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Cell-Mediated Cytotoxicity and Tumor Growth in Zinc-Deficient Mice[1] (41174)

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Abstract. Dietary-induced zinc deficiency results in an impaired cell-mediated immune response to non-H2 allogenic tumor cells in mice. Animals maintained on a zinc-deficient diet for as little as 2 weeks develop a severe impairment in their ability to generate a cytotoxic response in the face of tumor challenge. This impairment is totally reversible by returning zinc-deficient mice to normal dietary zinc intake. Such animals now demonstrate a normal cytotoxic response to tumor challenge. If mice are treated with toxic doses of dietary zinc, a similar impairment of the cell-mediated cytotoxic response occurs. This suggests that either a deficiency or toxic level of zinc impairs the immune response to allogeneic tumor cells.

The role of zinc in human metabolism is now well established (1). Zinc is essential for protein synthesis as well as for the production of both deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) (1). Since immune responses rely on cell replication, the role of zinc in the generation of these responses has become a subject of interest. We and others have already demonstrated that zinc deficiency results in a marked decrease in lymphoid tissue weight, associated with a concomitant decrease in the humoral immune response (2–5). More recently, Fernandes *et al.* (6) reported a decrease in both T-cell and natural killer cell activity in zinc-deficient animals. The purpose of this paper is to report our data with respect to the effect of zinc deficiency on the generation of a cell-mediated cytotoxic response and on tumor growth in mice. In addition, we also report here on the effect of toxic doses of zinc on the cell-mediated cytotoxic (CMC) response.

Materials and Methods. *Experimental design.* Male, Balb/c mice aged 5 weeks were obtained from Jackson Laboratory, Bar Harbor, Maine. The mice were maintained on Purina Lab Chow until placed on a zinc-deficient diet. The overall experimental design is presented in Table I. The animals were fed a semipurified diet containing 1.6% calcium, 0.6% phosphorus, 0.4% phytate, and 2 ppm zinc (7). The composition of the basal diet is presented in Table II. The zinc-deficient group (Group A) received (*ad libitum*) the basal diet with additional 0.2% phytic acid sodium salt (total phytate of 0.4%). Phytic acid at this level is not toxic and is known to accentuate deficiency of zinc and therefore has been routinely used for this purpose (7–9). Deionized water was supplied *ad libitum*.

The mice were randomly allotted by weight into four main experimental Groups A, C, E, and F, and two subgroups B and D (Table I). Group A received *ad libitum* zinc-deficient diet throughout. After 11 days, five mice from this group were randomly selected (Group B) and offered the control diet (110 ppm Zn) for the next 13 days. This was designed to determine if the defective cytotoxic response that was induced by zinc deficiency could be reversed by dietary zinc supplementation. Group C, the pair-fed (PF) control mice, received the 110 ppm zinc diet in an amount equal to the food consumed by the zinc-deficient group during the previous 24 hr. After 11 days, five mice were randomly selected from this

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TABLE I. EXPERIMENTAL DESIGN

Group	Number of mice per group	Diet[a]	Days on diet (range)	Injection day[b]	Assay day(s)[c]
A	30	−Zn	1−17	1	4,7,9,11, 14, and 17
B	5	−Zn	1−11	14	25
		+Zn(PF)	12−25	14	25
C	30	+Zn(PF)	1−17	1	4,7,9,11, 14, and 17
D	5	+Zn(PF)	1−11	14	25
		HiZn(PF)	12−25	14	25
E	30	+Zn(*ad lib*)	1−17	1	4,7,9,11, 14, and 17
F	30	+Zn(*ad lib*)	1−17	Not injected	4,7,9,11, 14, and 17

[a] −Zn: zinc-deficient diet (2 ppm Zn); +Zn: control diet (110 ppm Zn) HiZn: diet with toxic level of zinc (5000 ppm); PF: pair-fed.
[b] Subcutaneous injection of 5 × 10 MDAY-D2 tumor cells in 0.1 ml of saline.
[c] Tibias were also assayed for zinc.

group and offered the high zinc diet supplemented (with zinc sulfate) to contain 5000 ppm zinc (Group D). This was designed to examine the effect of toxic level of dietary zinc on cytotoxic ability of the animals. Group E was offered *ad libitum* control diet (110 ppm Zn) in order to determine the contribution of the amount of food consumed on the cytotoxic response of challenged mice. Group F consisted of *ad libitum*-fed (110 ppm zinc diet) unchallenged mice.

