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Research Article

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Serum Thymulin in Human Zinc Deficiency

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Abstract

The activity of thymulin (a thymic hormone) is dependent on the presence of zinc in the molecule. We assayed serum thymulin activity in three models of mildly zinc-deficient (ZD) human subjects before and after zinc supplementation: (*a*) two human volunteers in whom a specific and mild zinc deficiency was induced by dietary means; (*b*) six mildly ZD adult sickle cell anemia (SCA) subjects; and (*c*) six mildly ZD adult non-SCA subjects. Their plasma zinc levels were normal and they showed no overt clinical manifestations of zinc deficiency. The diagnosis of mild zinc deficiency was based on the assay of zinc in lymphocytes, granulocytes, and platelets. Serum thymulin activity was decreased as a result of mild zinc deficiency and was corrected by in vivo and in vitro zinc supplementation, suggesting that this parameter was a sensitive indicator of zinc deficiency in humans. An increase in T101-, sIg-cells, decrease in T4+/T8+ ratio, and decreased IL 2 activity were observed in the experimental human model during the zinc depletion phase, all of which were corrected after repletion with zinc. Similar changes in lymphocyte subpopulation, correctable with zinc supplementation, were also observed in mildly ZD SCA subjects. Inasmuch as thymulin is known to induce intra- and extrathymic T cell differentiation, our studies provide a possible mechanism for the role of zinc on T cell functions.

Introduction

Thymulin (formerly called the Facteur Thymique Serique [FTS]) is a well-defined thymic hormone with the following amino acid sequence: < Glu-Ala-Lys-Ser-Gln-Gly-Gly-Ser-Asn-OH (1). Since its initial isolation from pig serum, a number of actions of thymulin on the immune system have been recognized (2–6). Successful therapeutic use of this hormone in some human immunodeficiency diseases has also been observed (2–6). Dardenne et al. (7, 8) showed previously that thymulin requires the presence of zinc to express its biological activity.

The interaction between zinc and thymulin has been demonstrated directly by the chromatography of a mixture of Chelex 100-treated [^{3}H]thymulin and Zn-65 on Bio-Gel P-2. [^{3}H]-Thymulin and bound Zn-65 coelute precisely with the peak of thymulin biological activity and the binding affinity was calculated to be ~ 1 μM by equilibrium chromatography (9). More recently, by means of nuclear magnetic resonance, it has been shown that the conformation of the metal-deprived peptide is strikingly different from that of the zinc-peptide complex (10). These results indicate the existence of two forms of the hormone: the first one deprived of Zn and biologically inactive, the second one containing Zn and biologically active.

The zinc/thymulin relationship was previously investigated using two models of in vivo zinc deficiency. First, active thymulin levels were studied in sera from mice subjected to a long-term, marginally zinc-deficient diet. Despite the absence of thymic atrophy, a significant decrease in the serum levels of thymulin was observed as early as 2 mo after beginning the diet. However, these levels could be consistently restored after in vitro addition of $ZnCl_2$ (11).

Similar observations were made with sera from children suffering from nephrotic syndrome with zinc deficiency, a disease in which a low level of thymulin activity was observed and its level could be restored to normal after in vitro serum chelation and incubation with $ZnCl_2$ (12). These results confirm the presence of the inactive hormone in the serum of zinc-deficient individuals and its potential activation after zinc addition. The specificity of these results was confirmed by the lack of activation in experiments performed with sera from thymectomized mice or patients with DiGeorge's syndrome, in whom the hormone is nonexistent.

In this paper, we report the results of serum thymulin activity assay in three groups of zinc-deficient human subjects: (*a*) human volunteers in whom a specific and mild deficiency of zinc was induced by dietary means; (*b*) mildly zinc-deficient adult sickle cell anemia (SCA)[1] subjects; and (*c*) mildly zinc-deficient adult non-SCA subjects. The studies were repeated before and after oral zinc supplementation in all three groups. The zinc status in human subjects was assessed by using recently developed techniques in our laboratory for assay of zinc in lymphocytes, granulocytes, and platelets. Lymphocyte subpopulation studies and assay for IL 2 were performed in the experimental human model during baseline, and zinc depletion and repletion periods. Lymphocyte subpopulation studies were also done before and after zinc supplementation in zinc-deficient SCA subjects.

