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Experimental zinc deficiency: effects on cellular responses and the affinity of humoral antibody

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SUMMARY

Mice genetically selected for the production of either high- or low-affinity antibody were fed on diets sufficient or deficient in zinc. The effect of zinc deficiency on immune responses in these animals was analysed in terms of cell-mediated responses and the levels and affinity of antibody produced in response to immunization with T-dependent and T-independent antigens. In comparison with animals fed zinc-containing diets, mice fed zinc-deficient diets had reduced numbers of T cells and T-cell subsets, reduced proliferation to mitogens and specific antigen, and a decreased production of interleukin 2 (IL-2), but the number and affinity of IL-2 receptors were not affected. Furthermore, zinc-deficient animals produced reduced levels of antibody to the T-dependent antigen DNP-human serum albumin, but the affinity of this antibody was significantly elevated compared with that produced by zinc-sufficient animals. However, zinc deficiency had no effect on the levels and affinity of antibody produced to the T-independent antigen DNP-ficoll.

Keywords T cells zinc deficiency antibody affinity

INTRODUCTION

Experimental and clinical observations have highlighted the importance of zinc status in immune function (Chandra, 1980; Bach, 1981; Homsey, Morrow & Levey, 1986). Zinc deficiency has been implicated in immune deficiencies associated with acrodermatitis enteropathica and bovine lethal trait A46 (Brummerstedt *et al.*, 1977), with Down's syndrome (Bjorksten *et al.*, 1980) and with secondary zinc deficiencies following infectious disease (Beisel, 1976) and severe burns (Lennard *et al.*, 1974). Zinc deficiency is associated with increased susceptibility to a variety of infectious diseases and abnormalities of humoral and cellular immunity, many of which are ameliorated by zinc reconstitution (Oleske *et al.*, 1979).

T cells have been suggested to play an important role in the control of antibody affinity with the production of high-affinity (HA) antibody being dependent on the presence of T cells (Gershon & Paul, 1971). Furthermore, the study of T cell subsets has led to the proposal that helper-inducer T cells are necessary for the production of HA antibody (DeKruyff & Siskind, 1979) whereas T suppressor cells appear to selectively inhibit HA antibody production (Tada & Takemori, 1974) and so regulate the maturation of affinity (Steward, Stanley & Furlong, 1986).

The importance of zinc deficiency on T cell function has been shown in clinical studies where depressed responses to T cell mitogens and reduced levels of thymopoietin and serum thymic factor were observed (Cunningham-Rundles *et al.*, 1979). Zinc-deficient animals show thymic involution (Chandra, 1980), depressed responses to T cell mitogens, decreased cytotoxic T cell responses, natural killer (NK) cell activity and low levels of thymic hormone (Iwata *et al.*, 1979). Zinc deficiency may also selectively inhibit helper-inducer T cell function in mice (Fraker *et al.*, 1978) and inhibit interleukin 2 (IL-2) production and IL-2 receptor expression in rats (Dowd, Kelleher & Guillou 1986).

The apparently selective influence of zinc deficiency on the T cell compartment provides an opportunity to study the cellular regulation of antibody affinity. In this paper we report the results of experiments designed to assess the effect of zinc deficiency on cellular immune responses and antibody affinity.

MATERIALS AND METHODS

Mice

Female HA and low-affinity (LA) mice from generation 25–29 of selective breeding (Katz & Steward, 1975) aged 6–8 weeks were used. Animals were weighed at the beginning and the end of experiments (day 21).

Diets

Zinc-deficient (Zn^-) mice were fed a zinc test diet with egg albumin (USB 23566, United States Biochemical Corporation, Cleveland, OH; zinc content 0·8–2·0 ppm and containing a supplement of 2 mg/kg biotin) and distilled water *ad libitum.*

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Animals were kept in cages washed with 1 M EDTA and on low-zinc sawdust bedding. Control mice (Zn^+) were fed normal diet which contained 35·76 mg zinc/kg (86A, Dixon & Sons, Ware, UK).

Zinc analysis
The zinc content of serum and bone from mice fed on the diets for 21 days was measured by flame atomic absorbtion spectrometry using an air-acetylene flame with deuterium background correction, a slit width of 320 and a frequency of 213·9 nm (151 Thermoelectron, Warrington, UK).

Antigens
DNP_{10}-ovalbumin, DNP_2-HSA and DNP_{10}-*N*-(2-aminoethyl)carbamylated ficoll were synthesized and the degree of hapten substitution was determined, according to the methods of Eisen, Carsten & Belman (1953) and Inman (1975), respectively.

