

Functional capacity of the residual lymphocytes from zinc-deficient adult mice

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Zn deficiency has been shown to reduce host defence drastically. It was of interest to determine the capacity of the residual lymphocytes from Zn-deficient mice to proliferate and produce lymphokines in response to stimulation since there are many Zn-dependent metalloenzymes that might be altered by the deficiency. To address this question, young adult A/J mice were provided Zn-deficient or Zn-adequate diets or restricted amounts of a Zn-adequate diet for 30 d. Splenocytes from moderately or severely Zn-deficient adult A/J mice gave normal proliferative responses and generated adequate interleukin II (IL-2) activity when stimulated with the mitogen Concanavalin A. However, splenocytes from deficient mice exhibited a higher degree of proliferation (about 150%) and production of IL-2 in response to foreign target cells compared with T-cells prepared from mice provided a Zn-adequate diet. B-cells from deficient mice stimulated *in vivo* with sheep erythrocytes produced fewer total numbers of plaque-forming cells (PFC) per spleen. Nevertheless, the proportion or number of PFC/10⁶ viable splenocytes and the amounts of IgM and IgG antibody produced per PFC were equivalent to those of adequately-fed and restricted-fed controls. The previously described responses were not significantly affected by whether the level of Zn in the culture medium was adequate or limiting. Based on these tests it appeared that the residual splenic lymphocytes of Zn-deficient mice were able to carry out many fundamental immune processes.

Zinc: Lymphocyte function: Mouse

Zn deficiency is a prevalent human nutritional problem throughout the world, including the USA (Prasad, 1984). In both humans and animals, Zn deficiency causes rapid and severe depressions in immune function (Fraker *et al.* 1977, 1986; Fernandes *et al.* 1979). In the young adult mouse, antibody-mediated responses to both thymus-independent and thymus-dependent antigens exhibited marked depressions (50–70%) depending on the degree of the deficiency (Fraker *et al.* 1977). Cell-mediated responses such as delayed-type hypersensitivity and cytotoxicity of tumour cells were also substantially reduced (Fernandes *et al.* 1979; Fraker *et al.* 1982). The deficiency caused drastic reductions (40–50%) in the absolute numbers of lymphocytes and macrophages in the blood, thymus, and spleen (Wirth *et al.* 1984; Fraker *et al.* 1987). Yet the relative percentage of T- and B-cells and mononuclear phagocytic cells remained unchanged in deficient adults (Fraker *et al.* 1987). Thus, it was evident that part of the reduction in host defence capacity created by suboptimal Zn was due to the depression in overall numbers of leukocytes that could participate in host defence reactions.

Because of the reduced capacity of Zn-deficient adult mice to mount immune responses the question arose as to whether the depression in function was due only to the decreased numbers of lymphocytes or to a decrease in functional capacity of the residual lymphocytes, or both. There are over 100 enzymes that are dependent on Zn for function, including many

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of those associated with RNA and DNA synthesis. Indeed DNA synthesis, though not RNA synthesis, has been shown to be reduced in Zn-deficient animals (Chesters *et al.* 1988). Furthermore, Zn is thought to be essential for adequate membrane function, protein synthesis and the association of nuclear proteins with DNA (Prasad, 1984). Suboptimal dietary Zn, therefore, might significantly alter proliferation and the production of lymphokines by lymphocytes. These processes are the key to a successful immune response.

For this reason, the ability of T- and B-cells to respond to various stimulants was compared using splenocytes prepared from Zn-deficient, Zn-adequate and restricted-fed mice. The ability of T-cells to respond and proliferate to the mitogen Concanavalin A (Con A) and to foreign target cells was tested using a standard mixed lymphocyte culture system (MLC). In each case the ability of the responding T-cells to produce interleukin II (IL-2), a lymphokine critical to optimal proliferation of activated T-cells, was also quantified. IL-2 is produced primarily by activated T-helper cells and enhances their proliferation in part by increasing expression of IL-2 receptors. IL-2 also facilitates B-cell responses. Likewise, the ability of B-cells to produce antibodies was quantified by radioimmunoassay subsequent to *in vivo* immunization.

In contrast to other studies of this nature, care was taken to regulate the level of Zn available to the cells in culture by using either serum-free conditions, limiting amounts of serum (5 ml/l) prepared from Zn-deficient or Zn-adequate mice (autologous sera) or fetal calf serum (FCS; 50 ml/l) which contained higher amounts of Zn. In this way the possibility of *in vitro* repair of Zn-dependent functions, though not eliminated, was reduced. The findings presented here will demonstrate that the residual splenic T- and B-cells from Zn-deficient mice were able to perform a variety of essential functions *in vitro* even in those culture systems where Zn was limiting.