Methods

Human subjects. The protocol for all human studies was reviewed and approved by the Committee on Research in Human Subjects at Wayne

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1. *Abbreviations used in this paper:* AAS, atomic absorption spectrophotometer; Az, azathioprine; SCA, sickle cell anemia; SRBC, sheep red blood cells; Tx, thymectomized.

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We have succeeded in establishing an experimental human model for the study, by dietary means, of a mild state of zinc deficiency. Two adult male volunteers, ages 23 and 25 yr, were hospitalized at the Clinical Research Center of the University of Michigan Medical School Hospital. A semipurified diet that supplied ~ 3.0 mg of zinc on a daily basis was used to produce zinc deficiency. The details of methodology have been published elsewhere (13).

The volunteers were ambulatory and were encouraged to do daily moderate exercise throughout the study period. Before the study, a thorough history, physical examination, and routine laboratory tests including complete blood count, liver function, sequential multiple analyzer-12, and serum electrolytes were performed and found normal. Zinc in lymphocytes, granulocytes, and platelets was determined and found to be in the normal range.

They were given a hospital diet containing animal protein daily for 4 wk. This diet averaged 12 mg of zinc/d, consistent with the recommended dietary allowance of the National Research Council, National Academy of Sciences. After this, they received 3.0 mg of zinc a day while consuming a soy protein-based experimental diet. This regime was continued for 28 wk, at the end of which two cookies containing 27 mg of zinc supplement were added to the experimental diet. The supplementation was continued for 12 wk.

Throughout the study, the levels of all nutrients including protein, amino acids, vitamins, and minerals (both macro- and microelements) were kept constant, meeting the standards set by Recommended Dietary Allowances, except for zinc, which was varied as outlined above. By this technique, we were able to induce a specific zinc deficiency in human volunteers and were able to document thymulin activity during deficiency and after zinc supplementation.

Six zinc-deficient homozygous male SCA subjects were included in this study. Their ages ranged from 17 to 33 yr. They were ambulatory, had no neurological or psychiatric deficits, were not dependent on drugs, and had no other hemoglobinopathies such as alpha-thalassemia, beta-thalassemia, or Hb C disease, etc. These subjects have been followed regularly in the adult sickle-cell clinic of the University Health Center of Wayne State University, Detroit Medical Center. They had received no blood transfusion during at least the past 6 mo before this study. Before selection of these subjects for our study, zinc was assayed in lymphocytes, granulocytes, and platelets by flameless atomic absorption spectrophotometry. Plasma zinc levels ranged from 98 to 118 μg/dl and were considered to be within the normal range.

We also identified six human volunteers who showed a mild state of zinc deficiency. A mild state of zinc deficiency was defined by a decreased level (1 SD or more below the mean) of zinc in any two cell types (lymphocytes < 48, granulocytes < 42, and platelets < 1.7 $\mu g/10^{10}$ cells). By these criteria, the above subjects were diagnosed having a mild state of zinc deficiency. The normal levels of zinc (mean±SD) are: lymphocytes, 54.4±6.6; granulocytes, 49.8±7.5; and platelets, 2.2±0.5 μg per 10^{10} cells. The plasma zinc in these cases was within normal ranges (100 to 120 μg/dl), and there were no overt clinical manifestations of zinc deficiency. These subjects were laboratory personnel and medical students. Their ages ranged from 25 to 58 yr. Five were male, and one was female.

Reagents. Hanks' medium and sheep red blood cells (SRBC) were obtained from Pasteur Institute, Paris, France. Azathioprine (Az) used in its sodium salt form was obtained from Burroughs-Wellcome (Research Triangle Park, NC). Zinc chloride ($ZnCl_2$) of the highest reagent grade was purchased from E. Merck, Darmstadt, FRG. The metal ion-chelating agent, Chelex 100, was produced by Bio-Rad Laboratories, Richmond, CA. Anti-thymulin MAb were obtained by cell fusion according to a previously reported method (14).