TABLE II. COMPOSITION OF BASAL DIET

Ingredient	
Glucose hydrate (Cerelose)	57.55
Soybean assay protein[a]	21.25
Mineral premix[b]	10.00
Vitamins A, D, E, K, in corn oil[c]	1.00
Vitamin B complex[d]	1.00
Choline Cl (50% solution)	0.20
Corn oil	9.00

[a] Washed with EDTA for preparation of zinc-deficient diet.
[b] Supplied the following (g/kg of diet): $CaCo_3$, 10.62; $CaHPO_4$, $2H_2O$, 16.45; $MgCO_3$, 0.98; $MgSO_4$, $7H_2O$, 1.18; NaCl, 5.02; KCl, 0.83; $FeSO_4$, $2H_2O$, 0.78; KH_2PO_4, 12.48; $MnSO_4$, H_2O, 0.75; $CuSO_4$, 0.04; KIO_3, 0.032; $CoCl_2$, $6H_2O$, 0.0024; NaF, 0.039; $Cr_2(SO_4)_3$, nH_2O (19.4% Cr), 0.0257.
[c] Supplied the following (mg/kg of diet): α-tocopherol acetate, 30; menadione, 10; vitamin A acetate, 25,000 IU; vitamin D, 3300 IU, and butylated hydroxyanisole, 100 IU.
[d] Supplied the following (mg/kg of diet): thiamine hydrochloride, 16; riboflavin, 16; pyridoxine HCl, 16; Ca pantothenate, 40; biotin, 0.2; cyanocobalamin, 0.05; folic acid, 5; nicotinamide, 50; and methionine, 2 g/kg of diet.

Tumor. The tumor chosen for these experiments was the MDAY-D2, an undifferentiated metastasizing tumor of DBA/2 mice. This tumor was selected because it was recently derived in our laboratory and has been extensively evaluated with regard to its growth and metastatic characteristics (10, 11). The kinetics of the response of normal Balb/c mice against this tumor have been clearly defined (10, 12). A detailed description of the origin and characteristics of this tumor has been provided elsewhere (10−12).

Assay for cell-mediated cytotoxicity (CMC). The cytotoxic response of zinc-deficient (Group A) and pair-fed (Group C) Balb/c mice was assessed in animals that had received a subcutaneous injection of 5 × 10^6 tumor cells, 4, 7, 11, 14, or 17 days prior to being tested. *Ad libitum* fed control mice injected (Group E) and noninjected (Group F) were also tested for cell-mediated cytotoxicity in a similar fashion.

Two additional groups of mice (five in each group) were assayed for cell-mediated cytotoxicity response (see Table I). Five mice received zinc-deficient diet for 11 days

following which they received zinc supplementation (110 ppm) for the next 13 days (Group B). On the 14th day (3 days after zinc supplementation), they were challenged with tumor cells (5×10^6) and assayed for cytotoxic response on the 25th day (11 days after challenge). The other group (Group D) received zinc supplemented (110 ppm) diet for 11 days following which they received 5000 ppm of zinc containing diet for 13 days. On the 14th day (3 days after zinc supplementation) they were challenged with 5×10^6 tumor cells and assayed for cytotoxic responses on the 25th day (11 days after challenge).

CMC was measured by a modification of the isotope release method described by Brunner *et al.* (13) and involved the use of ^{111}Indium Oxine (^{111}InOx) (Mediphysics, Inc., Emeryville, Calif.) rather than ^{51}Cr, as the releasable isotopic label. ^{111}InOx has been shown to offer advantages over ^{51}Cr for long-term cytotoxicity assays (14). Effector cell suspensions were prepared by teasing the spleens of tumor challenged mice in RPMI 1640 medium with 10% fetal calf serum (FCS). Lymphocytes were washed three times in medium and resuspended to a concentration of 10^6 viable cells per ml in RPMI 1640 plus 10%, heat-inactivated FCS glutamine, and P/S (100 U penicillin/ml and 100 μg streptomycin/ml). MDAY-D2 tumor target cells were obtained by aspiration of ascites from the peritoneal cavity of tumor-bearing DBA/2 mice. These cells were washed twice in medium, and resuspended to a volume of 0.5 ml for ^{111}InOx labeling. The cytolytic assay was performed in round-bottomed microplates (Falcon Plastics, Oxnard, Calif.), as previously described (12).