MATERIALS AND METHODS

Animals and diets

A/J and C57Bl/6 adult female mice were purchased from Jackson Laboratory, Bar Harbor, ME, USA. Female Lewis rats were purchased from Charles River Breeding Laboratories, Portage, MI, USA. A/J female mice (6 weeks old) were placed in stainless-steel cages with mesh bottoms to reduce recycling of Zn and housed in a light (12 h/d) and temperature-controlled room (24°). They were provided *ad lib* with a biotin-fortified egg-white diet containing either deficient (0.8 µg Zn/g) or adequate (approximately 26–30 µg Zn/g) levels of Zn. The composition of the diet which has been used extensively with mice is described in Table 1 (Luecke *et al.* 1978; Wirth *et al.* 1984). Since inanition accompanies Zn deficiency, a restricted group which received a Zn-adequate diet equivalent to the average amount of food consumed the previous day by Zn-deficient mice was included (Luecke *et al.* 1978). All mice had free access to deionized distilled water (< 0.2 µg Zn/g). Diet intake was determined daily. Feed jars and water bottles were washed with 4 M-HCl and rinsed with deionized water to remove Zn. The mice were weighed at least once weekly. At the end of the dietary period those mice which received the Zn-deficient diet and weighed 65–68% of the average body weight of the mice fed on the Zn-adequate diet were designated as severely Zn-deficient mice. The latter also represented the lowest weight range for the deficient mice. Moderately deficient mice were defined as weighing 70–74% of the average body weight of Zn-adequate mice. Previous studies indicated that the latter group is only modestly affected by inanition and parakeratosis (Luecke *et al.* 1978). As will be discussed, the degree of parakeratosis was significant for the severely deficient mice. Although subjective in nature, the total degree of parakeratosis of the tail and ears per mouse was determined using an arbitrary scale of 0–4.

Table 1. *Composition of diets (g/kg)*

Ingredient*	
Glucose monohydrate	609
Egg-white solids (spray-dried)	200
Maize oil	100
Fibre†	30
Salt mix‡	40
Vitamin mix§	10
Ethoxyquin	1

* Except for maize oil (Michigan State University Food Services) and ethoxyquin, all ingredients purchased from Teklab Test Diets, Madison, WI, USA.

† Cellulose-type fibre.

‡ AIN-76 (American Institute of Nutrition, 1977) mineral mix without zinc carbonate, supplemented with appropriate amount of zinc carbonate.

§ Composition similar to AIN-76 (American Institute of Nutrition, 1977) mixture except that the biotin level was increased to provide an additional 2 mg/kg diet because of avidin present in egg white.

|| Santoquin or ethoxyquin; Monsanto Chemical Co., St Louis, MO, USA.

Collection of autologous serum

Serum was collected by severing the subclavian artery of the severely Zn-deficient or Zn-adequate mice. The blood was incubated for 15 min at 37° and for several hours at 4° before processing. Mouse sera (5 ml/l)-supplemented culture medium was filter sterilized using a 0.2- μ m Nalgene filter.

Zinc analysis

The diets and sera were analysed for Zn content by atomic absorption spectrophotometry (Techron AA-175; Varian, Springvale, CA, USA) as described previously (Fraker *et al.* 1977).

Cell culture and mitogenic stimulation

The culture medium consisted of RPMI-1640 (M.A. Bioproducts, Walkersville, MD, USA) buffered with 0.01 M-Hepes, pH 7.4 (Gibco Laboratories, Grand Island, NY, USA) and sodium bicarbonate (0.6 g/l), to which was added 0.1 mM-non-essential amino acids, 2 mM-glutamine, 1 mM-sodium pyruvate, 100 units penicillin, 100 μ g streptomycin/ml, 50 ml gentamycin (M.A. Bioproducts)/ml, 5×10^{-5} M-2-mercaptoethanol, and Eagle vitamin mix (M.A. Bioproducts). This medium was then supplemented with sera (5 ml/l) collected from deficient or control mice plus bovine serum albumin (BSA; 8 g/l; tissue culture grade; Sigma, St Louis, MO, USA), or with FCS (50 ml/l; M.A. Bioproducts). Splens were removed aseptically, minced, and pressed through a sterile stainless-steel mesh (100 gauge). The single-cell suspension was washed and cell viability was determined using the trypan blue dye exclusion method. These unfractionated or whole spleen cell preparations were used for all studies presented herein and are in keeping with standard procedure for these particular assays (Mishell & Shiigi, 1980). Phagocytic cells account for less than 5% of the population of nucleated cells of the spleen being unchanged in proportion by dietary treatment (Wirth *et al.* 1984). Cultures were incubated at 37° under a humidified atmosphere of CO₂:O₂:N₂ (10:7:83; by vol.) or of CO₂:ambient air (7:93; v/v).

Splenocytes (2.5×10^6 /ml) were cultured (100 μ l) in ninety-six-well, flat-bottom microtitre plates (Falcon Plastics, Oxnard, CA, USA) in serum-free medium containing Con A (Sigma), at concentrations ranging from 0.5 to 5 μ g/l. After 24 h, 1 μ Ci methyl-

[³H]thymidine (2 Ci/mM; Amersham International plc, Amersham, Bucks.) was added to each well. After 18 h the cultures were harvested using a multiple-sample harvester (Otto Hiller Co., Madison, WI, USA), and the DNA was precipitated with cold trichloroacetic acid onto glass-fibre filters to determine the amount of radioactivity incorporated into the DNA as measured by scintillation counting. Unstimulated A/J splenocytes incorporated less than 10 000 counts/min of methyl-[³H]thymidine. At the time of harvest, cell viability was 70 (SE 6)%, as determined using the trypan blue exclusion method. Supernatant fractions from identically stimulated cultures were collected for analyses of IL-2 activity at 24 h.

