

# Regeneration of T-cell helper function in zinc-deficient adult mice

(thymic atrophy/re-feeding of zinc-adequate diet/repair and repopulation of thymus/young adult A/J mice)

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**ABSTRACT** Diets deficient in zinc cause rapid atrophy of the thymus and loss of T-cell helper function in the young adult A/J mouse. Because zinc deficiency, as well as other nutritional deficiencies, causes extensive damage to the immune system, the question arose as to whether zinc-deficient mice could repair the thymus and fully regenerate T-cell helper function if returned to diets containing adequate amounts of zinc. Five-week-old A/J female mice were fed either a zinc-deficient ( $<1 \mu\text{g}$  of Zn per g) or a zinc-adequate ( $50 \mu\text{g}$  of Zn per g) diet for 31 days. Histological examination of thymuses from the zinc-deficient mice revealed that the cortex was preferentially involuted and the thymus was about one-third of normal size. The direct plaque-forming cells produced per mouse spleen in response to immunization with sheep erythrocytes was 34% of normal; indirect plaque-forming cells were 18% of normal (Jerne plaque assay). After the deficient mice had been fed a zinc-adequate diet for 1 week, their response was nearly normal, except that the indirect response was 68% of controls; in this same period, the thymuses of these mice had quadrupled in size and exhibited a greatly enlarged cortex repopulated with immature thymocytes. By 2 weeks, the thymuses of the previously zinc-deficient mice were normal in size and appearance; however, there was a slight increase in numbers of indirect plaque-forming cells. By 4 weeks, the thymus weights, direct and indirect plaque-forming cell counts, and secondary response of the previously deficient mice were normal. Mice that were nearly athymic after 45 days of dietary zinc deficiency were also able to fully reconstruct the thymus and regenerate T-cell helper function. The data show that the zinc-deficient young adult mouse has the capacity to fully restore the T-cell-dependent antibody-mediated responses upon nutritional repletion.

It has been known for some time that malnourished children are more susceptible to disease and infection (1, 2) and that the death rate from ordinary childhood diseases is extraordinarily high among these children compared to their normal counterparts (3). From available clinical data it is apparent that rapid atrophy of the thymus is a feature common to many types of deficiencies as shown by autopsies performed on children who suffered from protein-calorie malnutrition, kwashiorkor, or marasmus (4, 5). Laboratory animals made deficient in certain amino acids (6), vitamins (7), or trace elements (8) also exhibit marked atrophy of the thymus.

Fraker *et al.* (9) recently showed that dietary zinc deficiency also caused rapid wasting of the thymus. Young adult A/J mice fed zinc-deficient diets became athymic in 6 weeks. Analysis of the antibody-mediated response of these mice to sheep erythrocytes (SRBC) by the indirect Jerne plaque assay indicated that the deficient mice had  $1/10$ th the whole spleen response of mice fed zinc-adequate diets. Reconstitution of the zinc-deficient mice with thymocytes restored their response to near-normal levels. These data along with studies that showed a nearer normal IgM than IgG response among the zinc-deficient mice suggested that zinc deficiency primarily impaired T-cell helper function and had only a moderate effect on B-cell function in the young adult mouse.

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The severity of the loss of immune function and the swiftness with which zinc deficiency brought about destruction of the thymus brought to the fore the question of repair. Do nutritionally deficient animals have the capacity to repair damaged immune systems if re-fed nutritionally adequate diets and is there full restoration of immune function? Little information is available in the literature (10) on the ability of nutritionally deficient humans or animals to repair lymphocyte functions upon nutritional repletion. The question of repair capacity is of obvious importance to those concerned that dietary deficiency may cause permanent impairment of the immune system. Therefore, it was of interest to determine whether the zinc-deficient young adult mouse is capable of repopulating the atrophied thymus when re-fed a diet containing adequate amounts of zinc.

