Impairment of cell-mediated immunity functions by dietary zinc deficiency in mice
(thymic atrophy/T killer cells/natural killer cells)

GABRIEL FERNANDES, MADHAVAN NAIR, KAZUNORI ONOE, TOSHIO TANAKA, RACHAEL FLOYD, AND ROBERT A. GOOD

Memorial Sloan–Kettering Cancer Center, New York, New York 10021

Contributed by Robert A. Good, November 6, 1978

ABSTRACT Several immunologic features were analyzed in mice on a zinc-deficient diet (Zn–), in mice pair-fed a diet containing zinc (Zn+), in mice fed a Zn+ diet ad lib, and in mice fed laboratory chow ad lib. When placed on a Zn– diet, 6- to 8-week-old A/Jax, C57BL/Ks, and CBA/H mice showed loss of body weight, low lymphoid tissue weight, and profound involution of the thymus within 4–8 weeks after initiation of the regimen. Approximately 50% of the mice on the Zn– diet developed severe acrodermatitis enteropathica (lesions on tail and paws) and diarrhea. Pair-fed mice on the Zn+ diet did not show any of these symptoms. Mice on the Zn– diet showed the following immune deficiencies: (i) depressed plaque-forming cells against sheep erythrocytes after in vivo immunization; (ii) depressed T killer cell activity against EL-4 tumor cells after in vivo immunization; and (iii) low natural killer cell activity. However, antibody-dependent cell-mediated cytotoxicity against chicken erythrocytes was normal in the mice on the Zn– diet. Deficiency of T killer cell activity was not observed when immunization with EL-4 allogeneic lymphoma cells was carried out in vitro. Progressive loss of relative and absolute number of Thy 1.2+ cells and a proportionate relative increase in cells bearing Fc receptors was seen in spleen and lymph nodes of Zn– mice. It appears that zinc is an essential element for maintenance of normal T cell and other immune functions in vivo.

Zinc, an essential element in man and animals, is important for stabilization or function of numerous metalloenzymes involved in protein synthesis, protein catabolism, energy metabolism, and both DNA and RNA synthesis (1). Zinc deficiency in man and animals has been associated with hypogonadism, growth retardation, anorexia, gastrointestinal malfunction, and dermatitis characterized by hyperkeratosis and parakeratosis (2–7). In the A 46 lethal variant of Friesian cattle, dermatitis, diarrhea, failure of thymic development, and severe immunodeficiency occur due to defective absorption of zinc (8). This disease can be cured or prevented by giving relatively large amounts of Zn by mouth (9). Acrodermatitis enteropathica, a childhood disease of high morbidity and mortality that is transmitted genetically as an autosomal recessive trait and is associated with characteristic skin disease, bowel and central nervous system malfunction, and frequent immunodeficiency, is also completely correctable by administration of zinc (10, 11). In rats, zinc deficiency regularly leads to experimental acrodermatitis enteropathica and atrophy of lymphoid tissue and thymus, which can be promptly corrected by administration of zinc (12).

Abnormalities of the lymphoid cell distribution in lymph node and spleen and deficiency of thymic hormone have recently been reported in association with zinc deficiency in mice (13, 14). Prior immunologic studies have shown that zinc deficiency in mice interferes with their ability to produce anti-

body to sheep erythrocytes (SRBC) (15, 16). Fraker et al. (15) attributed this abnormality to failure of normal development of T helper cells. To date, no studies of other T cell functions in zinc-deficient rodents have been reported, although the A 46 lethal variant in cattle has been associated with T cell immunodeficiency and failure to develop delayed allergy which is correctable by zinc administration (8).

In this paper we report regular production of T cell immunodeficiency in zinc-deficient mice including defective development of T killer lymphocytes after in vivo sensitization with tumor cells as well as the defective development of natural killer (NK) lymphocytes; we also report defective development of both direct and indirect plaque-forming cells after immunization in vivo with SRBC. Antibody-dependent cell-mediated cytotoxicity (ADCC) was normal in zinc-deficient mice. However, upon in vitro immunization of lymphoid cells from zinc-deficient animals in the presence of zinc, T killer cell function developed normally.