MLC

A/J splenocytes (H-2^a; 2.5×10^5) prepared from each of the three dietary groups were incubated with 1×10^5 , 2.5×10^5 or 7.5×10^5 mitomycin C-treated C57Bl/6 splenocytes (H-2^b) using culture conditions described previously. From day 3 to 6 the cultures were pulsed with [³H]thymidine to determine the amount of proliferation as already described. Target cells incorporated less than 1000 counts/min of the radioisotope. The viability of all cultures at harvest time was 91 (SE 4)%. Supernatant fractions were collected from identically stimulated cultures at 48 h for analysis of IL-2 activity. Absorption of IL-2 to target cells or production of IL-2 by target cells was below limits of detection (values not shown).

Assay for IL-2 activity

CTLL-2 cells (1×10^4), an IL-2-dependent cytolytic T-cell line (Scripps Clinic, La Jolla, CA, USA), were supplemented with various dilutions of standard or test supernatant fractions from either Con A or allogeneic cell-stimulated splenocytes to analyse IL-2 activity as described by Gillis *et al.* (1978). The cultures were pulsed with 1 μ Ci methyl-[³H]thymidine for the final 18 h of a 42 h incubation with the IL-2 to determine the degree of proliferation of the CTLL cells. One unit IL-2 activity/ml was assigned to that dilution of the supernatant fraction from Zn-adequate splenocytes which induced 50% of the maximum methyl-[³H]thymidine incorporation as quantified by probit analysis.

Antibody production

Mice in each dietary group were immunized intraperitoneally with 1×10^6 sheep erythrocytes (SRBC) and 5 d later spleens were removed for the determination of the number of immunoglobulin-secreting cells and the average amount of immunoglobulin secreted per cell. The number of direct (IgM) and indirect (IgG) anti-SRBC plaque-forming cells (PFC) from each spleen was determined using a modification of the Jerne plaque assay as described in detail elsewhere (Fraker *et al.* 1977). Non-immunized mice, included as controls, produced less than one PFC per million splenocytes. For the determination of the average amount of immunoglobulin produced per PFC, 10^7 splenocytes were suspended in 1 ml culture medium supplemented with γ -globulin-free BSA (10 g/l) (Calbiochem, San Diego, CA, USA) and placed in a 12 \times 75 mm culture tube for 2 h at 37° under CO₂:ambient air (7:93; v/v). Supernatant fractions were collected and the amount of IgG and IgM produced was determined by radioimmunoassay as described below.

Radioimmunoassay

Mouse γ -globulin (Calbiochem) and mouse IgM (MOPC 104E; Bionetics, Charleston, SC, USA) were radioiodinated in the presence of Iodogen (Pierce, Rockford, IL, USA), as previously described by Fraker & Speck (1978). Specific activity of the iodinated immunoglobulin was 1.3×10^5 counts/min per μ g for IgM and 2.1×10^5 counts/min per μ g for IgG.

The amount of IgM or IgG produced in 2 h by cultured splenocytes from mice immunized with SRBC was determined by radioimmunoassay. Supernatant fractions to be tested or unlabelled immunoglobulins to be used as standards (murine IgM, MOPC 104E, or murine IgG) and 5×10^3 ng radioiodinated IgM or IgG were added to 10 μ l normal rabbit serum, along with either affinity purified rabbit anti-mouse μ or rabbit anti-mouse gamma (Zymed, San Francisco, CA, USA). The antibody complexes were allowed to form for 1 h at 37°, followed by precipitation with 90 μ l goat anti-rabbit serum which had been adsorbed with mouse immunoglobulins to remove cross-reaction. The precipitate was washed twice with borate-buffered saline (9 g NaCl/l; pH 8.0) and dissolved in 0.1 M-NaOH for gamma counting. There was no cross-reactivity as detected by this assay for mouse IgM with rabbit anti-mouse gamma or for mouse IgG with rabbit anti-mouse μ . The amount of immunoglobulin in the test supernatant fraction was determined from a standard curve using known amounts of IgM or IgG. The average amount of IgG or IgM produced per IgG or IgM PFC was calculated as follows: amount of immunoglobulin produced by 10^6 cells divided by PFC/ 10^6 cells = average amount of immunoglobulin produced per PFC. Splenocytes from non-immunized mice produced undetectable levels of antibody using these conditions.

Statistics

Mean values with their standard errors were calculated for each treatment group. Probability values for the comparison of the Zn-deficient and restricted groups to the Zn-adequate group were determined by a completely random ANOVA followed by Tukey's Test.

RESULTS

Nutritional status

After a 30 d dietary period the deficient mice had consumed 20% less diet than the adequately-fed mice (Table 2). For nutritional and immunological analysis the deficient mice were segregated into so-called severely deficient mice, which showed significant amounts of parakeratosis on the ears and tail and were 66% the weight of adequately fed mice ($P < 0.01$), and moderately deficient mice, which were those deficient mice which had limited amounts of parakeratosis and weighed 72% of control mice (Table 2; $P < 0.01$). Both the severely and moderately deficient mice exhibited a similar reduction (about 45%) in the numbers of viable splenic lymphocytes that were significantly different from both restricted fed and Zn-adequate mice (Table 2; $P < 0.01$). As is often the case for deficiencies in Zn, the serum Zn level of both the moderate and severely deficient mice were similar (about 500 μ g Zn/l), giving no distinction for the degree of deficiency but being significantly different than that of Zn-adequate mice (980 μ g/l; $P < 0.01$). Nevertheless, the thymus was 80% atrophied for severely deficient mice compared with 50% for moderates, being significantly different from each other and controls ($P < 0.01$). Restricted mice which were a control for inanition weighed 82% of adequate-fed mice and had a modest reduction in splenocyte numbers (about 18%). The restricted mice also exhibited non-significant changes in serum Zn levels and thymus weights compared with Zn-adequate mice (Table 2). All these variables were similar to previous results obtained from this laboratory (Fraker *et al.* 1982; Wirth *et al.* 1984).

