addition to its role as an inhibitor of macrophage function, AFP has been shown to inhibit antibody mediated responses to T cell dependent but not T independent antigens, as well as mitogenic responses to Con A, PHA, LPS, and a one way MLC (80). While some of the inhibition has been shown to be due to a direct action of AFP on Ia-dependent cell-cell interactions, AFP has also been shown to activate a neonatal suppressor cell of unique morphology. Unlike most T suppressor cells which are Lyt 1^-, Lyt $2,3^+$, and MHC Class II^-, this neonatal suppressor is Lyt 1^+, $2,3^-$, and class II $I\text{-}J^+$. These cells are found in large quantities in the thymus and are characteristically blast-like in appearance. They have been implicated in direct suppression of T helper cells and can be induced in adult splenocytes by culturing in the presence of antigen and AFP for four days.

The importance of immunosuppression as the principal reason for neonatal immunoincompetence has been challenged recently (97,119) particularly in regards to human neonates. These authors argue that the lack of immune responsiveness in neonates can be explained mostly by a lack of mature cells able to respond. This is in general agreement with flow cytometric data which indicate that adult-like percentages of neither T nor B cells are achieved in the murine spleen until sometime after six weeks of age (108,115). However, the existence of suppressor cells is acknowledged as a contributing factor even by these opponents. Proponents of fetal/neonatal

suppression (80,117) postulate that AFP and other suppressive mechanisms during ontogeny are necessary for two reasons: 1) the suppression of TD responses and the expression of Ia molecules on macrophages may be necessary for self tolerance. Circulating antigens during fetal life and to some extent neonatal life are primarily self antigens, which accordingly must not elicit immune responses in order for the animal to survive. The absence of this tolerizing period could conceivably lead to autoimmune states; 2), the fetus is basically an allograft and therefore subject to rejection by the mother. The presence of AFP and perhaps other immunosuppressive substances in the amniotic fluid are necessary then to reduce the chances of rejection and allow survival of the graft (i.e. the fetus). For in depth discussions on the immune interactions at the maternal-fetal joining at the placenta the reader is referred to two volumes on the subject edited by Gill and Wegmann (120,121). These adaptive processes of suppression then are necessary for survival even though they leave the neonate in a compromised state for some time after birth. Other adaptive mechanisms have been developed by the mother though to help protect the neonate from the onslaught of pathogens encountered in postpartum life.

Maternal contributions to neonatal immunocompetence

One mechanism developed in the pregnant female to protect the immunoincompetent fetus is the transfer of immunoglobulins via the placenta. Maternal IgG is transferred in increasing amounts with

gestational age, resulting in near adult serum levels of this immunoglobulin at birth in the neonate. Preterm infants, those born prior to 37 weeks of gestation in humans, are in part compromised immunologically due to the paucity of antibodies transferred prior to the premature birth (122). The role of IgG has not been completely identified as yet but is certainly related to boosting neonatal protection until endogenous production of Ig occurs. IgG is the longest-lived of all of the classes of Ig, remaining in the serum of the neonate for about one month after birth. Of particular interest is the theory that some maternal immunoglobulins transferred to the fetus via the placenta and to the neonate via breast milk may be anti-idiotypic to antibodies against certain pathogens (123,124). Anti-idiotypic antibodies are formed against the antigen-combining region of another antibody, and therefore are exact copies of the antigenic region the primary antibody was originally made to. If this is indeed the case, then maternal immunoglobulins may actually immunize the offspring without the pathogen actually being present.

Maternal contributions to immunity after birth in many animals including mice and humans are transferred via breast milk and are of both nonimmune and immune origin (125,126). In humans, growth factors for lactobacilli promote the growth of these harmless bacteria in the gut of the neonate. This creates a highly acidic pH environment which is suppressive for other more pathogenic bacteria (e.g. Enterobacteriaceae, Clostridia, and Bacteroides). Certain unsaturated free fatty acids, monoglycerides, proteins, and

oligosaccharides have been shown to act as antiviral and antibacterial factors in nonimmune mechanisms. Lactoferrin and lysozyme are also transferred in relatively high amounts during lactation. Lactoferrin binds iron, preventing bacterial use, while lysozyme is a potent lytic enzyme for bacterial cell wall proteins.

Extremely large amounts of IgA, mainly in the form of secretory IgA (SIgA), are contained in the milk, as well as some IgM, IgG, and IgD. SIgA is particularly well suited for survival in the hostile environment of the GI tract, being highly resistent to enzymatic degradation due to the presence of secretory component. Many of these antibodies are to bacterial viral antigens commonly found in the gut and respiratory tract. In addition to their role in immunity, breast milk antibodies also provide some protection against food allergies by binding certain proteins and preventing their intestinal absorption. Finally, there are large amounts of leukocytes in milk early in lactation. Lymphocytes comprise only 5-10% of these cells, the vast majority of which are T cells (125). The rest are made up of equal numbers of macrophages and neutrophils. Some studies in animals have indicated that these cells remain competent for some time despite the highly acidic conditions. Maternally derived T cells have been found in the serum of newborn mice and human neonates in significant numbers (up to 10%) (126). Cell-mediated immunity may be conferred by the T cells; phagocytosis and intracellular killing by the macrophages and neutrophils does occur, but not to the same extent as blood phagocytic cells. The role for these immune cells in protecting the neonate is not

completely understood. Thus, there exist several mechanisms by which passive immunity is conferred on the neonate to help defend against common pathogens.

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