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Suppression of Antibody Response by Excess Dietary Zinc Exposure during Certain Stages of Ontogeny (42202)

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Abstract. A study was made of the effects of excess dietary zinc on the antibody response to sheep red blood cells (SRBC) in mice. C57BL/6J mice were divided into 10 different dietary groups and exposed to diets containing zinc in normal (50 ppm) or excess (2000 ppm) concentrations during gestation/lactation/postweaning development in the sequences (1) 50/50/50; (2) 50/50/2000; (3) 2000/50/50; (4) 2000/2000/50; (5) 2000/50/2000; (6) 50/2000/50; (7) 50/2000/2000; (8) 2000/2000/2000; (9) 50/50/50 (pair-fed); and (10) chow/chow/chow. Mice in group 8 had severe signs of copper deficiency at 8 weeks of age, such as reduced plasma copper, lowered plasma hematocrit, and achromotrichia. Mice receiving 2000 ppm zinc during gestation had fewer offspring per litter (measured at 2 weeks of age) and more nonviable births than mice given 50 ppm zinc during gestation. The growth curve of mice exposed to excess zinc in the 50/50/2000 group was identical to that of the control (50/50/50) group. Growth curves for all other groups were reduced by varying amounts. The plaque-forming cell response to SRBC was reduced only in the groups receiving 50/2000/2000 and 2000/2000/2000 ppm zinc ($P < 0.05$); this reduced response was not associated with atrophy of the lymphoid organs. Splenic cell surface markers and mitogenic responsiveness were similar in the 50/50/50 and 2000/2000/2000 groups. These results suggest that the immune response is more susceptible to dietary manipulation during development than after the immune response has been developed.

Nutritional surveys have indicated that large numbers of children in developing countries are malnourished. The problem of malnutrition also exists in the developed world. Clinical studies have suggested that humans develop long-term immunologic defects after *in utero* or early postnatal malnutrition (1). Furthermore, the immunologic defects observed in children with intrauterine malnutrition can be reversed only with great difficulty, in contrast to the more readily reversible defects due to postweaning malnutrition (2, 3).

Studies with animals have also linked nutrient deprivation with impaired immunologic function. The advantage of animal studies lies in the ability to study single-nutrient deficiencies and toxicities. In addition, the timing of the impact of the deficiencies and toxicities can be carefully controlled. Another advantage in animal studies lies in the ability to use the inbred mouse to study the immunologic system. Since inbred mice are genetically identical, fluctuations in immune reactivity between individual animals are minimized. Studies with mice have demonstrated that deficient levels of some trace elements and vitamins during postweaning development lead to a suppressed immune response and an increased susceptibility to infection (4–6). Studies on perinatal malnutrition have yielded similar results (7–9). In addition, intrauterine malnutrition can persist for many generations (10, 11). Consequently, it is important to explore further the impact of nutritionally compromised diets on the immune response during different stages of development, since the timing of the nutritional deficiencies plays a significant role in determining the severity and reversibility of the resulting immunodeficiency.

The increased fortification of foods and the consumption of vitamin and mineral supplements, on the other hand, raise questions about the safety of excess nutrients. The safety of excess zinc is particularly important to determine because of the use of excess zinc in the form of single supplements rather than multiple supplements. Excess zinc is not only

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consumed in the form of supplements but is also used therapeutically for the treatment of coeliac disease (12), Wilson's disease (13), and in the prevention of copper toxicosis in sheep (14). One case of heritable elevated plasma zinc with no apparent clinical symptoms or abnormalities has been reported (15). Adults with sickle cell anemia developed copper deficiency when treated with zinc for 2 years; the hypocupremia was associated with microcytosis and relative neutropenia (16). Excess zinc has also been reported to cause immunosuppression in the mink (17).

Because of these uses of excess zinc and their possible harmful effect on humans and animals, we deemed it important to study the effect of excess zinc on the immune response. The present work was designed to determine which period is more susceptible to dietary manipulation: gestation, lactation, or postweaning development, or a combination of some or all of these periods.