In this study, 5-week-old A/J female mice were fed zinc-deficient diets for either 31 or 45 days, at which time their thymuses were 30 or 10% of normal size, respectively. At the end of these two feeding periods the antibody-mediated responses of deficient and control mice to a T-cell-dependent antigen were compared. The deficient mice were then re-fed zinc-adequate diets, and the regeneration of T-cell helper function and the degree of restoration of the thymus were assessed at 1, 2, and 4 weeks. The results show that the zinc-deficient adult mouse can fully restore the antibody-mediated response within 2 weeks after resumption of a zinc-adequate diet.

## MATERIALS AND METHODS

Sterile SRBC in Alsever's solution were from GIBCO; guinea pig complement was from Miles; immunoagarose was from Marine Colloids; vitamin and trace element mixtures were purchased from Teklabs; spray dried egg white was from General Biochemicals (Chagrin Falls, OH) and Paraplast was from Sherwood Medical Industries.

**Animals and Diets.** Female A/J mice (33-37 days old) purchased from Jackson Laboratory were weighed and distributed equitably into 14 groups. Each treatment group consisted of 12 mice housed 4 per cage; mean ( $\pm$ SEM) weight was  $17.1 \pm 0.2$  g. To minimize exposure to environmental zinc, the mice were housed in stainless steel cages with mesh bottoms that had been washed by hand and rinsed with distilled water. The mice had free access to deionized water ( $<0.3 \mu\text{g}$  of Zn per g). Feeder assemblies consisted of acid-washed glass jars fitted with stainless steel feed follow-through discs to reduce food loss.

All mice were fed ad lib a biotin-fortified egg white diet which contained either adequate ( $50 \mu\text{g}$  of Zn per g) or inadequate ( $<1 \mu\text{g}/\text{g}$ ) levels of dietary zinc as described (8, 9). The mice were fed a zinc-deficient diet for either 31 or 45 days before being returned to a zinc-adequate diet for assessment of their repair capacity. Diet consumption was monitored daily and the mice were weighed at least once a week. The zinc

Abbreviations: SRBC, sheep erythrocytes; PFC, plaque-forming cells.

content of the diets was determined by wet ashing in a perchloric acid/nitric acid mixture and atomic absorption spectrophotometry using the Varian AA-175 (9, 10).

**Jerne Plaque Assay.** Mice were immunized intraperitoneally with  $1 \times 10^8$  SRBC in sterile phosphate-buffered saline. The total number of direct (IgM) and indirect (IgG) plaque-forming cells (PFC) per mouse spleen was determined on day 5 by using a modification of the Jerne plaque assay (9). Briefly, the spleens were removed aseptically and pushed through an 80-mesh stainless steel screen. After washing, the cell suspension was brought to a final volume of 1 ml. Dilutions of the spleen cells along with  $2 \times 10^8$  SRBC were overlaid onto a 60-mm petri dish containing an agarose-medium base. After incubation for 1.5 hr at 37°C, nonhemolytic guinea pig complement was added to the direct plates. Rabbit anti-mouse IgG (11) was added to the indirect plates 30 min prior to the addition of complement. Corrections were made for the small number (9%) of direct plaques that developed on the indirect plates. Unimmunized mice gave a background of 30–40 plaques per spleen, which was insignificant.

**Histology.** Immediately after etherization, the thymuses of the zinc-deficient and control mice were removed, weighed, and placed in Bouin's fixative for 72 hr (12). The tissues were dehydrated in a series of dioxane/water washes (1:4, 1:2, 1:1, 2:1, vol/vol) and embedded in Paraplast. Sections 5  $\mu$ m thick were mounted on glass slides and stained by the Masson-trichrome procedure (12) which allows for differentiation of the medulla from the cortex. Midsections of each thymus were examined by light microscopy. A grid was superimposed over micrographs of these sections to allow quantitation of medulla and cortical areas.

**Statistical Methods.** All data were examined by analysis of variance with statistical significance of treatment differences being determined by Student's *t* test (13).