Evaluation of thymulin levels in the peripheral blood. The serum level of biologically active thymulin was evaluated by a rosette assay described in detail elsewhere (15), which we and several other investigators have shown to be strictly thymus specific (16, 17). The assay analyzes the conversion of relatively Az-resistant spleen of adult thymectomized mice to theta positive rosette-forming cells that are more sensitive to Az (15). Briefly, sera under study were filtered through membranes (YMT micropartition systems MPS-1; Amicon Corp., Danvers, MA) to eliminate high-molecular weight molecules that have been shown previously to interfere with the assay. The ultrafiltrates were incubated for 90 min at 37°C with spleen cells from C57BL/6 mice thymectomized (Tx) 10–15 d before the test and Az at the concentration of 10 μg/ml, a concentration that inhibits rosette formation in normal mice but not in Tx mice. Rosettes were then formed by centrifugation with SRBC and enumerated in a hematocytometer after gentle resuspension. In the presence of thymulin-containing sera, rosette formation was inhibited by Az. The results were expressed as the log 2 of the reciprocal highest serum dilution conferring sensitivity to Az inhibition upon spleen cells from adult Tx (ATx) mice. To confirm the specificity of the biological activity measured, all the determinations were repeated after preincubation of the sera under study with an anti-thymulin MAb or a specific antithymulin immunoabsorbent.

Chelation and metal treatment. Sera (100 μl) from patients and control subjects were incubated for 30 min at room temperature with an equal volume of Chelex 100 at 50 mg/ml in distilled water. At the end of the incubation, the mixture was centrifugated at 12,000 *g* for 2 min to eliminate the chelating resin and the biological activity in the supernatant of Chelex 100-treated serum was measured. 10 μg of $ZnCl_2$ were then added to 100 μl of Chelex-treated serum. The mixture was then incubated for 15 min at room temperature and its biological activity was measured by the rosette assay.

Zinc assay in lymphocytes, granulocytes, and platelets. 30 ml of venous blood was collected in the a.m. in a fasting state. Lymphocytes, neutrophils, and platelets were harvested simultaneously by a modification of a previous method (18). The entire isolation was done at room temperature. Precautions were taken to remove erythrocytes from neutrophils, platelets from lymphocytes, and trapped plasma from platelets, which are commonly associated as contaminants.

15 ml of blood was collected in a polystyrene centrifuge tube containing 300 μl of zinc-free and preservative-free heparin. The sample was centrifuged at 180 *g* for 10 min and platelet-rich plasma was removed and saved for zinc assay in the platelets. The residual blood sample was transferred to a 50-ml polypropylene centrifuge tube containing 30 ml of acid-citrate-dextran solution and the total volume was adjusted to 45 ml with PBS. The sample was mixed gently and allowed to settle for 15–20 min. The leukocyte-rich supernate was removed to a clean 50-ml polypropylene test tube and centrifuged at 180 *g* for 10 min. The supernate was discarded and 15 ml of PBS was then added to the leukocyte pellet and mixed gently.

The lymphocytes and granulocytes were fractionated with the use of discontinuous Histopaque gradients and digested with 500 μl of nitric acid (Ultrex) overnight at room temperature according to previously published methods (18).

For isolation of platelets, the platelet-rich plasma was centrifuged at 280 *g* for 5 min. The supernate was transferred to a polypropylene centrifuge tube and centrifuged at 3,700 *g* for 15 min. The supernate plasma was removed and 1 ml hypotonic PBS was added to the pellet. The pellet was partially dispersed by using a plastic transfer pipet. To lyse contaminating red cells, 4 ml of deionized water was added and mixed gently. After this, 5 ml of hypertonic PBS was added, the tube was capped, and the solution was mixed. The tube was then centrifuged at 3,700 *g* for 15 min. After removal of the supernate, the platelet pellet was resuspended and washed twice with PBS.

After the second wash, the supernate was discarded and 1 ml normal saline was added to the pellet. The platelet suspension was again centrifuged at 180 *g* for 1 min. 500 μl of the supernate was then transferred to a 1.5-ml prerinsed micro-Eppendorf tube and 200 μl of this solution was used for platelet counts (Baker 810 Platelet Analysis; Baker Instruments Corp., Allentown, PA).

The micro-Eppendorf tube was then centrifuged at 3,700 *g* and supernate was discarded. 500 μl of Ultrex concentrated nitric acid was then added to the pellet for digestion overnight at room temperature and at 65°C for 4 h.