Immunizations
Mice were fed either a Zn^+ or a Zn^- diet for 7 days, and groups of five mice were then immunized with either an i.p. injection of 1 mg DNP10-ovalbumin emulsified in Freund's complete adjuvant or with 100 μg DNP10-ficoll in Freund's complete adjuvant. Mice were bled weekly from the retro-orbital venous plexus under ether anaesthesia.

Antibody affinity assays
The affinity of anti-DNP antibodies was determined by a double-isotope radioimmunoassay. ^{125}I-DNP_2-HSA: anti-DNP immune complexes were precipitated with 20% polyethylene glycol (8000 Sigma). The affinity values were calculated in terms of M^{-1} and antibody levels in terms of pmoles of binding sites (Abt) in 10 μl serum (Stanley, Lew & Steward, 1983).

Lymphocyte-stimulation assays
Splenocytes from human serum albumin (HSA) immunized mice were cultured with HSA (0·078–5·0 μg), phytohaemagglutinin (PHA) (0·09–100 μg), concanavalin A (Con A) (0·13–73 μg) or lipopolysaccharide (LPS) (0·19–100 μg). Cells (2×10^5) were cultured with mitogen for 24 h or with antigen for 4 days, and proliferation was assessed by the measurement of ^{3}H-thymidine incorporation 24 h after the addition of radioactive thymidine. Results are expressed as the stimulation index that represents the ratio of the counts incorporated in stimulated cultures over the counts incorporated in unstimulated cultures.

IL-2 production
The IL-2 content of spleen cell supernatants was determined by a competitive binding assay using HUT cells and ^{125}I-labelled recombinant human IL-2 (0·01–500 ng, Glaxo, London, UK).

IL-2 receptor analysis
IL-2 receptor expression on splenocytes was determined by a binding assay in which 2×10^6 cells were incubated with a range of concentrations of recombinant IL-2 (9 nmoles–150 pmoles) containing a constant 0·043 nmoles of ^{125}I-IL-2 (DuPont, Boston, MA). Cell-bound radioactivity was measured in a gamma spectrometer.

Flow cytometry
Lymphocyte subsets in the spleen were quantified by flow cytometry employing three monoclonal anti-mouse T cell antibodies (FITC-labelled anti-Thy 1.2; FITC-labelled anti-Lyt 2, and phycoerythrin-labelled anti-L3T4) and an FITC-labelled monoclonal anti-mouse Ig directed towards B cells expressing immunoglobulin. Cells were analysed on a fluorescence activated cell sorter (Becton Dickinson) with a detection limit of 5000 events at a flow rate of 300. Gates were applied during data analysis and maintained for each individual stain and results are expressed as a percentage of cells staining positive for a given conjugate.

RESULTS

Clinical effects of Zn^- diet
The Zn^- diet led to a 30–50% loss in body weight and a significant reduction in bone and serum zinc compared with Zn^+ control mice. Serum albumin levels were not affected (Table 1).

The effect of zinc deficiency on antibody responses to T-dependent and T-independent antigens in HA and LA mice
Zinc deficiency had different effects on the antibody response of HA and LA mice from the T-dependent antigen DNP–HSA compared with those of Zn^+ animals. Antibody levels in LA mice fed the Zn^- diet were significantly reduced compared with those in Zn^+ fed mice (*P* values ranged from 0·001 to 0·005) (Fig. 1a). In addition, antibody affinity values in Zn^- LA mice were significantly enhanced in all breeds tested compared with Zn^+ mice (Fig. 1b). In HA mice, neither antibody levels nor affinity were significantly affected by changes in dietary zinc (Figure 1c, d). Zinc deficiency did not significantly affect the levels and affinity of the antibody response of either HA or LA mice to a T-independent antigen DNP-ficoll (Fig. 2). To confirm that these effects were the result of the absence of zinc in the Zn^- diet, LA mice fed the Zn^- diet were given zinc sulphate in their drinking water (100 μg/ml) and their antibody responses to DNP–HSA were assessed. The results obtained showed that the LA mice receiving the Zn^- diet with zinc supplementation mounted antibody responses which were similar in terms of levels and affinity to those in mice receiving the Zn^+ diet (anti-DNP affinity in Zn^+ mice: $1{\cdot}25 \times 10^6\ M^{-1}$ and in Zn^- reconstituted $1{\cdot}16 \times 10^6\ M^{-1}$; $P > 0{\cdot}20$). In addition, these mice showed a weight gain similar to mice receiving the normal Zn^+ diet. Zinc

Table 1. The effect of Zn^- diet on LA mice

Diet	Weight (g)	Serum albumin (mg/ml)	Bone zinc (μg/g)	Serum zinc (μg/ml)
Zn^+	31·1±3·7	57±10·9	3·19±0·86	0·12
Zn^-	16·8±3·2	69±13·4	1·10±3·4	0·07
P	0·001	0·5	0·001	—*

Data obtained from animals after 21 days on the diet.