Materials and Methods. *Animals.* Female weanling C57BL/6J mice (first generation) were obtained from the Jackson Laboratory (Bar Harbor, Maine) at 21 days of age, matched by weight, and placed into three different dietary groups receiving chow or either 50 or 2000 ppm zinc. At approximately 6 weeks of age they were bred with age-matched C57BL/6J males. To study the effect of excess zinc during gestation/lactation/postweaning development, the dams and offspring (second generation) were further distributed into ten different dietary groups which received the following diets during gestation/lactation/postweaning development: (1) 50/50/50 (control); (2) 50/50/2000; (3) 2000/50/50; (4) 2000/2000/50; (5) 2000/50/2000; (6) 50/2000/50; (7) 50/2000/2000; (8) 2000/2000/2000; (9) 50/50/50 (pair-fed during postweaning development with group 8); and (10) chow/chow/chow. The dams exposed to the 50- or 2000-ppm diets during postweaning development were placed into groups that required the same diet during gestation. The exposure periods of the dams before mating are not included in the sequence of exposure periods of the offspring. The method of assignment was by animal weight on an APL random numbers generator.

Housing of animals. First generation mice were housed in plastic cages equipped with stainless-steel covers and stainless-steel raised wire floors. Pregnant dams were housed individually in plastic cages equipped with stainless-steel covers and pine chips on the floor. The room was equipped with laminar flow systems. Water bottles were fitted with polyethylene hollow stoppers and stainless-steel sipper tubes; double-distilled water was used throughout the study.

Diets. All mice were fed *ad libitum* (except the pair-fed group) a biotin-fortified egg white diet that contained either normal (50 ppm) or excess (2000 ppm) levels of zinc. The diets, which were formulated and prepared by Teklad Test Diets (Madison, Wisconsin), consisted of egg white solid, spray-dried, 200 g/kg; biotin, 0.004 g/kg; L-tryptophan, 0.6 g/kg; dextrose, technical, monohydrate, 627.37 g/kg; corn oil, 100 g/kg; nonnutritive fiber (cellulose), 30 g/kg; and vitamin mixture AIN-76, 1%. Since this vitamin mixture did not include a source of choline, choline bitartrate was added at 10 g/kg. Salts were added to supply the mineral requirements of the mouse as recommended by the National Research Council (18). Both diets were analyzed for zinc by atomic absorption spectrophotometry. Certified rodent chow 5002 (Ralston Purina Company, St. Louis, Missouri) containing 52 ppm zinc was fed to group 10.

Copper, zinc, and hematocrit determinations. Tibia zinc was determined by neutron activation analysis. Gamma-ray spectra were recorded by using a Model 6620 minicomputer-based analyzer system (19) (Nuclear Data, Inc., Schaumburg, Ill.). Plasma copper was determined by atomic absorption spectrophotometry (Model 5000, flame absorption, equipped with a bead impact spoiler, Perkin-Elmer Corp., Norwalk, Conn.). Hematocrit determinations were made by an Adams microhematocrit centrifuge and reader.

Plaque-forming cell assay. At approximately 8 weeks of age, mice were injected ip with 0.5 ml of a 2% solution of sheep red blood cells (SRBC). Erythrocytes from a single sheep, chosen from among several tested for its ability to produce a good antibody response, were supplied from Cleveland Scientific (Cleveland, Ohio). After 5 days of exposure to SRBC, the mice were sacrificed by cervical dislocation and individual spleens were removed and

teased in RPMI-1640 culture medium. Cell concentrations were determined on a Coulter Counter Model Z_F (Coulter Electronics, Hialeah, Fla.). Staining with trypan blue showed that suspensions contained 90–95% viable cells. Individual spleen cells were assayed for SRBC-specific IgM response by the Cunningham–Szenberg modification of Jerne's hemolytic plaque assay (20). Initial studies demonstrated that the response to SRBC was optimal at Day 5. The background PFC response for mice not immunized with SRBC was 60 PFC/10^6 spleen cells.