MATERIAL AND METHODS

Animals. Four- to 8-week-old A/Jax female and male inbred mice, obtained from Jackson Laboratory, and C57BL/Ks and CBA/H mice, maintained at the SKI mouse colony, were used. Breeding stock of the two latter strains was obtained from the University of Minnesota mouse colony.

Diets. Zinc-deficient (Zn–) and zinc-supplemented (50 ppm) (Zn+) diets in pellet form were obtained from Teklad Test Diets (Madison, WI). These diets were prepared according to the original formula of Luecke et al. (17). Animals were marked individually and housed in stainless steel cages with wire mesh bottoms. Double-distilled drinking water was provided in glass bottles with siliconized rubber stoppers and stainless steel tubing. Food pellets were placed in stainless steel feeders, and a record of daily food consumption was maintained. Extreme care was taken to eliminate sources of zinc contamination. Because animals on Zn– diets are known to consume less food than average for mice, an equal number of pair-fed mice on the Zn+ diet were also included for comparison. In addition, mice fed Zn+ diets ad lib were included in the study. Body weight and signs of skin lesions, other external abnormalities, and diarrhea were recorded regularly.

Response to In Vivo Immunization with SRBC. Each mouse was injected intraperitoneally with 0.2 ml of a 10% suspension of SRBC (GIBCO). Ninety-six hours later the spleens were removed and gently homogenized, and the viable cell concentration was adjusted to 107 cells per ml. The primary

Abbreviations: Zn(+), zinc-supplemented; Zn(–), zinc-deficient; FPC, plaque-forming cell; SRBC, sheep erythrocytes; NK, natural killer; ADCC, antibody-dependent cell-mediated cytotoxicity; CRBC, chicken erythrocytes; RFC, rosette-forming cell; FeR, receptor for the Fe part of IgG.

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immunoresponse (primarily IgM) was quantified by the plaque-forming cell (PFC) assay using a modification of the method of Bullock and Möller (18) as described (19). To measure secondary immune response, which is largely IgG responsive, mice were reimmunized 7 days after the first injection of SRBC, and the PFC assay was carried out on day 11. To enumerate indirect PFC (IgG), rabbit anti-mouse IgG plus guinea pig complement was added in appropriate dilutions.

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Cytotoxicity Response to Immunization with EL-4 Tumor Cells. In vivo immunization was carried out by injecting 10⁶ viable EL-4 tumor cells intraperitoneally; 12 days later, the immunized mice were sacrificed and the cytotoxic activity of spleen cells was determined in a microplate ⁵¹Cr-release assay as described (20).

In vitro sensitization to allogeneic cells was performed in plastic multiwell dishes (FB 16–24 TC, Linbro Chemical Co., Hamden, CT) by a modification of the method of Hodges et al. (21). During the period of incubation, the cells were exposed to zinc contained in the fetal calf serum and in the RPMI medium (19).

Assay for NK Cell Activity. NK activity was determined in a direct ⁵¹Cr-release assay modified as described (22). Briefly, spleen cells from mice in different dietary groups were prepared on the same day; the RL3.1 lymphoma mouse cell line, obtained from R. Herberman (National Institutes of Health, Bethesda, MD) and maintained in vivo as a tissue culture cell line, was used as a target. Details of this microcytotoxicity have been described (23).

Antibody-Dependent Cell-Mediated Cytotoxicity. The ADCC activity for spleen cells was determined after removing the plastic-adherent cells by the method of Handwerger and Koren (24); ⁵¹Cr-labeled chicken erythrocytes (CRBC) were used as targets. Details of this assay have been described (24).

Complement-Dependent Cytotoxicity Assay. Anti-Thy 1.2 serum, prepared in congenic strains, was kindly supplied by E. Stockert (Sloan–Kettering Institute). The cytotoxicity test was based on the method of Amos et al. (25) with slight modifications as described (22).

EA-Rosette-Forming Cell (RFC) Assay. The EA-rosette assay with lymphoid cell suspensions was performed as described (26, 27). Lymphocytes with three or more erythrocytes attached per cell as seen under the light microscope at ×100 were considered to be rosettes. The results were expressed as percentages of rosettes in relation to the total number of untreated cells.