## RESULTS

**Regeneration of T-Cell Helper Function in Mice Fed Zinc-Deficient Diet for 31 Days.** The first experiment in this series is outlined in Table 1. In this case, the mice were divided into eight groups of 12 mice each. Four groups were fed a zinc-adequate diet and four groups were fed a zinc-deficient diet ad lib. After 31 days, one group fed the zinc-deficient diet (group 0) and one group fed the zinc-adequate diet (0C) were immunized intraperitoneally with  $1 \times 10^8$  SRBC. The number of direct and indirect, PFC per mouse spleen was determined 5 days later by the Jerne plaque assay. The wet weight of various tissues was also obtained at this time; this served as a measure of the loss of immune capacity and degree of tissue atrophy incurred during the 31 days of zinc deficiency. To assess their potential for repair, the three remaining zinc-deficient

groups were returned to zinc-adequate diets and were immunized with SRBC at intervals of 1, 2, and 4 weeks. The number of PFC produced per mouse spleen at day 5 was compared to controls to determine the degree of restoration of immune function.

The body and organ weight data for the first experiment are shown in Table 2. Before the return to zinc-adequate diets, the body weight of the zinc-deficient mice (0) was only 69% (6 g less) of that of the controls (0C). The livers of the deficient mice were moderately lighter than those of the controls; the kidneys weighed 83% as much as those of the controls. As in previous experiments (9), the lymphatic organs showed the greatest signs of atrophy; the spleen was one-half normal weight and the thymus was only one-third normal. The effect of zinc deficiency on the T-cell-dependent antibody-mediated response is shown in Table 3. The deficient mice produced only 34% as many IgM PFC and 18% as many IgG PFC per spleen as control mice.

The repair capacity of the zinc-deficient young adult mouse proved to be exceptionally good. After 1 week (plus 5 days) on the zinc-adequate diet, the formerly deficient mice had normal organ and body weights (Table 2). In addition, their diet consumption, on a gram per gram of body weight basis, was 40% greater than that of controls during the first week of re-feeding a zinc-adequate diet. Even more remarkable was the regenerative capacity of the thymus which nearly quadrupled in size, increasing from 34% to 125% of normal wet weight. A greatly enlarged cortex packed with immature thymocytes (see below) was responsible for this temporary enlargement. The previously zinc-deficient mice (group 1) had nearly normal numbers of IgM PFC but only 68% of normal IgG PFC (Table 3), indicating that restoration of T-cell helper function was not yet complete (14). After 2 weeks of the re-feeding regimen, the thymuses of the previously zinc-deficient mice were normal in weight and appearance and there was a slight increase ( $P < 0.20$ ) in the total number of indirect PFC, perhaps as a result of the hyperactivity and enlargement of the thymus during the previous week. Thus, repopulation of the spleen with mature helper T cells required about 2 weeks. By week 4, organ weights and numbers of PFC in the repair group were identical to those of the control group.

**Secondary Response of Mice Maintained on Zinc-Deficient Diet for 31 Days and Re-fed Zinc-Adequate Diet for 4 Weeks.** After 4 weeks of re-feeding of a zinc-adequate diet, the capacity of the zinc-deficient mice to mount a secondary response to SRBC was tested. Concurrent with the previous experiment, an additional group of 12 mice was also fed the zinc-deficient diet for 31 days. A companion control group was fed the zinc-adequate diet. As in the case of groups 4 and 4C (Table 1), both groups were then fed a zinc-adequate diet for 4 weeks to allow maximal time for repair. Both groups were given two intraperitoneal injections of  $1 \times 10^8$  SRBC spaced 1 week apart. A week after the second injection the number of indirect PFC per mouse spleen was assessed by the Jerne plaque assay. There was no statistically significant difference in the responses of the formerly zinc-deficient mice (mean  $\pm$  SEM,  $75,800 \pm 5,900$  PFC per mouse spleen) and of the control mice ( $63,900 \pm 5,300$  PFC per mouse spleen), indicating full response of T-memory cells. Body and organ weights were also equivalent.