Capacity to proliferate and produce IL-2 in response to Con A

The capacity of splenic T-cells to respond to Con A was measured since the latter is a potent mitogen that activates a broad spectrum of T-cells. The response was evaluated in serum-free medium which provided excellent results and good viability. This did not eliminate but,

Table 2. *Body weight, diet consumption, thymus weights, and serum Zn levels of mice after 30 d on a Zn-deficient or a Zn-adequate diet*

(Mean values with their standard errors for six to eight mice)

Dietary group† ...	Severely Zn-deficient		Moderately Zn-deficient		Restricted		Zn-adequate	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Initial body wt (g)	16.9	0.1	16.9	0.1	17.0	0.3	17.1	0.2
Final body wt (g)	14.3**	0.5	15.8**	0.2	17.7**	0.1	21.8	0.2
Food consumption (g)‡			73.4**	4.5	73.4**	4.5	92.7	4.8
Thymus wt (mg)	6.5**	0.8	17.6**	1.3	30.4	1.3	33.7	1.0
Cells per spleen ($\times 10^{-7}$)	1.09**	0.1	1.6**	0.1	2.7	0.2	3.3	0.3
Serum Zn levels ($\mu\text{g/l}$)	524**	16	485**	22	1019	27	985	21

Mean values were significantly different from those of Zn-adequate group. ** $P < 0.01$.

† For details of diets, see Table 1 and p. 836.

‡ Average diet consumption by entire Zn-deficient group shown before separation into severely and moderately deficient groups.

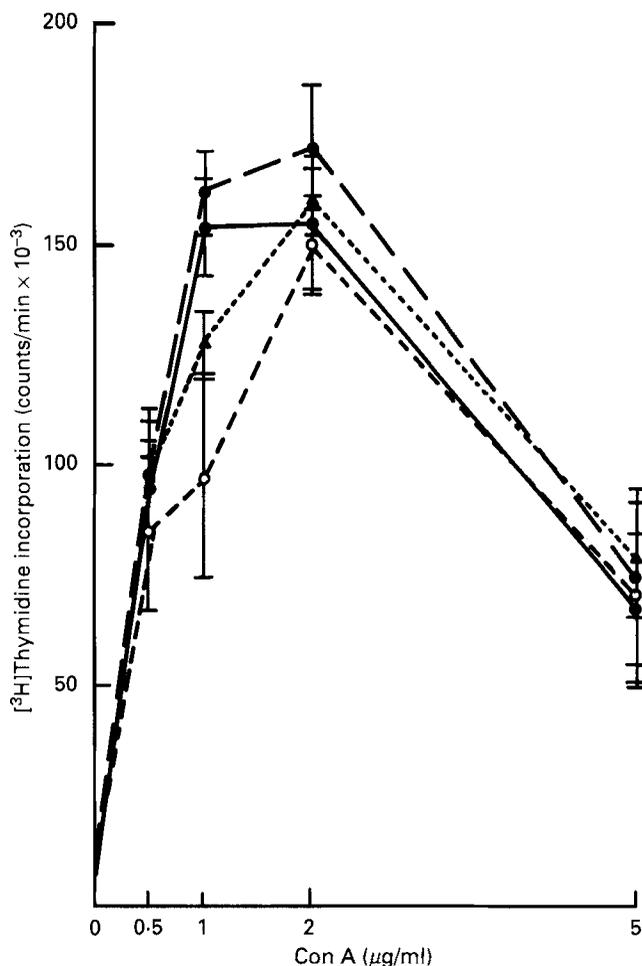


Fig. 1. Dose curve for the proliferative response in a serum-free system ($< 80 \mu\text{g Zn/l}$) to the mitogen, Concanavalin A (Con A) by splenocytes prepared from mice maintained on Zn-deficient (severe (●—●), moderate (●---●)), restricted (○---○) and Zn-adequate (▲---▲) diets for 30 d (for details of diets, see Table 1 and p. 836). Points are means with their standard errors, represented by vertical bars, for six mice.

nevertheless, reduced the opportunity for extensive repair *in vitro* since the levels of Zn in the culture medium ($< 80 \mu\text{g Zn/l}$) were below physiological levels ($800\text{--}1000 \mu\text{g Zn/l}$ for adult murine serum). Using these conditions the production of IL-2, an important T-cell lymphokine or growth factor produced by activated T-cells, was also quantified for all dietary groups. The optimal degree of proliferation in response to Con A was obtained on day 2 with maximum IL-2 production being at 24 h for all treatment groups (dose curves not shown). It is apparent from Fig. 1 that T-cells from moderately and severely deficient mice had similar rates of proliferation from 0.5 to $5 \mu\text{g Con A/ml}$ as splenocytes from adequately fed mice. Restricted-fed mice gave normal responses, with the exception of an unexplained depression in degree of proliferation at $1 \mu\text{g/ml}$ by splenocytes from restricted mice. Likewise, from Fig. 2(a) it is apparent that the activity of IL-2 produced by the moderately deficient mice is equivalent to restricted- and Zn-adequate-fed mice. There was a modest but statistically non-significant reduction in IL-2 activity in the culture