Results

Effect of Zn(−) Diet on Growth and on Serum Zinc Levels of C57BL/Ks Female Mice. Five- to 6-week-old female mice on the Zn(−) diet gained weight only during the first week; from the second week onward, a gradual loss of body weight occurred (Fig. 1). After 10 weeks on the Zn(−) diet, most of the animals died. Pair-fed mice on the Zn(+) diet, although growing, gained less weight than did mice on the ad lib Zn(+) diet. Nearly 50% of the mice on the Zn(−) diet had anorexia, diarrhea, and typical skin lesions on the tail and paws. No such lesions occurred in the pair-fed mice on the Zn(+) diet. As described elsewhere (13, 15), most of the mice examined showed a decline of thymus weight from 2 weeks onward, and by 6 weeks virtually complete involution of the thymus had occurred. By contrast, the thymus weight of the pair-fed animals was higher than that of mice on the Zn(−) diet but was less than that of the mice on the ad lib Zn(+) diet. A similar influence of the Zn(−) diet was observed in three other inbred strains, A/Jax, CBA/H, and AKR/J. Serum zinc concentration was measured in a Perkin–Elmer atomic absorption spectrophotometer (28) on pooled sera. Zinc levels ranged from 110 to 125 μg/dl in pair-fed and ad lib fed mice; in mice fed Zn(−) diets they ranged from 35 to 60 μg/dl after the animals had been on the diet for 4–8 weeks.

PFC Response to In Vivo Immunization with SRBC. Table 1 summarizes PFC response of C57BL/Ks mice challenged with SRBC at 3, 4, and 6 weeks on different dietary regimens. PFC responses in the spleen were not altered in Zn(−) mice after 2 weeks on the diet when compared to pair-fed mice. However, the response was significantly lower after 4 weeks on the Zn(−) diet, and a pronounced deficiency of PFC response was seen after 6 weeks on the Zn(−) diet. The pair-fed mice showed significantly higher PFC responses than did mice on the Zn(−) diet. The mice on the Zn(+) ad lib diet and those fed regular laboratory chow ad lib showed significantly higher PFC response than did the Zn(−) group and slightly higher responses than the Zn(+) pair-fed controls. The development of indirect PFC response in Zn(−) animals undergoing primary response remained lower than the direct PFC response. A second set of experiments, carried out on A/J mice, was designed to test both direct and indirect PFC responses after primary and secondary exposure to the antigen (SRBC). Here again, the mice on the Zn(−) diet showed defective formation of direct PFC

Table 1. PFC response in C57BL/Ks mice maintained on various diets

<table>
<thead>
<tr>
<th>Diet*</th>
<th>Duration, weeks</th>
<th>Direct PFC per spleen</th>
<th>Indirect PFC per spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zn(−)</td>
<td>2</td>
<td>29,127 ± 9,682</td>
<td>23,149 ± 8,402</td>
</tr>
<tr>
<td>Zn(+) PF</td>
<td>2</td>
<td>25,428 ± 1,276</td>
<td>19,699 ± 920</td>
</tr>
<tr>
<td>Zn(−)</td>
<td>4</td>
<td>18,502 ± 5,606</td>
<td>14,913 ± 5,444</td>
</tr>
<tr>
<td>Zn(+) PF</td>
<td>4</td>
<td>38,392 ± 9,902</td>
<td>31,934 ± 8,549</td>
</tr>
<tr>
<td>Lab chow</td>
<td>6</td>
<td>64,281 ± 7,152</td>
<td>53,294 ± 3,144</td>
</tr>
<tr>
<td>Zn(−)</td>
<td>6</td>
<td>8,278 ± 2,651</td>
<td>5,128 ± 2,312</td>
</tr>
<tr>
<td>Zn(+) PF</td>
<td>6</td>
<td>80,983 ± 2,772</td>
<td>69,071 ± 6,749</td>
</tr>
<tr>
<td>Zn(+) ad</td>
<td>lib</td>
<td>117,968 ± 15,858</td>
<td>95,446 ± 7,393</td>
</tr>
<tr>
<td>Lab chow</td>
<td>8</td>
<td>104,662 ± 18,957</td>
<td>74,647 ± 6,202</td>
</tr>
</tbody>
</table>

*PF, pair-fed.

At least four mice were immunized with SRBC in each group. Data are shown as mean ± SEM.
in the primary response compared to pair-fed mice (Table 2).