**Regeneration of T-Cell Helper Function in Athymic or Nearly Athymic Zinc-Deficient Mice.** To determine whether full immune repair was also possible in cases of prolonged zinc deficiency, which leaves only an involuted rudiment or no visible sign of a thymus, an additional four groups of mice were incorporated into the initial experiment (two zinc-deficient and two zinc-adequate dietary groups). In this case the mice were maintained on a zinc-deficient diet for 45 days before being

Table 1. Outline of first experiment

Dietary treatment*		Repair period, weeks†
0	–Zn	0
0C	+Zn	0
1	–Zn	1
1C	+Zn	1
2	–Zn	2
2C	+Zn	2
4	–Zn	4
4C	+Zn	4

\* For 31 days.

† Period during which mice received zinc-adequate diets.

Table 2. Body and organ weights upon re-feeding of zinc-adequate diets

Dietary group	Repair period, weeks	Weights*				
		Body, g	Liver, g	Kidney, mg	Spleen, mg	Thymus, mg
0	0	14.3 ± 0.6	0.79 ± 0.04	254 ± 10	43 ± 3	11 ± 2
		(69%)†	(67%)†	(83%)†	(50%)†	(34%)†
0C		20.7 ± 0.3	1.18 ± 0.03	307 ± 9	86 ± 4	32 ± 2
1	1	19.2 ± 0.5	1.15 ± 0.32	297 ± 10	89 ± 3	36 ± 1
		(91%)	(91%)	(104%)	(100%)	(125%)
1C		21.2 ± 0.5	1.26 ± 0.06	287 ± 8	90 ± 5	29 ± 1
2	2	18.9 ± 0.9	1.12 ± 0.07	279 ± 13	96 ± 6	30 ± 4
		(92%)	(95%)	(98%)	(96%)	(100%)
2C		20.5 ± 0.6	1.18 ± 0.34	284 ± 6	100 ± 5	30 ± 1
4	4	20.8 ± 0.8	1.21 ± 0.05	295 ± 10	86 ± 9	31 ± 1
		(92%)	(95%)	(93%)	(106%)	(110%)
4C		22.7 ± 0.5	1.28 ± 0.04	318 ± 5	81 ± 2	28 ± 1

Five-week-old A/J female mice were fed a zinc-deficient diet (<1 µg/g) for 31 days. One group of deficient mice (0) and one group of control mice fed a zinc-adequate diet (0C) were immunized at this time with  $1 \times 10^8$  SRBC. The remaining groups of zinc-deficient mice were fed a zinc-adequate diet for 1, 2, or 4 weeks and then studied.

\* Mean ± SEM;  $n = 12$ . In parentheses are shown the -Zn values as percentage of the +Zn values.

†  $P < 0.001$ , Student's  $t$  test.

returned to a zinc-adequate diet. As in the first experiment, one zinc-deficient group (0) and one control group (0C) were immunized on day 45 with  $1 \times 10^8$  SRBC and the number of direct and indirect PFC per mouse spleen were determined 5 days later. The thymuses of the zinc-deficient mice were about  $1/10$ th normal size (Table 4). Three of the 12 zinc-deficient mice died (group 0) and the remainder weighed only 57% as much as the controls. Such prolongation of zinc deficiency results in inanition which Luecke *et al.* (11) have shown contributed substantially to loss in T-cell helper function and immune responsiveness. Among the zinc-deficient mice, the direct PFC response was 13.7% of normal and the indirect PFC were 5.6% of normal. Such a reduction in immune response represents a severe loss in immune capacity.

The thymuses of the deficient mice had no visible cortex and a greatly reduced medulla. However, even in this extreme case

the surviving zinc-deficient mice showed full recovery of the antibody-mediated response when examined after 4 weeks of nutritional repletion (Table 4). Body and thymus weights and the number of PFC per spleen were identical in both the control and formerly zinc deficient mice (Table 4).