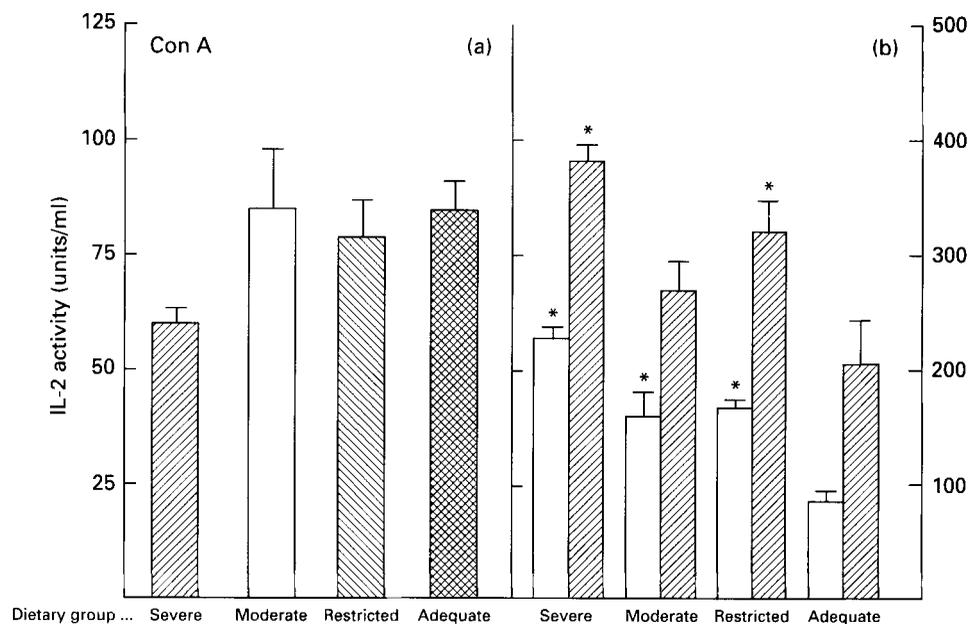


Fig. 2. Interleukin II (IL-2) activity of splenocytes prepared from A/J mice maintained on Zn-deficient (severe or moderate), restricted, or Zn-adequate diets for 30 d (for details of diets, see Table 1 and p. 836). (a) IL-2 activity-generated culture supernatant fractions to $2 \mu\text{g}$ Con A/ml after 24 h in a serum-free culture system; (b) IL-2 activity generated to 1.0×10^5 (\square) or 2.5×10^5 (hatched) foreign target cells (mitomycin c-treated C57Bl/6 splenocytes) after 48 h in culture. Values are means with their standard errors, represented by vertical bars, for six mice. Mean values were significantly different from those of Zn-adequate group: * $P < 0.05$ or greater.

supernatant fractions from severely deficient mice. Thus, the optimal dose, kinetics of proliferation and activity of IL-2 produced in response to Con A were similar for splenocytes for all dietary groups (Fig. 2(a)).

Capacity to proliferate and produce IL-2 in response to allogeneic or foreign target cells

The ability of residual A/J T-helper cells (H-2^a) to proliferate and produce IL-2 in response to allogeneic or foreign target cells was assessed by MLC. This assay is a facsimile of the response the immune system might make to tumour cells or a foreign graft. To obtain good cell viability and proliferation the MLC medium was supplemented with FCS (50 ml/l) with optimal proliferation achieved for all dietary groups on day 4 using 2.5×10^5 mitomycin C-treated C57Bl/6 (H-2^b) target cells (Fig. 3). The proliferation of splenocytes from severely and moderately Zn-deficient A/J mice were 146 and 151% respectively of Zn-adequate controls at the optimum point (day 4; Fig. 3). This heightened degree of proliferation was also apparent at day 3, but faded by day 5 as the response dropped off. Splenocytes from the restricted mice exhibited modest increases in proliferation but were only significantly different from Zn-adequate mice at day 3 ($P < 0.05$).

Since the FCS contained high levels of Zn (3.51 mg Zn/l), the higher response of the deficient groups might have been due in part to *in vitro* repair of Zn-dependent functions. To test this possibility we minimized the presence of Zn by supplementing a subsequent MLC with BSA (8 g/l) in combination with one-tenth the amount of serum (5 ml/l) prepared from Zn-deficient mice (480 μg Zn/l) or serum (5 ml/l) from control mice (980 μg Zn/l). It should be noted that this level of sera (5 ml/l) was the lowest amount that would ensure satisfactory rates of proliferation. MLC cultures cannot be maintained in serum-free