When reimmunized, the Zn(-) animals developed a high number of IgM plaques and showed a modest increase in IgG PFC response as well. In contrast, both pair-fed and ad lib Zn(+) animals, as well as laboratory chow-fed animals, showed a 3- to 4-fold increase in IgG PFC per spleen after secondary immunization.

Cell-Mediated Response to Immunization with EL-4 Tumor Cells. Cytotoxic response of spleen cells from Zn(-) animals immunized in vivo was consistently lower than that of all the Zn(+) groups (Table 3). Mice on the Zn(-) diet showed a marked deficiency in generating T killer cells against allogeneic tumors when immunization was carried out as early as 2 weeks after initiation of the Zn(-) diet. On the other hand, pair-fed animals responded as vigorously in this critical T cell immune response as did animals fed either a Zn(+) diet ad lib or a standard laboratory chow diet. Animals maintained on the Zn(-) diet for 8 weeks, by which time skin lesions had developed in many, responded minimally to the generation of T killer cells after in vivo immunization with EL-4 cells.

In Vitro Immunization of Spleen Cells with EL-4 Cells. As in the case of C57BL/Ks mice, in vivo immunization with EL-4 cells of CBA/H mice fed a Zn(-) diet revealed a gross deficit in production of T killer cells (Table 4). In contrast to these results, after in vitro immunization, spleen cells of CBA/H mice fed a Zn(-) diet for 8 weeks had a capacity to develop killer cells comparable to that shown by pair-fed animals on the Zn(+) diet. During in vitro immunization the T cells of the Zn(-) mice were exposed, over the entire 5-day span of incubation, to medium and fetal calf serum containing zinc.

NK and ADCC Activity in Zn(-) Animals. A significant deficiency of NK activity was also observed in animals on the Zn(-) diet, and this deficiency appeared within 2 weeks after the start of the Zn(-) diet (Fig. 2). From 4 weeks onward, a marked depression of NK activity was observed, although the animals did not develop skin lesions attributable to Zn deficiency until somewhat later. It is of interest that, after 8 weeks on the Zn(+), the pair-fed animals showed somewhat lower NK activity than did the two other control groups (data not shown). In contrast to the low NK activity, ADCC capacity was not depressed in Zn(-) animals. Indeed, a modest increase in ADCC was seen in these animals between the second and eighth weeks on the Zn(-) diet compared to the pair-fed group. Mice of the group fed Zn(+) diet ad lib had ADCC activity equal to that of the pair-fed animals (data not shown)

Thy 1.2 and FcR* Lymphocytes in Zn(-) Animals. Table 5 summarizes our results quantifying the number of Thy 1.2 and Fc-receptor bearing lymphocytes in spleen and lymph nodes. The proportion of T lymphocytes in both spleen and lymph nodes decreased modestly over the period of feeding the Zn(-) diet. The decline in proportion of T lymphocytes was greater in lymph nodes than in the spleen. When the total number of T lymphocytes was calculated, however, a substantial reduction was found in the Zn(-) animals. By contrast, cells bearing Fc receptors increased slightly in proportion in the spleen but not in the lymph nodes in animals fed the Zn(-) diet. The total number of Fc receptor cells, however, was not increased in either spleen or lymph nodes.

**DISCUSSION**

Deficiency of dietary zinc in man and animals produces growth retardation, gastrointestinal malfunction, hypogonadism, and skin disease characterized by parakeratosis (1, 2). If care is taken to avoid all sources of zinc contamination, profound deficiency of this vital trace element can regularly be produced within 4–8 weeks in young growing rats and mice of several inbred strains (12–16). Thus, readily available model systems for studying the influence of dietary zinc on the body economy have now been developed. In the zinc deficiency of inbred rats and mice, acromegaly-like skin lesions, especially of the paws and tail, gastrointestinal malfunction, growth failure, loss of appetite, thymic hypoplasia, decreased thymic hormone function, and morphological abnormalities reflecting T cell deficiency of lymph nodes and spleen are regularly seen (13–15). Of major importance in studying the influence of zinc deficiency on immunity functions is the pair-fed control, because Zn(-) an-