* No statistical analysis possible; pooled serum samples used.

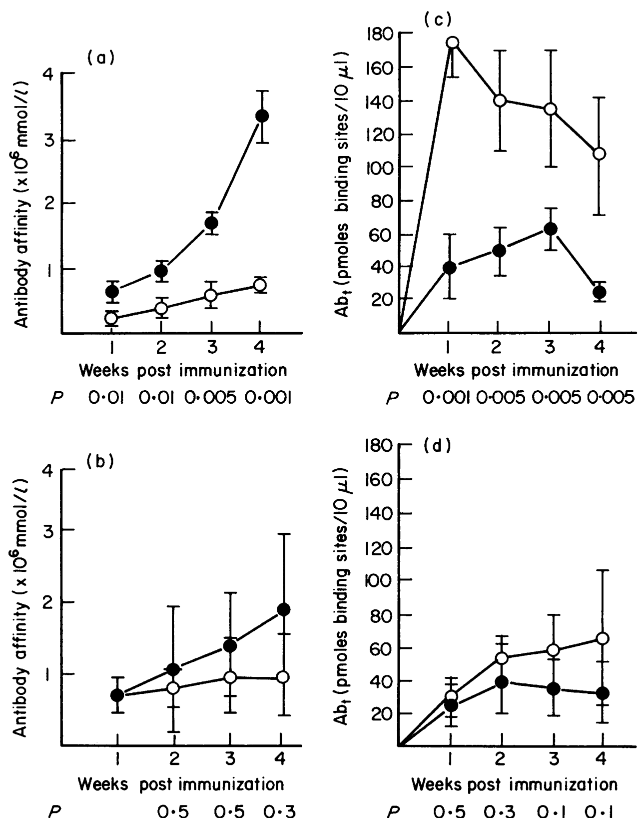

Fig. 1. The effect of Zn^- diet on the antibody response of HA and LA mice following immunization with the T-dependent antigen DNP-ovalbumin. Results are expressed as affinity, and levels of anti-DNP antibody in Zn^- mice (●) and Zn^+ mice (○). (a) affinity in LA Mice; (b) affinity in HA mice; (c) antibody levels in LA mice; and (d) antibody levels in HA mice.

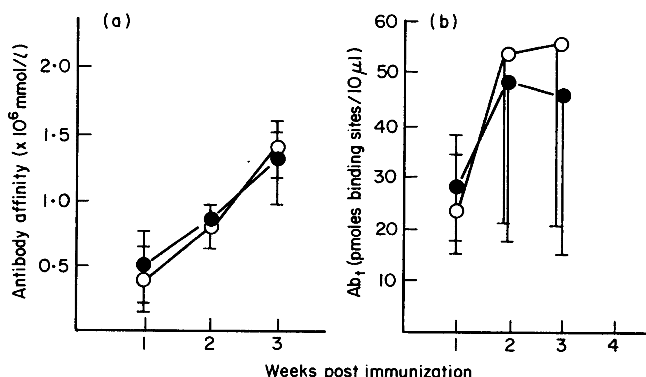

Fig. 2. The effect of Zn^- diet on the antibody response of LA mice following immunization with the T-independent antigen DNP-ficoll. (a) affinity; and (b) levels of anti-DNP antibody in Zn^- (●) and Zn^+ (○) mice.