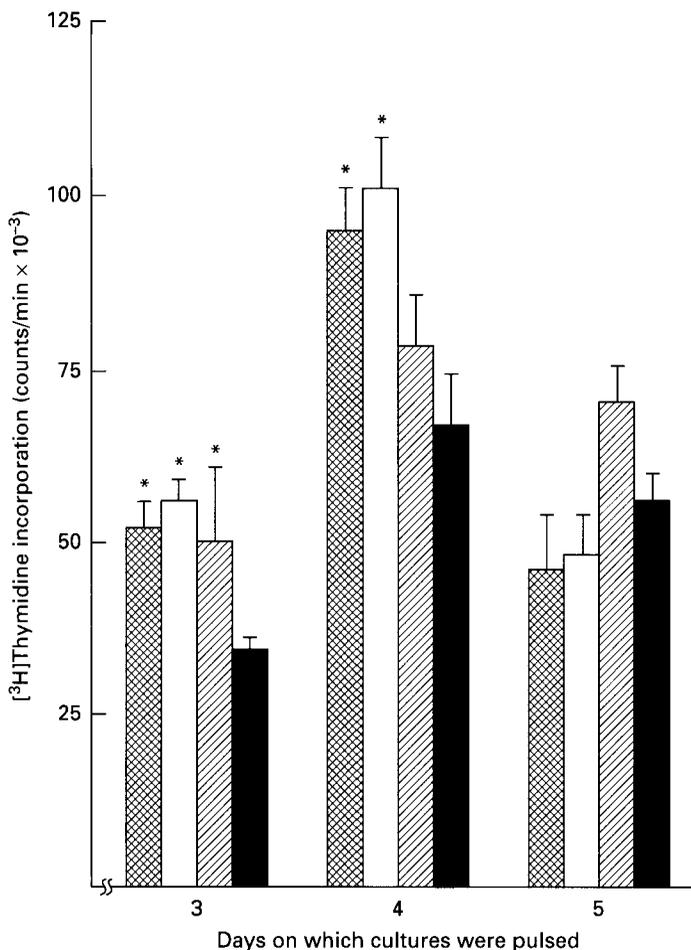


Fig. 3. Kinetics of mixed lymphocyte culture proliferative response in medium supplemented with fetal calf serum (50 ml/l). A/J splenocytes from all dietary groups (severely zinc-deficient (▨), moderately Zn-deficient (□), restricted-fed (▩), Zn-adequate (■)) were cultured with mitomycin c-treated C57Bl/6 splenocytes which served as foreign, allogeneic target cells. Values are means with their standard errors, represented by vertical bars for six mice. Mean values were significantly different from those of Zn-adequate group: * $P < 0.05$ or greater. For details of dietary treatments, see Table 1 and p. 836.

conditions. The optimal proliferation for these conditions was day 5. Once again, proliferation by splenocytes from moderately or severely Zn-deficient A/J mice was as much as 128 and 146% of control values even when cultured in Zn-deficient sera. Differences between severely and moderately deficient mice were not significant. Little differences were observed between supplementing media with the deficient sera *v.* sera from Zn-adequate mice (Fig. 4(a and b)). Restricted-fed mice also exhibited heightened degrees of proliferation incorporating about 30% more [³H]thymidine at optimal stimulation than splenocytes from Zn-adequate mice ($P < 0.05$).

IL-2 activity which was optimal at 48 h for all groups was measured in supernatant fractions from the MLC supplemented with FCS at 48 h which was optimal for all groups (values not shown). Fig. 2(b) shows that IL-2 activity was increased in the supernatant fractions of splenocytes prepared from the severe, moderate, and restricted groups (about 185, 131, and 155% of controls respectively) compared with Zn-adequate controls at