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**Table 2.** Development of direct or indirect PFC response after immunization with SRBC in A/Jax mice maintained on Zn(-) and Zn(+) diets for 8 weeks

<table>
<thead>
<tr>
<th>Diet</th>
<th>n</th>
<th>Direct PFC per spleen</th>
<th>Indirect PFC per spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zn(-)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary</td>
<td>11</td>
<td>6,515 ± 2,261</td>
<td>5,949 ± 2,158</td>
</tr>
<tr>
<td>Secondary</td>
<td>7</td>
<td>13,938 ± 3,848</td>
<td>22,669 ± 7,996</td>
</tr>
<tr>
<td>Zn(+) PF</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary</td>
<td>9</td>
<td>18,501 ± 2,321</td>
<td>17,406 ± 1,960</td>
</tr>
<tr>
<td>Secondary</td>
<td>7</td>
<td>8,929 ± 2,756</td>
<td>75,535 ± 17,930</td>
</tr>
<tr>
<td>Zn(+) ad lib</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary</td>
<td>7</td>
<td>20,436 ± 3,438</td>
<td>18,870 ± 3,704</td>
</tr>
<tr>
<td>Secondary</td>
<td>6</td>
<td>8,737 ± 2,254</td>
<td>63,756 ± 19,679</td>
</tr>
<tr>
<td>Lab chow</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary</td>
<td>5</td>
<td>21,355 ± 5,762</td>
<td>22,625 ± 7,897</td>
</tr>
<tr>
<td>Secondary</td>
<td>5</td>
<td>10,174 ± 3,374</td>
<td>82,070 ± 6,116</td>
</tr>
</tbody>
</table>

n, Number of mice immunized; PF, pair-fed. Data are shown as mean ± SEM.

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**Table 3.** Cell-mediated cytotoxicity of spleen cells of C57BL/Ks mice on Zn(-) diet immunized in vivo with EL-4 tumor cells

<table>
<thead>
<tr>
<th>Duration, weeks</th>
<th>% 51Cr release*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Zn(-) diet</td>
</tr>
<tr>
<td>2</td>
<td>33.8 ± 2.8 (4)</td>
</tr>
<tr>
<td>4</td>
<td>34.6 ± 4.3 (4)</td>
</tr>
<tr>
<td>6</td>
<td>26.4 ± 6.8 (4)</td>
</tr>
<tr>
<td>8</td>
<td>14.7 ± 3.9 (8)</td>
</tr>
</tbody>
</table>

* At 4 hr with 1:100 target-to-spleen cell ratio. Numbers of animals tested are shown in parentheses. Data are shown as mean ± SEM.

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**Fig. 2.** Effect of Zn(-) (open bars) and pair-fed Zn(+) (hatched bars) diets on NK and ADCC activity in C57BL/Ks female mice after 2, 4, and 8 weeks. Data are shown as mean ± SEM obtained with a 1:100 target:effector ratio.
The present studies of immunological function in Zn(-) mice confirm and extend the findings of Fraker et al. (15) who found that Zn(-) mice fail to produce a normal number of PFC after immunization with SRBC. In the analyses reported in this paper, a significant depression of both direct and indirect PFC responses has been demonstrated. The deficiency of indirect plaque formation during the secondary response was even more profound than the deficiency of direct plaque formation in the Zn(-) animals, as might be anticipated if deficiency of T helper cells were the basis (32).

In addition, T killer cell formation in response to immunization with EL-4 target cells in vitro and NK cells capable of destroying RL6 1 target cells were progressively depressed as the zinc deficiency developed. These alterations of immunologic function were accompanied by a progressive gradual decrease in the proportion of T lymphocytes and an even more striking reduction in the total number of T lymphocytes in spleens of the Zn(-) mice. In contrast to the decline of T cell numbers, ADCC was found to be much better preserved in a nonadherent spleen cell population from Zn(-) animals than were other T cell functions. Of special interest in this regard were the findings that T killer cells generated after in vitro immunization with EL-4 cells were normal even when the cells being stimulated were taken from mice that were grossly deficient in zinc and whose immune responses to in vivo immunization with the same target cells were grossly deficient. Although it must be recognized that the in vitro immunizations were carried out over a 5-day period in the presence of medium and fetal calf serum which contain considerable amounts of zinc, the possibility that events essential to presentation of antigen in vivo—macrophage functions, lymphocyte helper functions, serum factors, or other inflammatory cells or cell distribution—might be abnormal in zinc deficiency that could play a crucial role in the defective immune responses in vivo. In studies of the immunological abnormalities observed in diabetic mice, a similar discrepancy between in vitro and in vivo generation of T cell cytotoxicity was observed (19). Because zinc deficiency in rats and mice affects protein and nucleic acid synthesis (1, 38), spleen cells may not respond to in vivo antigenic stimulation, whereas the zinc in the culture medium might be sufficient to permit these important events and make possible sensitization in vivo.