The percentage specific release (SR) of ^{111}InOx was calculated by the following formula:

$$\% \text{ SR} = \frac{\begin{array}{c}\text{release by} \\ \text{sensitized} \\ \text{lymphocytes}\end{array} - \begin{array}{c}\text{release by} \\ \text{normal} \\ \text{lymphocytes}\end{array}}{0.8 \times \text{total incorporated radioactivity}} \times 100.$$

Assay for tibial zinc. The tibias were lyophilized, digested with concentrated nitric acid, and their zinc content was determined with an atomic absorption spectrophotometer (Perkin Elmer, Model 460, Norwalk, Conn.), according to techniques reported earlier (7). Student's *t* test was used for statistical analysis (18).

Results. *Effect of zinc deficiency on body weight and zinc concentration of tibia.* The growth rate of zinc-deficient animals was significantly lower than that of the pair-fed controls (Fig. 1). A significant decrease in zinc concentration in the tibia was observed in zinc-deficient animals when compared to pair-fed controls (Fig. 2). Animals returned to control diet containing 110 ppm zinc (Group B) showed both a rapid increase in body weight and tissue zinc levels (Figs. 1 and 2). Body weight increased by 83% (12 to 22 g) in 10 days while zinc levels in the tibia increased by 32.6%. The animals receiving toxic levels of zinc (Group D) showed a decrease in growth rate and an increase in zinc content of the tibia (Fig. 2). On the 25th day *ad libitum*-fed groups had

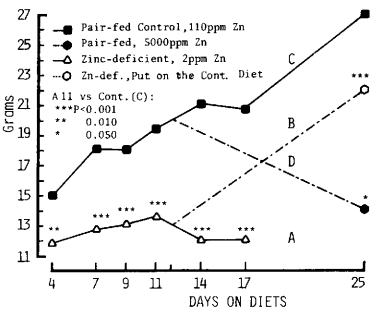

FIG. 1. Effect of zinc deficiency and toxicity on body weight of male mice. (A) Zinc-deficient mice (2 ppm Zn); body weights (mean ± SD) in grams at 4, 7, 9, 11, 14, and 17 days were as follows: 11.8 ± 2, 12.7 ± 0.5, 13.3 ± 1, 13.5 ± 1, 12.2 ± 2 and 12.3 ± 2, respectively. (B) Zinc deficient for 11 days and then fed the control diet (110 ppm Zn); at 25 days the body weight (mean ± SD) in grams was 22.2 ± 1 (C) Pair-fed control (110 ppm Zn); Group C body weights (mean ± SD) in grams at 4, 7, 9, 11, 14, 17, and 25 days were as follows: 14.9 ± 3, 18.3 ± 1, 18 ± 1, 19.4 ± 2, 21 ± 1, 20.6 ± 3 and 27.3 ± 2, respectively. (D) On control diet for 11 days and then fed the high Zn diet (5000 ppm Zn); at 25 days the body weight (mean ± SD) in grams was 14 ± 0.1. Statistically, A, B, and D were compared with C.

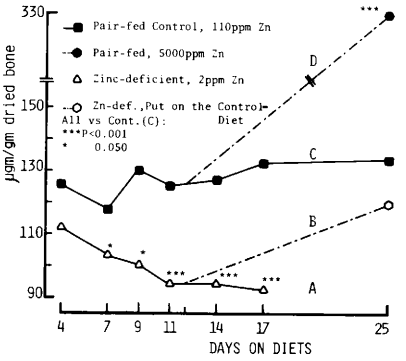

FIG. 2. Tibia zinc concentration as affected by low and high zinc diets. (A) Zinc-deficient mice (2 ppm Zn); zinc concentration (mean ± SD µg/g dry wt) at 4, 7, 9, 11, 14, and 17 days were as follows: 112 ± 12, 103 ± 12, 100 ± 14, 94 ± 8, 94 ± 8, and 92 ± 8, respectively. (B) Zinc deficient for 11 days and then fed the control diet (110 ppm Zn); at 25 days zinc concentration (mean ± SD µg/g dry wt) was 121 ± 4. (C) Pair-fed control (110 ppm Zn); zinc concentration (mean ± SD µg/g dry wt) at 4, 7, 9, 11, 14, and 17 days were as follows: 125 ± 9, 117 ± 5, 123 ± 21, 125 ± 8, 127 ± 10, and 133 ± 14, respectively. (D) On control diet for 11 days and then fed the high zinc diet (5000 ppm Zn); at 25 days zinc concentration (mean ± SD µg/g dry wt) was 329 ± 34. Statistically, A, B, and D were compared with C.

mean body weights of 26.8 ± 2.1 g. Tibia zinc levels were in the same range as those of the pair-fed controls.