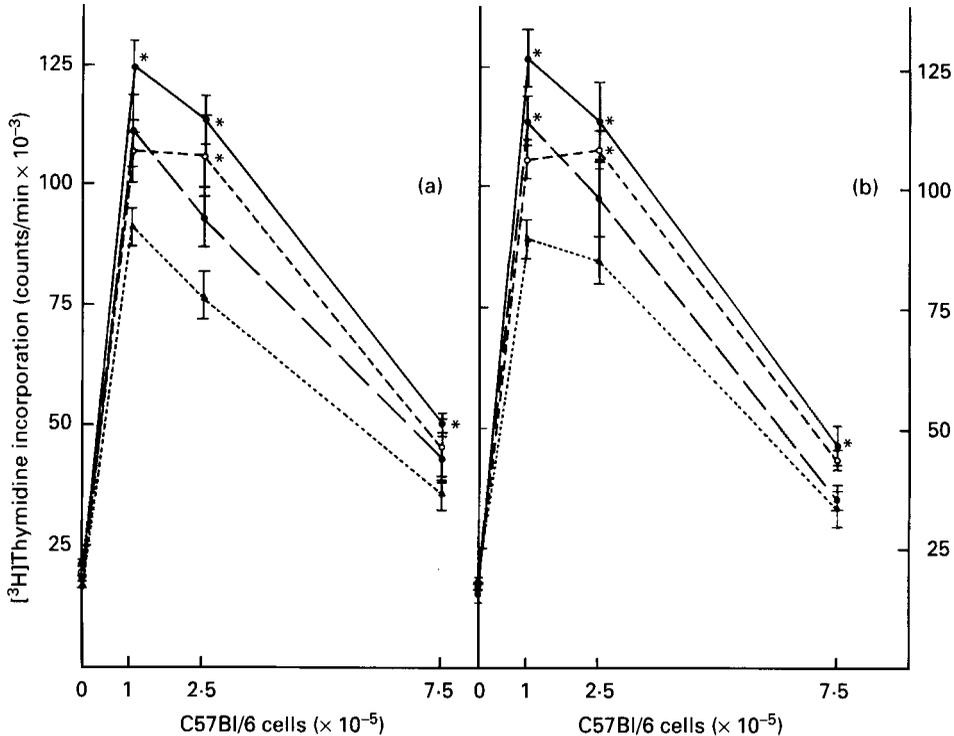


Fig. 4. Dose curve for mixed lymphocyte culture proliferative response in medium supplemented with (a) serum from Zn-deficient mice (5 ml/l), or (b) serum from Zn-adequate mice (5 ml/l). A/J splenocytes from all dietary groups (severely Zn-deficient (●—●), moderately Zn-deficient (●—●), restricted-fed (○—○), Zn-adequate (▲·▲)) were cultured with mitomycin c-treated C57Bl/6 splenocytes which served as foreign, allogeneic target cells. Values are means with their standard errors, represented by vertical bars for six mice. Mean values were significantly different from those of Zn-adequate group: * $P < 0.05$ or greater. For details of dietary treatments, see Table 1 and p. 836.

optimal conditions (2.5×10^5 C57Bl/6 target cells). The enhanced production of IL-2 by restricted-fed mice was somewhat surprising since this proliferation was not much different from the controls. However, this phenomenon was observed on several occasions. The activity of IL-2 was also greater in the cultures containing splenocytes from severely and moderately deficient mice but correlated with the heightened degree of proliferation of T-cells observed for these groups.

Antibody-mediated responses of B-cells

The functional status of residual B-cells of deficient mice was assessed by examining their ability to respond and proliferate to antigens and produce immunoglobulins on a per well basis (PFC). To our knowledge the latter determination had never been made for a nutrition-immunological model. Splenocytes were removed from mice injected 5 d earlier with SRBC and analysed for the numbers of cells or plaques producing anti-SRBC antibody (PFC) using the Jerne plaque assay. The amount of IgM or IgG antibody produced per PFC was determined using a radioimmunoassay. The level of Zn introduced *in vitro* was again minimized. The cells were incubated in medium supplemented only with BSA (10 g/l) for the production of antibodies ($< 80 \mu\text{g Zn/l}$). As in several previous studies (Fraker *et al.* 1987) the total number of PFC produced per spleen in response to SRBC was reduced 50% in the moderately deficient groups (Table 3) ($P < 0.05$). The severely deficient

Table 3. *Antibody-mediated response to sheep erythrocytes (SRBC) of mice after 30 d on a Zn-deficient or Zn-adequate diet*

(Mean values with their standard errors for four to six mice)

Dietary group†	Total IgG PFC		IgG PFC (10 ⁶ splenocytes)	IgM (pg/IgM PFC)		IgG (pg/IgG PFC)	
	Mean	SE		Mean	SE	Mean	SE
Severely Zn-deficient	18 300*	4200	1678	22.1	4.7	15.5	3.1
Moderately Zn-deficient	26 719*	4800	1609	29.6	5.2	16.9	2.8
Restricted	46 048	5400	1705	18.3	3.5	17.0	5.8
Zn-adequate	56 850	5750	1722	25.4	2.2	12.9	3.4

IgM and IgG, direct and indirect anti-SRBC PFC respectively; PFC, plaque-forming cells.

Mean values were significantly different from those of Zn-adequate dietary group: * $P < 0.05$ or greater.

† For details, see Table 1 and p. 836.

mice were suppressed almost 70%, being significantly different from all other groups including the moderately deficient mice ($P < 0.05$ or greater). Restricted mice, though not significantly different from Zn-adequate controls, showed some decline in response. However, the proportion of B-cells responding as determined by the number of PFC per million viable splenocytes remained unaltered in the Zn-deficient groups compared with adequate and restricted-fed mice (Table 3). Table 3 shows that the average amount of IgM produced per IgM-producing anti-SRBC PFC was normal for the Zn-deficient and restricted groups, as was IgG production per IgG-producing anti-SRBC PFC. Thus, although there were fewer total numbers of splenic B-cells responding in the deficient mice, the proportion per million viable lymphocytes was normal, as was the amount of antibody secreted per cell.

DISCUSSION

A variety of *in vitro* tests performed previously (Fraker, 1983; Fraker *et al.* 1987) and in the present study suggest that many of the residual splenic lymphocytes of the Zn-deficient mouse can adequately perform a variety of immune functions. Such results, however, must be interpreted with caution when studying nutritional-immunological systems. First, nutrients such as Zn which are limiting in the host may be readily available in culture systems supplemented with 50–100 ml sera/l. Second, changes created by Zn deficiency, such as the production of chronically elevated levels of corticosteroids during the course of the deficiency, cannot be properly replicated *in vitro* (DePasquale-Jardieu & Fraker, 1980). Yet the corticosteroids are widely recognized as immunosuppressants and, thus, may greatly alter lymphocytic responses *in vivo* (Fauci *et al.* 1976). Furthermore, host defence to T-cell dependent and independent antigens are reduced *in vivo* in the Zn-deficient host, partly because of the reduction in total numbers of leukocytes (Fraker *et al.* 1982; Wirth *et al.* 1984). These changes are offset or normalized for *in vitro* studies because of the need to place equal numbers of viable cells in culture. Failure to consider these important factors and the use of lymphocytes from a variety of tissues may account for the highly variable results obtained by investigators when studying the effects of Zn deficiency on immune function *in vitro* (Gross *et al.* 1979; Pekarek *et al.* 1979; Allen *et al.* 1982; Kramer, 1984; Carlomagno *et al.* 1986).