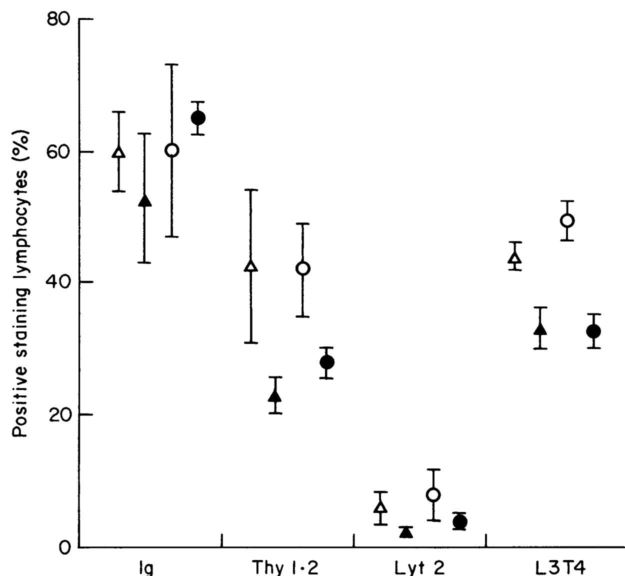

Fig. 3. Lymphocyte subsets assessed by flow cytometry following reaction with fluorescence-labelled antibodies to Ig, Thy 1.2, Lyt 2 and L3T4, in Zn^- (▲) and Zn^+ (△) HA mice; and in Zn^- (●) and Zn^+ (○) LA mice.

supplementation of either HA or LA mice fed a normal diet had no effect on either levels or affinity of antibody (data not shown).

The effect of zinc deficiency on lymphocytes and lymphocyte function

The results of flow cytometry analysis of splenocytes stained by the FITC- and phycoerythrin-labelled antibodies to lymphocyte surface molecules are shown in Fig. 3. Significant differences were observed in the number of cells staining positive for Thy 1.2, Lyt 2 and L3T4 in both HA and LA mice on Zn^- diets, compared with Zn^+ animals. However, zinc deficiency did not affect the number of cells staining for immunoglobulin in either HA or LA mice. The only significant difference between Zn^+ HA and LA mice demonstrable by flow cytometry was a lower number of L3T4 staining cells in HA mice.

The results of LPS, PHA and Con A stimulation of Zn^+ and Zn^- splenocytes from LA mice are given in Fig. 4. The optimum concentrations for stimulation were similar in both diet groups for each mitogen. At the optimum dose, a significant reduction in the stimulation index following culture with ConA was observed with splenocytes from the Zn^- diet ($P < 0{\cdot}0005$). With PHA, although the standard deviations for the Zn^- group were large, the differences were statistically significant ($P < 0{\cdot}010$). Following culture with LPS, there were no demonstrable differences in the stimulation indices between the two diet groups.

The results of *in vitro* culture with HSA of spleen cells from Zn^+ and Zn^- HA and LA mice immunized with HSA are given in Fig. 5. It can be seen that for both lines of mice, zinc deficiency resulted in a reduced proliferative response to antigen which was particularly marked in LA mice.

There was a significant reduction ($P < 0{\cdot}005$) in IL-2 levels in spleen cell supernatants from Zn^- LA mice compared with supernatants from Zn^+ animals. However, the number and