Mitogen-induced proliferation. Aliquots consisting of 0.1 ml mitogens and 0.1 ml of 2×10^5 lymphocytes in medium (RPMI-1640 supplemented with 10% fetal calf serum, 2 m*M* glutamine, 25 m*M* Hepes buffer, 100 units penicillin/ml, and 100 μg streptomycin/ml) were added to triplicate wells of 96-well Linbro round-bottomed microtiter trays (Flow Laboratories, McLean, Va.). The cultures were incubated for 72 hr at 37°C in a humidified atmosphere of 5% CO_2 in air. After a 5-hr pulse with 1.0 μCi of [^{3}H]methyl thymidine (sp act 6.17 Ci/mmole, New England Nuclear, Boston, Mass.), the cells were harvested onto glass fiber filter paper. The amount of incorporated radioactivity was determined with a Packard liquid scintillation counter and expressed as counts per minute.

Cell sorter analysis. Briefly, 3×10^6 spleen cells were prepared in 150 μl of RPMI-1640 containing 1 m*M* Hepes, 0.01% NaN_3, and 10% fetal calf serum and incubated with fluorescein-conjugated specific antibody (goat) to mouse IgG (H + L) (Litton Bionetics, Kensington, Md.) or directly fluorescein-conjugated anti-Thy-1.2, anti-Lyt-1, or anti-Lyt-2 (Becton Dickinson, Mountain View, Calif.) for 20 min on ice. After washing two times, antibody-conjugated cells were analyzed on a Coulter EPICS V system (Coulter Electronics).

Statistical analysis. The data were tested by analysis of variance (ANOVA). If the outcome of the ANOVA was significant (i.e., the *P* value of the *F*-ratio was <0.10), a "protected" least significant difference (LSD) test (21), a two-sided test with $P < 0.05$ for significance, was performed.

Results. *Reproductive performance of C57-BL/6J mice exposed to normal or excess zinc during gestation.* The results shown in Table I demonstrate that the mean time to produce a litter, the percentage of productive matings, and the sex ratios of offspring in both groups were equivalent. The average weight of the mothers at the time of conception was 18 g. The number of litters that died for any reason within 4 days after birth was significantly higher and the litter size at approximately 2 weeks of age was significantly lower in the group receiving the excess zinc diet. The litter size was not determined until the offspring were 2 weeks of age so as not to disturb the mother before that time. Most of the nonviable litters were observed as stillbirths or were cannibalized by the mother within 24 hr after birth. No anatomical malformations were evident in any of the offspring.

Growth curves of second generation female mice exposed to normal or excess zinc during different stages of ontogeny. Mice were first weighed at 1–3 weeks of age. As Fig. 1 indi-

TABLE I. REPRODUCTIVE PERFORMANCE OF FIRST GENERATION FEMALE MICE[a]

Dietary Zn (ppm)	Mating to retirement (weeks)[b]	Productive matings (%)[c]	Nonviable litters (%)[d]	Litter size (2 weeks)[e]	Sex ratio at weaning (% males)
50	10	18	25	7	51
2000	10	17	59[f]	4.5[f]	52

[a] Values are based on 284 and 235 female mice given diets containing 50 and 2000 ppm zinc, respectively, during gestation.

[b] Weeks from the time of mating (6 weeks) to the time of sacrifice (when offspring were 4 weeks old).

[c] Matings were determined by palpation after the tenth day of gestation.

[d] A nonviable litter is defined as a litter which was aborted, stillborn, killed by the mother, or died for any other reason within 4 days of birth.

[e] Litter size was observed at approximatley 2 weeks of age.

[f] Significantly different from value for the control group (50 ppm Zn), $P < 0.05$.

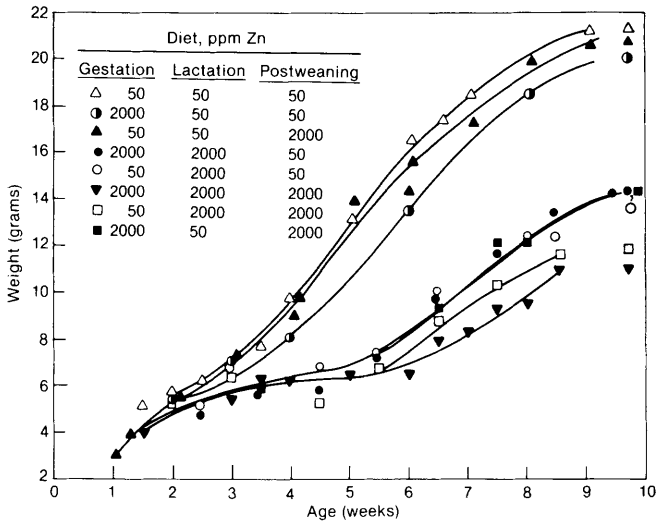

FIG. 1. Mean body weight of C57BL/6J second generation mice exposed to 2000 and 50 ppm zinc during different stages of ontogeny. Mice were exposed to diets containing zinc in normal (50 ppm) or excess (2000 ppm) levels during gestation/lactation/postweaning development in the sequences indicated in the figure. The symbol on the far right corresponds to the curve for that sequence. Each point represents the mean of at least seven females from three to six litters per treatment group.