The mitogen Con A was chosen as a test of T-cell function because it is thought to stimulate a high proportion of various subsets of T-cells. Kramer (1984), Carlomagno *et al.* (1986) and Dowd *et al.* (1986) also observed that splenocytes from Zn-deficient rodents

gave normal proliferative responses to Con A. However, only Dowd *et al.* (1986) took the precaution of also culturing splenocytes in autologous sera, as was done in the present study. The latter group also found IL-2 production in response to Con A stimulation to be normal for Zn-deficient rats in agreement with the present finding for both severely and moderately deficient mice. Thus, our findings confirm those of Dowd *et al.* (1986) that T-cell helper cells *in vitro* appear to function normally, even those from Zn-deficient mice, and that reduced responses to T-cell dependent antigens *in vivo* are probably due to overall reductions in T-helper cells and other leukocytes. It is, nevertheless, acknowledged that other investigators who used fetal bovine serum-supplemented cultures report depressed responses for lymphocytes from Zn-deficient rodents and patients to Con A and other T-cell mitogens (Gross *et al.* 1979; Pekarek *et al.* 1979; Allen *et al.* 1982).

In contrast to Con A, the MLC test assessed the functional capacity of a narrow subset of T-cells, namely those that recognized H-2^b target cells (C57Bl/6). Surprisingly, the proliferation of T-cells and production of IL-2 by splenocytes from moderately and severely deficient mice exceeded that of mice from the Zn-adequate group. Information collected in this laboratory (L. King and P. J. Fraker, unpublished results) and that of Dowd *et al.* (1986), indicates no significant change in the proportion of T-cells or helper T-cells created by suboptimal Zn that would account for the enhanced MLC. Modest reductions in the proportion or function of suppressor T-cells in Zn-deficient mice might also account for an enhanced response, although it would seem that the Con A response should also have been elevated. Although corticosteroids are generally immunosuppressive, a few investigators have noted these steroids can in some instances cause elevated MLC responses for yet unknown reason (Blomgren & Svedmyr, 1971; Lee, 1977). Since splenocytes from these Zn-deficient mice have been exposed to chronically elevated levels of corticosteroids (DePasquale-Jardieu & Fraker, 1980), this might explain the altered response. It also remains unclear as to why splenocytes from restricted-fed mice exhibited more enhanced proliferative capacity in autologous mouse sera but not FCS (50 ml/l). In any event, these tests indicate that many of the residual T-cells of the Zn-deficient mouse, especially those of the helper variety, are normal or augmented in terms of capacity to proliferate and produce IL-2. It is, however, acknowledged that none of these assays directly tested T-cell cytotoxic function (T_c) or suppressor function (T_s).

Finally, it is apparent that B-cells from deficient mice, though producing fewer total numbers of antibody-producing cells per spleen in response to antigenic stimulants, nevertheless produced proportional numbers of antibody-producing cells (PFC/10⁶ viable splenocytes). The latter fact has been previously noted by us and others (Frost *et al.* 1977; Carlomagno & Murray, 1983; Fraker *et al.* 1987). However, it was also demonstrated in the present study that similar amounts of antibody per activated B-cell were produced by all cells from all dietary groups. Furthermore, other studies examining splenic B-cell responses from Zn-deficient mice to a variety of mitogens (Fraker, 1983) also suggested that the proliferative capacity of the residual B-cells was normal, even when Zn was limiting in the culture system.

Although it is readily acknowledged that not all subsets of T- and B-lymphocytes were directly tested, the variety of assessments performed here and in past studies (Fraker *et al.* 1983) suggests that many of the residual splenic lymphocytes of the Zn-deficient mouse are functional. Although complete assurance that repair of Zn-dependent functions did not occur *in vitro* cannot be given, care was taken to conduct each test with an amount of Zn that was well below physiological levels. That many of the residual lymphocytes of the deficient mouse are functional is also supported by *in vivo* findings which indicated that the proportion of splenocytes responding to antigenic stimulation *in vivo* (e.g. PFC/10⁶ viable splenocytes) was normal for deficient mice (Frost *et al.* 1977; Carlomagno & Murray, 1983;

Fraker *et al.* 1987). In this regard it is interesting to note that on a per cell basis functionality also remained intact in mice that exhibited higher degrees of parakeratosis and weight loss that were associated with the severely deficient mice. Nevertheless, the severely deficient mice always exhibited greater thymic atrophy, greater loss of splenocyte numbers and more substantial losses in total defence capacity (e.g. PFC responses per spleen) than mice which were moderately Zn deficient. Thus, the reduction in the total numbers of leukocytes created by suboptimal Zn appears to be a significant factor in the demise of host defence. Interestingly, recent analysis of the phenotypic distribution of the lymphocytes of the spleen using flow cytometry indicated that Zn-deficiency did not significantly alter the relative ratio or proportion of the major subsets of T- and B-cells (King & Fraker, 1991). Ascertaining the mechanism whereby Zn deficiency causes lymphopenia, thymus atrophy and reductions in phagocytic cells will be instrumental in understanding the nature of nutritional-immunological interactions.

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