The present studies also show that in Zn(-) animals there is a sharp dissociation between NK cell function and function of cells responsible for ADCC. Indirect evidence favors the presence of Fc receptors on both cell populations, but some investigators insist that their role on NK cells is much less certain than for ADCC (34, 35). The deficiency of NK activity could be due to failure to develop NK cells in Zn(-) animals, whereas the cells responsible for ADCC develop normally, thus indicating that the two populations are indeed distinct. The possibility also exists that functions responsible for NK activity are interfered with by zinc deficiency but functional activities involved in ADCC are not. It has been shown previously that zinc ions in excess can inhibit macrophage migration capacity, whereas zinc deficiency can cause significant enhancement of this capacity (36). Thus, either deficiency or excess of zinc can interfere selectively with functions of different cells or cell subpopulations. Further studies will be needed to dissect this fascinating dissociation of NK and ADCC cytolytic cell functions in the presence of zinc deficiency.

Table 5. Thy 1.2 and FcR positive cells in spleen and lymph nodes of C57BL/Ks mice fed Zn(-) and Zn(+) diets

<table>
<thead>
<tr>
<th>Group</th>
<th>Total cells*</th>
<th>Thy 1.2*, %</th>
<th>FcR*, %</th>
<th>Thy 1.2*, %</th>
<th>FcR*, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>At 4 weeks:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zn(-)</td>
<td>83 ± 4</td>
<td>25.2 ± 4</td>
<td>64.6 ± 4</td>
<td>52.0 ± 1</td>
<td>31.2 ± 5</td>
</tr>
<tr>
<td>Zn(+) PF</td>
<td>99 ± 11</td>
<td>27.4 ± 3</td>
<td>62.6 ± 4</td>
<td>63.7 ± 1</td>
<td>30.0 ± 1</td>
</tr>
<tr>
<td>At 8 weeks:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zn(-)</td>
<td>35 ± 5</td>
<td>19.3 ± 6†</td>
<td>73.2 ± 2</td>
<td>45.6 ± 2</td>
<td>37.8 ± 2</td>
</tr>
<tr>
<td>Zn(+) PF</td>
<td>88 ± 9</td>
<td>26.1 ± 2†</td>
<td>64.1 ± 2</td>
<td>56.0 ± 3</td>
<td>35.0 ± 1</td>
</tr>
</tbody>
</table>

Mean ± SEM.
* Viable cell recovery (×10^6) per spleen.
† Total Thy 1 cells per spleen, 6.7 × 10^8.
‡ Total Thy 1 cells per spleen, 22.9 × 10^8.
Immunologic function in acrodermatitis enteropathica, a genetically determined failure of zinc absorption in man, seems to be deficient (ref. 37, R. A. Good, unpublished observations), although, in our view, studies of this point have not yet been definitive. Because protein and protein/calorie malnutrition syndromes of children are often accompanied by low concentration of zinc in plasma (38) and profound T cell immunodeficiency (39), the recent evidence that zinc deficiencies may account for the cell-mediated immunodeficiencies in protein/calorie malnutrition syndromes of man seems attractive (40). The present findings would be consistent with this view. Because decreased food intake accompanies many chronic human diseases and because diets deficient or somewhat deficient in zinc are commonplace in the United States (1, 41), one must question how frequently zinc deficiency with or without deficiency of other trace metals contributes to secondary immunodeficiencies seen in cancer patients and patients with chronic infections or other debilitating illnesses. Studies directed toward answering these questions are very much in order.

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28. Analytical Methods for Atomic Absorption Spectrophotometry (Perkin-Elmer, Norwalk, CT).