Zinc was assayed in the samples by means of an atomic absorption spectrophotometer (AAS) equipped with a furnace and auto-sampling system (Instrumentation Laboratory 555 AA Spectrophotometer with a 655 furnace and 254 Fastac Auto Sampler; Instrumentation Laboratory, Inc., Lexington, MA). Standards were prepared as follows: (*a*) elemental zinc standards containing 60, 120, 180, and 240 ng/ml in Ultrex concentrated nitric acid; (*b*) concentrated nitric acid digests of bovine liver standard 1577A (National Bureau of Standards, Washington, DC) diluted to contain 60, 120, 180, and 240 ng/nl; and (*c*) a third set of standards was prepared by mixing at a 1:1 ratio the standards prepared in steps *a* and *b*.

For the assay, the three sets of standards were diluted 1:40 with deionized water in triplicate. The peak height and peak area absorbances of the three sets of standards were read at 213.9 nm on the AAS. The peak area absorbances of the three sets of standards read the same; and, as such, the combined zinc/bovine liver standard was used for the reference curve.

The AAS was programmed to read the samples in concentration units. The machine was recalibrated at frequent intervals. The coefficient of variation ranged from 4 to 5%, and the recovery of zinc ranged from 99 to 106% for these assays.

Lymphocyte subpopulation studies. PBMC were isolated by density-gradient centrifugation with Ficoll-Hypaque (Sigma Chemical Co., St. Louis, MO). Cell surface markers were studied with the use of color-coded, antibody-coated microbeads (Quantigen; Bio-Rad Laboratories, Richmond, CA). In brief, 1×10^6 mononuclear cells were incubated with 200 μl of one of two microbead preparations. For the detection of surface immunoglobulins (sIg), in B lymphocytes, beads coated with antihuman immunoglobulin antisera were used. In the same preparation, beads coated with the T101 MAb were used for the quantification of T lymphocytes. The T101 MAb reacts with a 65,000-D antigen present on the surface of mature and immature T cells, but not mature B cells. Simultaneously, an identical cell suspension was incubated with a preparation of the MAb T4- and T8-coated microbeads for the enumeration of T helper and T suppressor lymphocytes, respectively. Cells with at least three identical microbeads attached were scored as positive for the particular antibody. Cells with no beads were counted as T101−, sIg−. Macrophages were identified by the presence of intracytoplasmic beads. 200 cells were counted from each preparation. The percentage of lymphocytes reactive with each antibody was calculated and corrected for macrophages. Absolute numbers of lymphocytes in each subclass were then calculated from the peripheral blood absolute lymphocyte count.

In the experimental human model studies, lymphocyte subpopulation data were obtained once a month throughout the study, during baseline, zinc-depletion, and zinc-repletion phases.

Lymphocyte subpopulation data were obtained in four SCA subjects, twice before and twice after zinc supplementation. A paired *t* test was done to determine the effect of zinc supplementation on lymphocyte subpopulation.

IL 2 activity. IL 2 activity was assayed in duplicate in our normal volunteer studies once a month during baseline, zinc-depletion, and zinc-repletion phases. PBMC obtained by Ficoll-Hypaque separation were tested for production of IL 2 by culturing them for 48 h with 1% PHA-M, a T cell mitogen, at 10^6 cells/ml in 2 ml final volume. The IL 2 content of supernatants harvested after 48 h culture was measured in a proliferation assay as previously described (19) using a murine IL 2-dependent T cell line, cTLL-20, and a standard reference IL 2 preparation arbitrarily assigned an IL 2 activity of 100 U/ml.

Statistical analysis. For the analysis of data in the experimental human model, the statistical test used was the Mann-Whitney-Wilcoxon two-sample rank sum test. For a single individual and a given parameter, observations during the baseline period, the beginning of the depletion phase, and the latter part of the repletion phase, were all considered as observations from one sample. Observations during the latter part of the depletion phase and the beginning of the repletion phase made up the second sample. This was true for zinc concentrations in plasma, lymphocytes, and granulocytes, lymphocyte subpopulations, and IL 2 activity. Because the platelets showed rapid changes in zinc levels during the zinc-restricted and repletion phases, observations during the baseline period and the repletion period were considered as observations from one sample, whereas observations during the entire depletion phase made up the second sample.