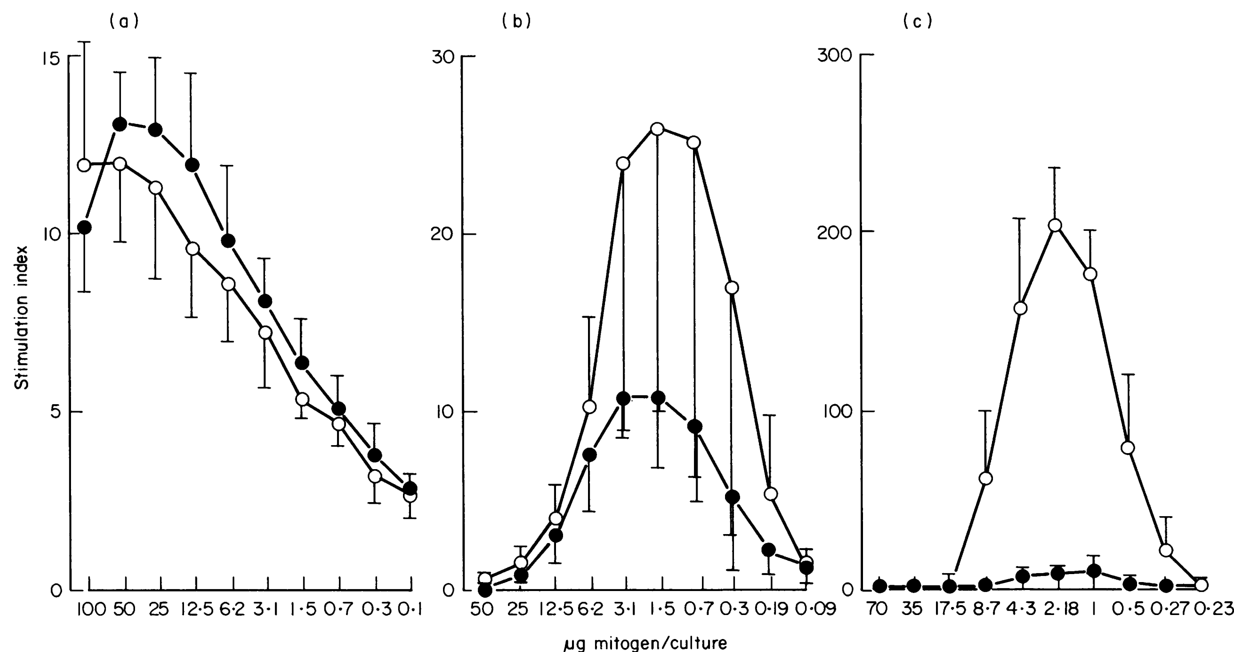

Fig. 4. Lymphocyte stimulation by LPS (a); PHA (b); and Con A (c) of splenocytes from LA mice fed a Zn^+ (○) or Zn^- (●) diet.

affinity of IL-2 receptors in spleen cells from Zn^+ and Zn^- LA mice 24 h after immunization were not significantly different (Table 2).

DISCUSSION

The zinc deficiency induced in the mice in this study was of a clinically mild form, and the only observed symptom was failure to gain weight. Flow cytometry revealed that the mild zinc deficiency induced had important effects on the T cell compartment of the immune system. Thus Zn^- mice had reduced numbers of Thy 1.2, Lyt 2 and L3T4-bearing cells, reduced IL-2 production but unchanged IL-2 receptor expression and affinity. The reduction in Thy 1.2 cells has been previously reported (Fernandes *et al.*, 1979) but the increase in B cells reported by these authors was not seen in our study. Furthermore, the reduction in both L3T4 and Lyt 2 cells contrasts with the report of Fraker *et al.* (1978) that only the L3T4 subset is affected in zinc deficiency. These changes in phenotypic expression in the lymphocyte populations of Zn^- animals may well have influenced the reduced proliferative responses to mitogens and specific antigen in these animals. Con A is thought to stimulate both helper/inducer and suppressor T cells, whereas PHA stimulates T suppressor cells (Miller, 1983) and the reduced proliferation may therefore arise from the presence of fewer differentiated or functional T cells in the assay. However, the reduction in T helper/inducer cells as assessed by flow cytometry (1.5-fold) is significantly lower than the decrease in ConA stimulation (40-fold), indicating an effect on the proliferative potential of T cells independent of their reduced numbers. The possibility that changes in diet affected antigen presenting cells cannot, however, be ruled out.

The antibody responses of Zn^- animals to both a T-dependent and T-independent antigen were investigated. The results showed a decrease in antibody levels only in LA mice immunized with a T-dependent antigen and this was associated with a significant enhancement of antibody affinity. However, the affinity of antibody produced to the T-dependent antigen by Zn^- LA mice was significantly elevated compared with that produced by Zn^+ animals. A decrease in antibody levels in Zn^- animals has previously been shown (Fraker, Hass & Lvecke, 1977) but this is the first report of such a decrease in antibody levels being associated with an increase in affinity. Furthermore, the antibody response of mice already capable of producing HA antibody (HA mice) is not qualitatively altered by zinc deficiency.

The precise cellular mechanisms by which antibody affinity is controlled are not known but it has been suggested that the selective proliferation of B cells of a particular affinity is driven by cellular competition for limited amounts of antigen (Siskind & Benacerraf, 1969). T cells have been shown to be important in the control of affinity but the roles played by lymphocyte subsets are not clear. However, helper cells appear to be important for a HA response (DeKruyff & Siskind, 1979) and it has been suggested that suppressor cells inhibit HA antibody production (Tada & Takemori, 1974) and prevent affinity maturation (Steward, Stanley & Furlong, 1986). The increase in affinity in Zn^- mice is difficult to explain; yet, in the light of previously published findings, it would appear that the lack of zinc had altered the relative ratios of Th to Ts, to favour HA production. Indeed, if it is assumed that the dramatic (40-fold) reduction in proliferation in response to Con A represents a significant reduction in T suppressor cells, then the observed increase in affinity may arise from the reduction of suppression.