Normal cytotoxic response. The subcutaneous injection of the non-H2 allogeneic MDAY-D2 metastatic tumor into Balb/c mice, resulted in initial tumor growth followed by immune rejection of the tumor. The peak cytotoxic response, as measured in the draining peripheral lymph nodes (PLN) or in the spleen, occurred at Days 9–11 and quickly subsided. Tumors were palpable (1–2 cm) at Day 8 but were no longer detectable at Day 14. The rejection of this tumor correlated directly with a measurable T-cell cytotoxic response (10, 12). There is no evidence that antibodies play any role in the rejection of MDAY-D2 by Balb/c mice (10, 12).

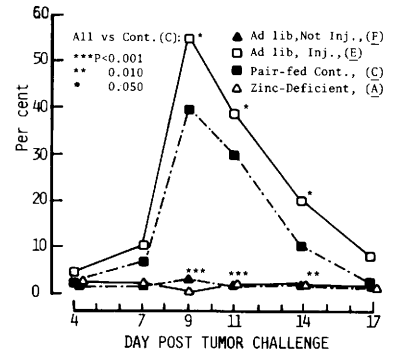

FIG. 3. Kinetics of the cytotoxic response against MDAY-D2 tumor cells. Also see Table I.

Effect of zinc deficiency on the generation of a T-cell cytotoxic response. While *ad libitum*-fed control and pair-fed control (Groups E and C) Balb/c mice mounted a considerable cytotoxic response against MDAY-D2, zinc-deficient animals (Group A) were totally incapable of generating any response against this tumor. Pair-fed control animals (Group C) responded to a lesser degree than *ad libitum*-fed control mice (Group E). Of interest as well, was our inability to detect any tumor growth in the zinc-deficient animals. In a series of four experiments, no tumor growth was detectable by palpation. Anatomic examination of the tumor injection site 8 days after challenge, when growth is maximal in normal animals, revealed only minimal residual tumor in zinc-deficient animals.

The reinstitution of 110 ppm of zinc in the diet to zinc-deficient animals (Group B) increased the cytotoxic response from 3 ± 1 to 43 ± 4% (not shown in the graph) 11 days following tumor challenge.

Cytotoxic response in Group D animals (toxic zinc) was 4 ± 6%, 11 days following tumor challenge (not shown in the graph).

Discussion. It is now clear that a diet deficient in zinc has severe effects on many biochemical and physiologic functions as well as on the ability of the zinc-deficient host to generate an immune response. Animals deficient in zinc generate a poor humoral response upon antigenic challenge (2) and an inadequate cytotoxic response upon tumor challenge.

Pories *et al.* (15) reported a marked reduction in tumor growth in mice and rats on zinc-deficient diets. This diminished tumor growth was clearly associated with prolonged survival among these animals. These authors argued that the failure of tumor growth resulted from zinc deficiency in the host. Tumor inhibition is a general

effect of deficiency of any nutrient which is involved in protein synthesis, irrespective of cell type, cell growth rate, species, or site of growth (15). Thus, it is not surprising, in view of the fact that zinc is required for cell division and protein synthesis, to observe a decrease in tumor growth as a result of deficiency.

In a recent paper, however, it was reported that a high zinc diet (200 ppm) initially protected against the development of oral cancer induced by a chemical carcinogen in rats and eventually accelerated the tumor growth (16). In our experiments we observed that toxic doses of zinc adversely affected the normal cytotoxic response of mice. This effect was demonstrated when mice were given doses *50* times greater than the level of intake of our pair-fed controls (110 ppm).

Since replicating cells provide a greater antigenic stimulus to the host than cells in a resting state, it could be argued that in zinc-deficient animals the failure of the cellular immune response is a result of deficient antigenic stimulation. While this question may not be answered directly, there is evidence from other experiments to indicate that the defect is indeed in the effector arm of the immune response rather than in the diminished antigenic load. Other workers as well as ourselves have demonstrated defects in T-cell function in animals on zinc-deficient diet (2–6). In addition, Pekarek *et al.* (17) have demonstrated similar defects of cell-mediated immunity in a human who had developed an acquired zinc deficiency. Thus, we conclude that either deficiency or toxic level of zinc may impair immune response to allogenic tumor cells most likely by affecting adversely T-cell functions.

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