For the analysis of other group data, the *t* test was used to determine significance.

Results

Zinc in plasma and cells. Despite a 2-mo zinc-restricted diet, plasma zinc levels in normal volunteers remained > 100 μg/dl. A significant decrease was observed in the third mo of the zinc-depletion phase (Fig. 1).

The zinc in platelets decreased within 1 mo. However, the zinc concentration of lymphocytes and granulocytes decreased only after 2 mo of zinc-restricted dietary intake (Fig. 2). The changes in granulocyte zinc in subject 2 were nonsignificant because of a smaller number of samples. The maximum decline in zinc in the cells was observed at the end of 6 mo of restricted dietary zinc intake.

Table I shows the data for zinc in the cells before and after zinc supplementation in zinc-deficient (SCA and non-SCA) subjects. Plasma zinc levels were within normal ranges before zinc supplementation in these subjects. After zinc supplementation, the plasma zinc levels ranged between 120 and 140 μg/dl. The zinc concentration of cells increased significantly and became normal after zinc supplementation.

Thymulin activity in volunteers submitted to zinc restriction. Activity of circulating thymulin observed in the two healthy young volunteers before the onset of dietary zinc restriction was normal as compared with a group of age-matched healthy controls. Thymulin activity began to decrease 3 mo after the beginning of zinc deprivation, and was undetectable after 6 mo. In vivo zinc supplementation induced a rapid return to a normal level of thymulin activity within 1 mo. The final level of activity was even higher than those observed before zinc restriction (Fig. 3).

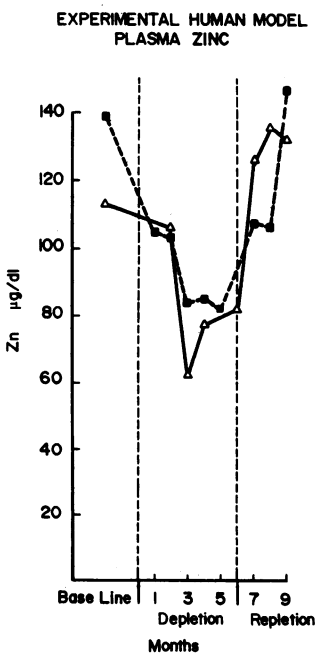

Figure 1. Plasma zinc concentrations in a sequential study of human volunteers submitted to zinc-restricted diet for 6 mo followed by zinc supplementation are shown here. Each data point represents the averages of two separate determinations. ■, subject 1; $P < 0.05$. △, subject 2; $P < 0.05$.