In a pilot experiment identical to the one just described, the deficiency period was extended to 49 days which resulted in the death of half or more of the mice in the zinc-deficient groups. Prior to refeeding of zinc-adequate diets, six of the mice from one of the zinc deficient groups were examined and found to be athymic. The five remaining mice from the second group of zinc deficient mice were re-fed a zinc-adequate diet for 4 weeks. These mice also were capable of restoring fully the T-cell-dependent antibody-mediated responses. These results indicate that the young adult is still capable of regenerating the thymus even in those cases in which no visible thymus can be observed.

**Histology of Thymuses from Zinc-Deficient Mice and Deficient Mice Re-fed Zinc-Adequate Diet.** Histological examination of the thymus during the progression of zinc deficiency revealed a preferential involution of the cortex of the thymus, which is the region populated by immature thymocytes (Fig. 1A and B). After 36 days of zinc deficiency, the average cortical area was 0.75 times the area of the medulla (Fig. 1A) whereas mice of the same age fed zinc-adequate diet had a cortex-to-medulla ratio of 1.9. As the period of zinc deficiency was extended, the cortex continued to involute, fewer thymocytes were visible, the normally densely staining area gradually lightened, and the cortical epithelial cells became more prominent. The medulla exhibited no visible changes in appearance and only a minimal decrease in area during the 36-day period of zinc deficiency. However, with prolonged deprivation, the medulla also underwent atrophy and Hassel's bodies appeared more numerous, possibly as a result of the shrinkage of the medulla.

The histology and repopulation of the atrophied thymus was followed in mice made zinc deficient for 31 days prior to nutritional repletion (first experiment). Prior to re-feeding, the average thymus had the appearance of that shown in Fig. 1A. After 1 week (plus 5 days) on a zinc-adequate diet, an enlarged thymus similar to that in Fig. 1C was found. The previously

Table 3. Effects of re-feeding zinc adequate diet on plaque-forming response of A/J mice made zinc deficient for 31 days

Dietary group	Repair period, weeks	Plaques per spleen*	
		Direct (IgM)	Indirect (IgG)
0	0	4,490 ± 920*	4,120 ± 740
		(34%)†	(18%)†
0C		13,100 ± 1,390	22,250 ± 4,750
1	1	12,860 ± 1,240	15,260 ± 1,670
		(94%)	(68%)
1C		13,670 ± 730	22,410 ± 2,240
2	2	14,590 ± 2,150	27,720 ± 2,770
		(107%)	(137%)
2C		13,700 ± 1,150	20,270 ± 2,930
4	4	13,640 ± 1,400	20,200 ± 3,040
		(116%)	(94%)
4C		11,780 ± 730	21,400 ± 1,600

Conditions of this experiment are explained in Table 2. Total numbers of direct and indirect PFC per spleen were determined by the Jerne plaque assay 5 days after immunization with  $1 \times 10^8$  SRBC.

\* Mean ± SEM;  $n = 12$ . In parentheses are shown the -Zn values as percentage of the +Zn values.

†  $P < 0.001$ , Student's  $t$  test.