cates, the mice exposed to 50/50/50 and 50/50/2000 ppm zinc had nearly identical growth curves. Mice exposed to the chow diet also had growth curves similar to these groups (data not shown). Mice exposed to 2000/50/50 ppm zinc had a slightly reduced postweaning growth curve, indicating that exposure to excess zinc during gestation reduced postweaning growth. The growth curve was reduced to a greater extent for mice exposed to 2000/50/2000, 50/2000/50, and 2000/2000/50 ppm zinc, and was lowest for mice exposed to 2000/2000/2000 and 50/2000/2000 ppm zinc. Similar results were obtained with second generation male mice; however, the absolute weight for each male group was slightly higher than that of the equivalent female group.

Hair pigmentation in second generation mice exposed to excess zinc during different stages of ontogeny. Mice exposed to the different dietary regimens all had normal hair pigmentation at 2 weeks of age. However, at 8 weeks of age, mice in groups 2, 4, 5, 6, 7, and 8 had varying degrees of achromotrichia. The achromotrichia was most severe in the 50/2000/2000 and 2000/2000/2000 groups.

Effect of excess dietary zinc on body and organ weights of second generation mice. As shown in Table II, 8-week-old second generation mice exposed to 2000 ppm zinc throughout gestation, lactation, and postweaning development had reduced body and spleen weights compared with mice exposed to 50 ppm zinc. However, the spleen weight of the second generation mice was reduced in proportion to the reduction in body weight, indicating that this lymphoid organ was not atrophied. In contrast, the weights of the thymuses were greater than those of age-matched control mice, but the difference was not significant.

Effect of excess dietary zinc on second generation tibia zinc levels, plasma copper levels, and hematocrit values of second generation mice. The levels of tibia zinc in mice exposed to diets containing either 50 or 2000 ppm zinc during gestation, lactation, and postweaning development reflected dietary zinc intake (Table III). Plasma mineral analysis indicated that the mice exposed to excess zinc (2000 ppm) throughout development had reduced copper levels and hematocrit values ($P < 0.05$).

Direct plaque-forming cell response of second generation mice to sheep red blood cells. The *in vivo* primary responses to SRBC of mice exposed to excess dietary zinc during dif-

TABLE II. EFFECT OF EXCESS DIETARY ZINC DURING GESTATION/LACTATION/POSTWEANING ON BODY, SPLEEN, AND THYMUS WEIGHTS OF SECOND GENERATION MICE[a]

Dietary Zn (ppm)	Body weight (g)	Thymus weight (g)	Spleen weight (g)
50/50/50	21.27 ± 2.03 (M) 17.25 ± 0.64 (F)	0.0293 ± 0.0066	0.0954 ± 0.0280
2000/2000/2000	14.05 ± 2.55[b] (M) 12.07 ± 2.54[b] (F)	0.0499 ± 0.0199	0.0445 ± 0.0166[b]

[a] Values are means ± SD of seven 8-week-old males or females for body weights and 14 males and females for thymus and spleen weights. These values represent the average of two litters for the 50/50/50 group and three litters for the 2000/2000/2000 group. The groups contained equal numbers of males and females. These were the only groups on which thymus and spleen weights were determined.

[b] Significantly different from value for the control group (50/50/50), $P < 0.05$.

ferent stages of ontogeny are shown in Fig. 2. Each group receiving experimental diets was assayed with an age-matched control (50/50/50) ppm zinc group to correct for any day-to-day variability in the assay. The data presented in Fig. 2 indicate that the PFC/10^6 spleen cells of mice exposed to diets containing either 2000/2000/2000 or 50/2000/2000 ppm zinc sequence were reduced in comparison to the PFC response of the other groups ($P < 0.05$).