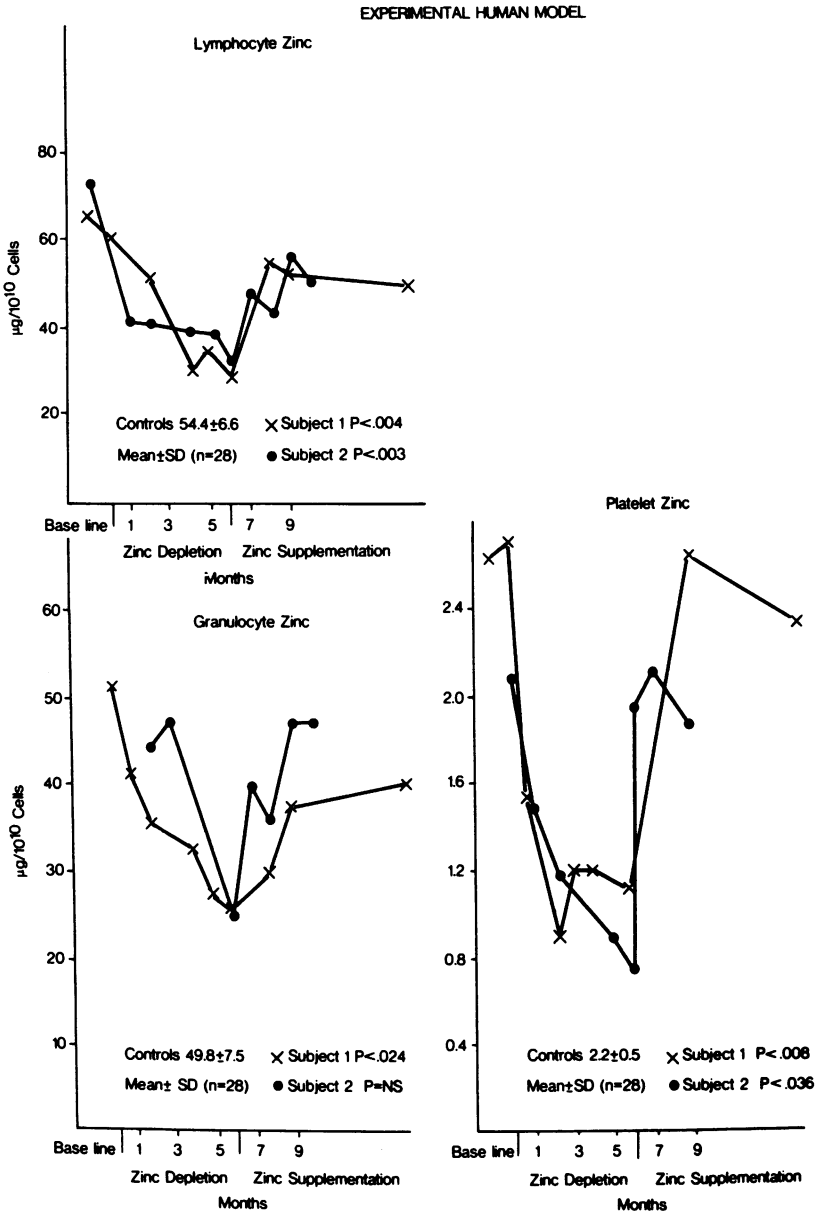

Figure 2. Cellular zinc concentrations in a sequential study of young human volunteers submitted to a zinc-restricted diet for 6 mo followed by zinc supplementation are shown here.

Effect of zinc supplementation on levels of thymulin activity in zinc-deficient SCA and non-SCA subjects. Low levels of active thymulin were found in the zinc-deficient SCA patients and non-SCA zinc-deficient subjects as compared with age-matched healthy individuals. In both cases, zinc therapy restored thymulin levels to normal levels as shown in Fig. 4.

Effect of in vitro zinc addition on thymulin activity. Recent data suggested that the natural thymulin molecule could exist in two forms, one biologically active, containing zinc, and the other inactive and deprived of the metal ion (9–10). To confirm the possibility that the peptide could be present in the serum from zinc-deprived individuals in an inactive form as previously shown in zinc-deficient mice and patients with nephrotic syndrome (12), we performed supplementary experiments, adding $ZnCl_2$ to the sera under study. This addition was done after serum chelation to eliminate other metals, which could compete with zinc for the binding site on the peptide, present in the sera (9).

As observed in Fig. 5, after chelation and incubation with $ZnCl_2$, the activity of thymulin in the serum of zinc-deficient individuals reached normal levels similar to those observed after in vivo zinc supplementation. In contrast, no change in the activity of the hormone was observed after incubation of serum with $ZnCl_2$, when these experiments were done with the sera obtained from patients after zinc supplementation, i.e., at a time when thymulin activity had already reached normal levels, or in healthy volunteers before the beginning of zinc restriction at a time when thymulin was fully active (data not shown). These data demonstrate that, during zinc depletion, thymulin from zinc-deficient subjects contained less zinc on a mole/mole basis than thymulin from zinc-sufficient subjects.

Lymphocyte subpopulation. The significant changes in lymphocyte subpopulation in the experimental human model as a result of zinc depletion and zinc repletion are shown in Fig. 6. The T4+/T8+ cells ratio declined as a result of zinc-restricted diet, and this was corrected by zinc repletion. A signifi-

Table I. Cellular Zinc in Zinc-deficient SCA and non-SCA Subjects before and after Zinc Supplementation

	Zinc		
	Lymphocytes	Granulocytes	Platelets
		$\mu g/10^{10}$ cells	
SCA (n = 6)			
Before	51.8±4.6	35.7±4.2	1.35±0.26
After Zn supplementation*	61.4±5.5	42.8±3.37	2.24±0.71
P	<0.01	<0.01	<0.03
Non-SCA (n = 6)			
Before	47.7±8.4	37.8±1.95	1.6±0.17
After Zn supplementation‡	62.6±5.7	44.0±2.9	1.9±0.15
P	<0.005	<0.002	<0.01
Controls (n = 28)	54.4±6.6	49.8±7.5	2.2±0.5