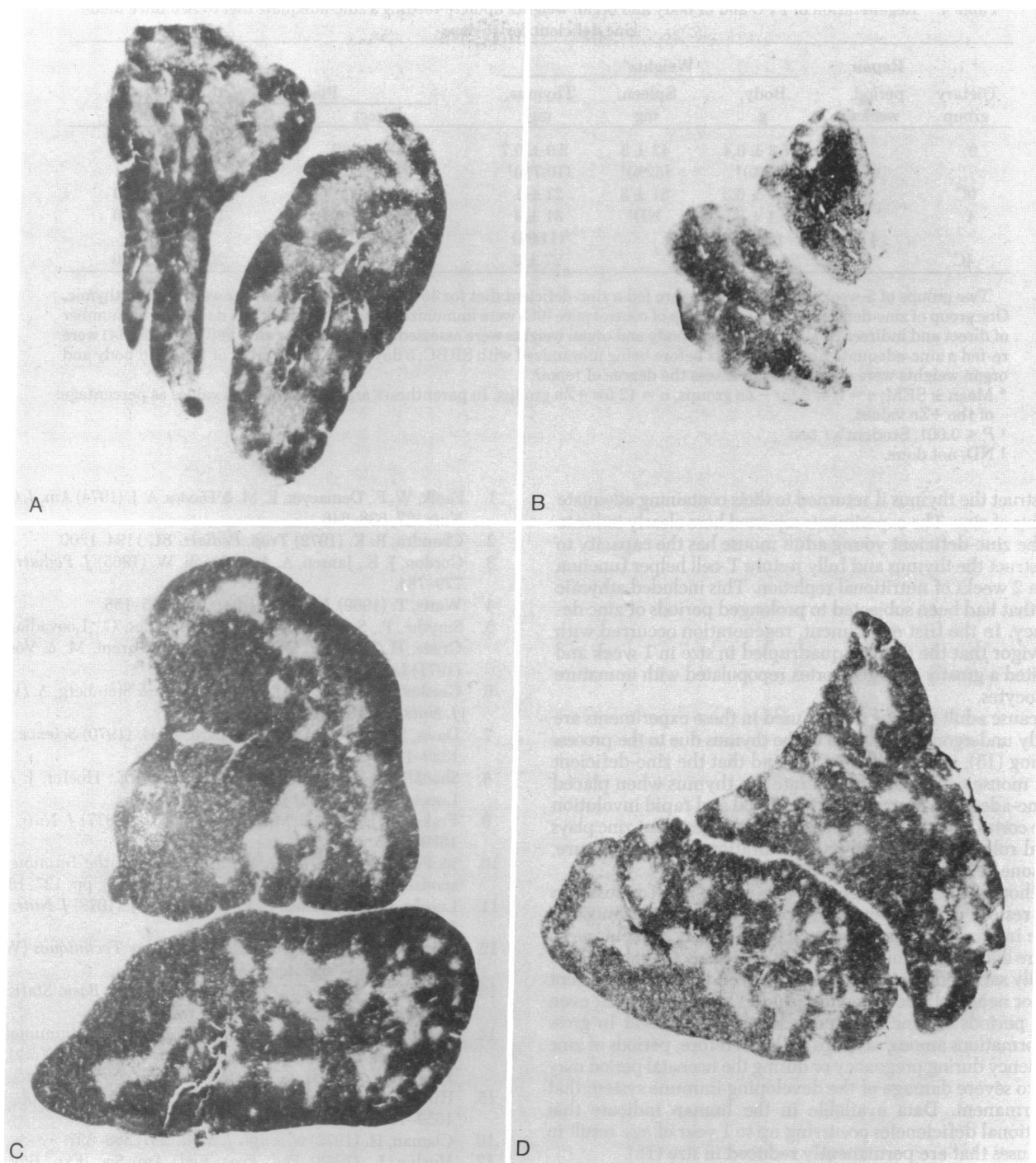


FIG. 1. Thymuses of A/J female mice. (Masson-trichrome;  $\times 6.5$ .) (A) After 36 days on a zinc-deficient diet (from the first experiment). Note rim of cortex surrounding intact medulla. (B) After 45 days on a zinc-deficient diet. Cortex is completely atrophied. Medulla stains darker than cortex due to destruction of most of the cortical lymphocytes. (C) Previously deficient animal from the first experiment after 1 week (+5 days) of re-feeding a zinc-adequate diet. Note enlarged cortical area, repopulated with lymphocytes. (D) Thymus of control mouse taken during week 5 of the first experiment.

involved cortex had enlarged to an area 3.0 times that of the medulla compared to a ratio of 1.9 for controls of the same age. As shown in Table 2, the thymus had quadrupled in wet weight. The enlarged cortex was densely packed with large, immature thymocytes, which appeared to be a sign of hyperactivity on the part of the thymus in its attempt to quickly replenish the peripheral T-cell population. After 2 weeks on a zinc-adequate diet, the thymus was normal in appearance and size (Fig. 1D) and remained so throughout the experiment.