Deficiencies of trace metals, particularly zinc, have been proposed as underlying reasons for the immune dysfunction in protein energy malnutrition (Golden *et al.*, 1978). Zinc deficiency consistently accompanies inadequate dietary protein in humans and experimental animals (Golden & Golden, 1981; Filteau & Woodward, 1982) and zinc administration to mal-

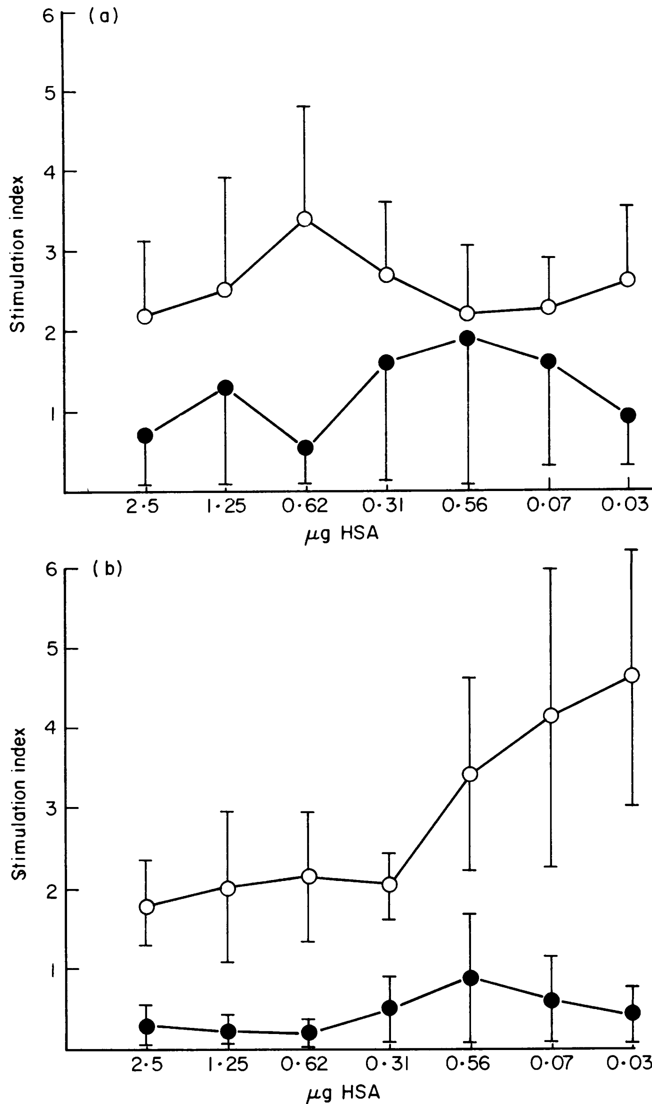

Fig. 5. Lymphocyte stimulation by specific antigen of splenocytes from HSA-immunized LA mice (a) and HA mice (b) fed a Zn^+ (○) or Zn^- (●) diet.

Table 2. IL-2 concentration and IL-2 receptor number and affinity in Zn^+ and Zn^- mice

Diet	Splenic IL-2 concentration (ng/50μl)	IL-2 receptor number/cell	IL-2 receptor affinity ($\times 10^{-12}$ M)
Zn^+	22·6+2·1	8000+ 940	61·8+12·3
Zn^-	14·7+3·6	8700+1250	64·4+12·8

nourished children accelerates thymus growth (Golden *et al.*, 1978). Reconstitution of Zn^- animals with zinc sulphate in the present studies restored the antibody response to that seen in Zn^+ animals, suggesting that those animals were only deficient in zinc.

The mechanism(s) by which zinc deficiency affects the immune system and T cell function in particular is unknown. However, a number of possible explanations have been proposed: (1) the elevation of corticosteroid levels in zinc deficiency and their immunosuppressive action; (2) the role of zinc in lymphocyte trafficking, a process influenced by metal binding proteins (DeSousa, 1978); and (3) the influence of zinc on cell membrane integrity (Chvapil *et al.*, 1976). However, few of the postulated mechanisms of zinc action appear to reflect the T cell specificity of the effect of zinc deficiency described in this study. In contrast with the data presented here, studies by Dowd, Kelleher & Guillou (1986) suggest that the influence of zinc deficiency in rats is expressed via a decrease in IL-2 production and IL-2 receptor expression perhaps through effects on zinc-dependent thymic hormones (Cunningham-Rundles *et al.*, 1980).

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