* SCA subjects were supplemented with 50 mg of zinc (as acetate) orally in two divided doses daily for a 3–6-mo period.
‡ Non-SCA subjects were supplemented with 50 mg of zinc (as acetate) orally in two divided doses daily for 3 mo.
n = number of subjects.

cant increase in T101−, sIg− cells during zinc depletion was observed. This was also correctable with zinc repletion. No significant changes in absolute counts of lymphocytes, T101+, sIg+, T4+, or T8+ cells were noted. The changes in the ratios of T4+/T8+ during zinc depletion were related to slight decrease in T4+ cells and a similar slight increase in T8+ cells that were reversed after zinc supplementation (see Table II).

Table III shows lymphocyte subpopulations in four SCA subjects before and after zinc supplementation. In general, SCA subjects before zinc supplementation had increased levels of absolute lymphocyte count, increased levels of sIg+ and T101−, sIg− cells, and a decreased ratio of T4/T8 cells compared with the normal controls. After zinc supplementation, a significant increase in sIg+ and T4+ cells were observed. The ratio of T4+/T8+ cells also increased significantly. The number of T101−, sIg− cells decreased significantly after zinc supplementation.

IL 2 activity. Our data in the experimental human model show that IL 2 activity declined as a result of zinc restriction toward the end of the zinc-depletion period and in the early zinc-repletion period (Fig. 7), thus suggesting that changes in IL 2 activity may be a late effect of mild zinc deficiency in humans. Unfortunately we lost two samples during the third and fourth months of our experiment; as such, we observed no statistical significance by using the Mann-Whitney-Wilcoxon two-sample rank sum test in IL 2 activity between samples obtained at baseline, during zinc-depletion, and during zinc-repletion periods. The lowest levels of IL 2 activity, as a result of zinc depletion in two subjects, were 3 and 7 U/ml. The IL 2 activity in controls (n = 12) in our laboratory (mean±SD) is 32±8 U/ml. IL 2 activity was promptly corrected during the zinc-repletion period.

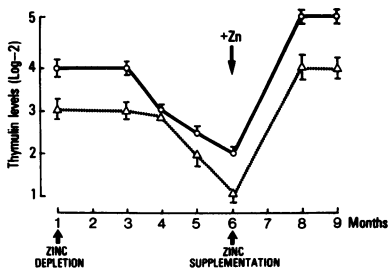

Figure 3. Levels of thymulin activity in sequential study of young human volunteers submitted to a zinc-restricted diet for 6 mo followed by zinc supplementation are shown here. Results are expressed as log-2 reciprocal titers (mean±SEM). Each determination was performed in triplicate.

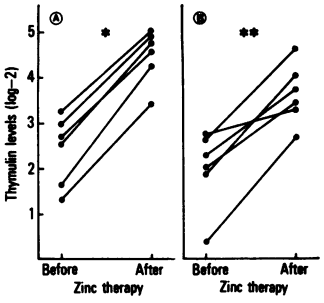

Figure 4. Effect of zinc therapy on the levels of thymulin activity in (*A*) SCA subjects and (*B*) non-SCA deficient patients are shown here. Results are expressed as log-2 reciprocal titers. Each point represents the mean of three determinations. *$P < 0.01$; **$P < 0.001$.

Discussion

Although several polypeptides have been extracted from the thymus, and a number of them have been characterized chemically, from the physiological point of view, only two of these are true thymic hormones, actually produced by the thymus and biologically active on T cells (1, 2, 14). These are thymopoietin and thymulin, both of which are produced by the thymic epithelium (1, 2). Recent data demonstrate that thymulin binds to high-affinity receptors, induces several T cell markers, and promotes T cell function including allogenic cytotoxicity,