Mitogen-induced lymphocyte proliferation of second generation mice exposed to 50 or 2000 ppm zinc. Lymphocyte stimulation of second generation mice exposed to 50 or 2000 ppm zinc during gestation, lactation, and postweaning development is shown in Fig. 3. Lipopolysaccharide (LPS) stimulates B cells (22) and staphylococcal enterotoxin A (SEA) and phytohemagglutinin P (PHA) stimulate T_1 and T_2 cells, respectively (23), whereas concanavalin A (ConA) stimulates all T cells. The background incorporation in all unstimulated cultures was less than 500 cpm. The results indicate that there was no significant alteration ($P < 0.05$) in the response of the splenic B and T cells from second generation mice fed the diets containing the two different levels of zinc.

Quantitative cellular expression of B and T cell markers on splenic lymphocytes of second generation mice exposed to 50 or 2000 ppm zinc. The directly fluorescein-conjugated anti-Lyt-1, anti-Lyt-2, anti-Thy-1.2, and anti-mouse IgG were incubated with splenic lymphocytes of mice exposed to 50 or 2000 ppm zinc throughout gestation, lactation, and postweaning development and analyzed on the EPICS V system. The results summarized in Table IV show that the proportion of cells expressing these surface markers was similar in both groups. The histograms were also similar (data not shown), indicating that the density of these markers was similar on the lymphocytes from each of these two groups.

TABLE III. EFFECT OF EXCESS DIETARY ZINC ON TIBIA ZINC LEVELS, PLASMA COPPER LEVELS, AND HEMATOCRIT VALUES OF 8-WEEK-OLD SECOND GENERATION MICE[a]

Measurement	Dietary Zn (ppm) 50/50/50	2000/2000/2000
Zinc, μg/g dry bone	179.29 ± 13.29	661.00 ± 29.83[b]
Copper, μg/100 ml	137.00 ± 29.55	47.67 ± 45.84[b]
Hematocrit, %	37.40 ± 4.13	22.25 ± 2.06[b]

[a] In the 50/50/50 group, values are means ± SD of 12 mice from three litters for zinc and copper and of six mice from two litters for hematocrit. In the 2000/2000/2000 group, values are means ± SD of 13 mice from four litters for each statistical analysis.

[b] Significantly different from control values ($P < 0.05$).

Discussion. Mice exposed to the excess zinc during gestation, lactation, and postweaning development had severe signs of copper deficiency, such as reduced copper plasma, lowered hematocrit, and achromotrichia at 8 weeks of age. It must be noted that the dams of this group were exposed to excess zinc from the time they were weaned; this was done in order to produce a mineral deficiency during gestation. If these dams had been exposed to excess zinc only from the time of gestation, their copper stores may not have been as depleted since they would have been exposed to excess zinc for a shorter period of time. The added exposure period used in this study could