## DISCUSSION

Previous studies in our laboratories (9) indicated that zinc deficiency in the young adult mouse caused rapid atrophy of the thymus and a substantial depression of the antibody-mediated response. Reconstitution studies indicated that this was primarily due to a loss in T-cell helper function. The severity of the loss in immunity which could result in complete atrophy of the thymus and  $1/10$ th normal PFC per spleen (9) raised the question as to whether or not the zinc-deficient mice could

Table 4. Regeneration of PFC and of body and organ weights upon re-feeding a zinc-adequate diet to A/J mice made zinc deficient for 45 days

Dietary group	Repair period, weeks	Weights*			Plaques/spleen*	
		Body, g	Spleen, mg	Thymus, mg	Direct	Indirect
0	0	11.3 ± 0.4	42 ± 3	2.9 ± 0.7	2,900 ± 630	2,170 ± 640
		(57%)†	(52%)†	(10.7%)†	(13.7%)†	(5.6%)†
0C	4	19.9 ± 0.3	81 ± 3	27 ± 1	21,180 ± 1,230	38,900 ± 3,370
4		20.4 ± 0.5	ND†	31 ± 4	15,500 ± 1,430	19,050 ± 2,300
		(90%)		(114%)	(113%)	(94%)
4C		22.7 ± 0.4		27 ± 3	13,710 ± 1,200	20,340 ± 2,740

Two groups of 5-week-old A/J females were fed a zinc-deficient diet for 45 days, by which time they were nearly athymic. One group of zinc-deficient (0) and one group of control mice (0C) were immunized with  $1 \times 10^8$  SRBC; 5 days later the number of direct and indirect PFC per spleen and body and organ weights were assessed. The remaining zinc-deficient mice (4) were re-fed a zinc-adequate diet for 4 weeks before being immunized with SRBC; 5 days later the number of PFC and body and organ weights were determined to assess the degree of repair.

\* Mean ± SEM;  $n = 8$  or  $9$  for  $-Zn$  groups,  $n = 12$  for  $+Zn$  groups. In parentheses are shown the  $-Zn$  values as percentage of the  $+Zn$  values.

†  $P < 0.001$ , Student's  $t$  test.

‡ ND, not done.

reconstruct the thymus if returned to diets containing adequate amounts of zinc. The experiments reported here clearly indicate that the zinc-deficient young adult mouse has the capacity to reconstruct the thymus and fully restore T-cell helper function within 2 weeks of nutritional repletion. This included athymic mice that had been subjected to prolonged periods of zinc deficiency. In the first experiment, regeneration occurred with such vigor that the thymus quadrupled in size in 1 week and exhibited a greatly enlarged cortex repopulated with immature thymocytes.

Because adult mice of the age used in these experiments are already undergoing involution of the thymus due to the process of aging (15), it was of interest to find that the zinc-deficient adult mouse could fully regenerate the thymus when placed on zinc-adequate diets. The preferential and rapid involution of the cortex of the thymus in these mice suggests that zinc plays a vital role in the survival and maturation of the immature, cortisone-sensitive  $T_1$  thymocytes (16).

Although the knowledge that the young adult mouse can fully restore its T-helper cell response is assuring, it must be borne in mind that the mice used for these experiments were mature and fully immunocompetent. We cannot be certain that equally satisfying results will be achieved with zinc-deficient fetal or neonatal mice. Previous studies have shown that even short periods of zinc deficiency late in term result in gross malformations among rat pups (17). Therefore, periods of zinc deficiency during pregnancy or during the neonatal period may lead to severe damage of the developing immune system that is permanent. Data available in the human indicate that nutritional deficiencies occurring up to 1 year of age result in thymuses that are permanently reduced in size (18).

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