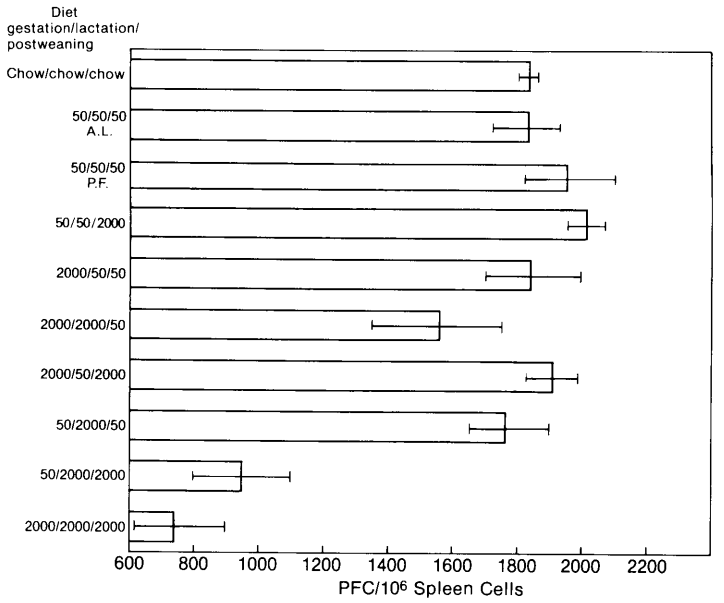

FIG. 2. IgM plaque-forming cell (PFC) response of second generation C57BL/6J mice exposed to a chow diet or diets containing 50 and/or 2000 ppm zinc during different stages of ontogeny. Each bar represents the mean ± SEM of at least 11 mice with an average of 13 mice from three to six litters per treatment group. The PFC responses of mice exposed to the dietary sequences 50/2000/2000 and 2000/2000/2000 ppm zinc were significantly reduced ($P < 0.05$) when compared with the value from the control (50/50/50 ppm zinc) group.

therefore have resulted in lower copper levels available to the offspring during gestation and lactation. All of the other groups exposed to excess zinc at some period during development (with the exception of the group exposed only during gestation) had achromotrichia by 8 weeks of age; the achromotrichia was most severe in the two groups that had the reduced immune response. Achromotrichia is thought to be due to the effect of copper deficiency on the activity of tyrosinase, the first enzyme in the melanin biosynthesis pathway (24). High maternal dietary zinc resulted in a reduction in both the viability and litter size of offspring. Fetal resorptions were not determined but the smaller litter size of offspring of dams exposed to excess dietary zinc could be due to resorptions. Neonatal mortality could also account for this observation since the litter size was not determined until 2 weeks of age. All groups exposed to excess dietary zinc except the 50/50/2000 ppm zinc group had reduced growth rates, although the weights in all of the groups were approximately the same until the animals were approximately 2 weeks of age.

From the antibody response data in this study, excess dietary zinc is detrimental to the immune response only when administered during gestation/lactation/postweaning development or lactation/postweaning development. Since restricted dietary intake has no significant effect on the antibody response to SRBC, the reduced antibody response cannot be due to reduced feed intake. From the achromotrichia data, it is reasonable to assume that these two groups had the most severe mineral deficiency at 8 weeks of age. Since the pigmentation was normal in these groups at 2 weeks of age, the dam (gestation/lactation/postweaning) may have had adequate copper stores to supply the fetus during gestation but not throughout lactation. In addition, both groups of nurslings could have received adequate nutrition at the beginning of lactation, but a reduction in milk supply and lowered absorption of copper due to the excess dietary zinc (25) could account for these observations. At this point it is not possible to determine if the reduced immune response is due to one or a combination of nutrient deficiencies and

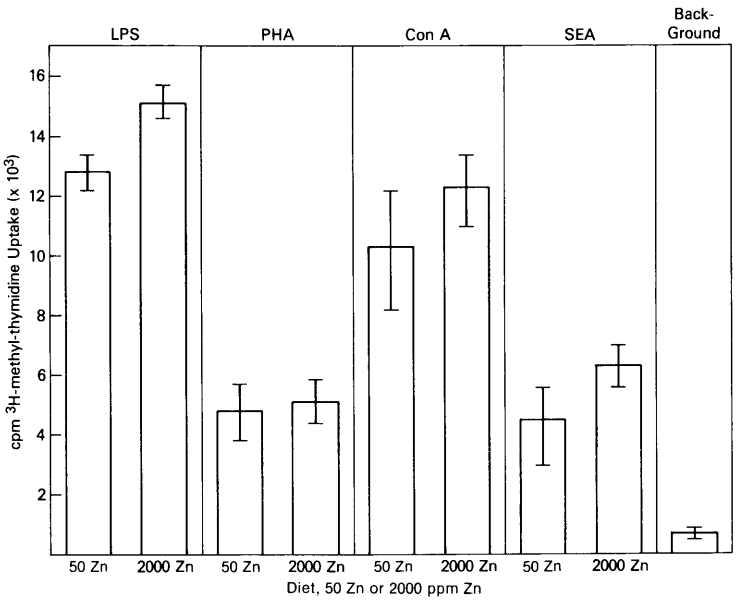

FIG. 3. Lymphocyte stimulation of 8-week-old mice exposed to 50 or 2000 ppm zinc during gestation, lactation, and postweaning development. Mitogen stimulation was performed as described under Materials and Methods. Each bar represents the mean ± SE for six or seven mice from two to three litters per group. This figure represents one of three experiments with similar results. All mitogens were added to triplicate wells. The following mitogen concentrations per well were determined by titration to produce maximum lymphocyte stimulation: 25 μg lipopolysaccharide (LPS) (*Escherichia coli* 055:B5 Westphal, Difco Laboratories, Detroit, Mich.); 25 μg phytohemagglutinin P (PHA) (Burroughs-Wellcome, Research Triangle Park, N.C.); 1.0 μg concanavalin A (ConA), 3 times crystallized (Miles Biochemicals, Elkart, Ind.); and 0.1 μg staphylococcal enterotoxin A (SEA) (kindly provided by R. Bennett, FDA). There is no significant difference ($P < 0.05$) between the two different dietary groups.

toxicities caused by excess zinc. Trace element analysis of all the groups must be performed in order to establish this.

The mechanism by which the immune response is suppressed in mice exposed to excess zinc throughout development appears to be different from that of zinc deficiency. Zinc-deprived mice have reduced mitogen responsiveness (26) and an alteration in the various subsets of B and T cells (7), in contrast to the mice in this study. Also, mice exposed to excess zinc do not have an atrophied thymic organ, as do mice given either zinc-deficient diets or diets with adequate zinc but restricted in amount of food intake (27). Since the weights of the thymuses in mice exposed to excess zinc were actually greater (as a percentage of body weight) than the thymus weights of age-matched control mice, it can be postulated that the normal process of involution has not yet occurred in these mice, which suggests an undeveloped immune system. If the dam were unable to provide adequate nutrition during the latter part of lactation, the immune response in these mice may have developed normally until approximately 2 weeks of age, after which time an arrest or delay in the normal development may have occurred. If maturation of the immune response is delayed, the immune response in these mice would be more like that of the young mouse whose response to SRBC does not appear until approximately 2 weeks of age and gradually increases until adult levels are reached (28). Studies have demonstrated that the cell surface markers Thy, Lyt-1, Lyt-2, and Lyt-3 are similar in spleen cell populations of 14-day-old and adult mice (29). In addition, splenic lymphocytes from 2-week-old mice respond to both B and T cell-dependent mitogens and there are no striking changes in mitogenic response between the two groups with time (30). The data

TABLE IV. FREQUENCY (%) OF QUANTITATIVE CELLULAR EXPRESSION OF SPLENIC B AND T CELL SURFACE MARKERS IN MICE EXPOSED TO EXCESS ZINC[a]

	Dietary Zn (ppm)	
Antibody	50/50/50	2000/2000/2000
anti-IgG	64.8 ± 1.5	60.0 ± 2.8
anti-Thy-1.2	37.5 ± 2.5	39.5 ± 1.9
anti-Lyt-1	31.6 ± 2.1	31.0 ± 3.6
anti-Lyt-2	13.7 ± 2.1	12.8 ± 1.4

[a] Spleen cells from 8-week-old mice were stained directly with fluorescein-conjugated antibodies and analyzed on a Coulter EPICS V cell sorter. Cell frequencies were determined by integration of cells under the positive peak in Coulter EPICS V profiles. The histogram for each surface marker was the same in both groups. Values are means ± SD of 16 mice from three litters and 14 mice from four litters in the 50/50/50 and 2000/2000/2000 groups, respectively.

presented in this paper support the hypothesis that splenic lymphocytes from 8-week-old mice in the excess zinc group are similar to normal young mice with respect to antibody response to a T-dependent antigen, distribution of cell surface markers (Ig, Thy, Lyt-1, and Lyt-2), and mitogen responsiveness. At this point, more work is required with the use of B and T cell differentiation and maturation markers to discern if the defect in the immune response of the mice exposed to excess dietary zinc resides in a B and/or T cell or one of their products. In addition, studies on the effects of the aging process on lymphocyte reactivity are required to discern if the defect is due to a delay in maturation or a total arrest in maturation.

Further studies on the effect of nutrient excesses during ontogeny could lead to the ability to determine the most critical period for nutrient supplementation. In addition, the levels of nutrient supplementation which are deleterious to the immune response need to be established to avoid the possibility that early supplementation may result in a suppressed immune response that could persist for many generations.

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