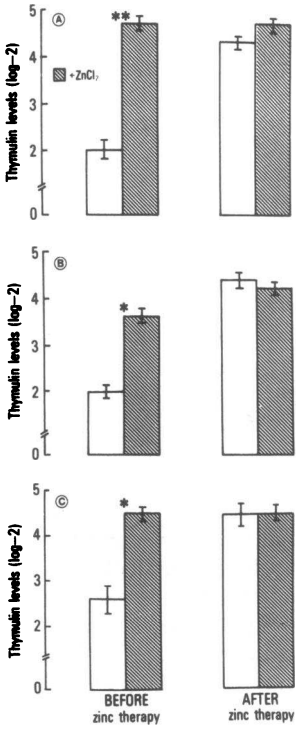

Figure 5. Restoration of normal thymulin activity in sera of zinc-deficient patients after in vitro addition of $ZnCl_2$ is shown. After chelation, 200 μl of serum was incubated for 1 h at 37°C with 10 ng of $ZnCl_2$. Thymulin activity determination was performed before and after in vitro zinc addition on individual samples. *$P < 0.01$; **$P < 0.001$. (*A*) Healthy volunteers submitted to zinc restriction. (*B*) Non-SCA zinc-deficient subjects. (*C*) SCA zinc-deficient subjects.

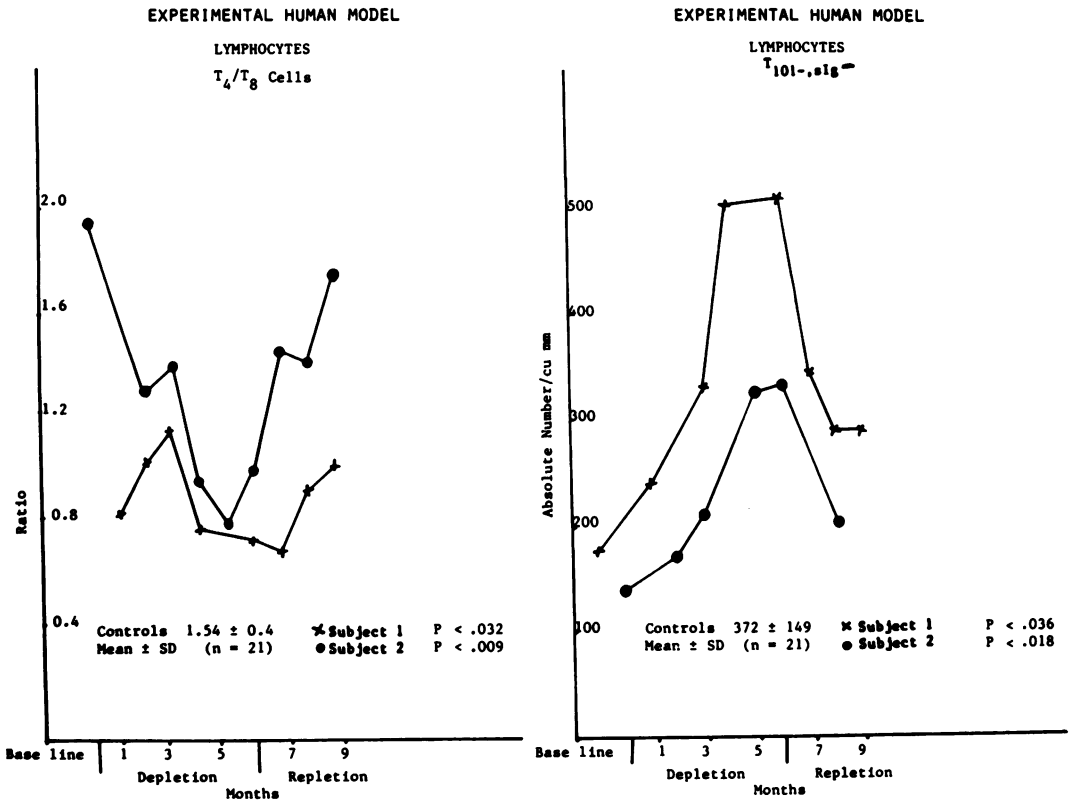

Figure 6. Changes in lymphocyte subpopulations (T4/T8 and T101−, sIg− cells) as a result of zinc restriction and zinc repletion in the experimental human model subjects are shown here. Each data point represents the averages of two separate determinations.

suppressor function, and IL 2 production (20). For the first time, our study has provided evidence that even a mild deficiency of zinc may affect serum thymulin level.

Interestingly, not only oral zinc supplementation in vivo corrected serum thymulin activity in all zinc-deficient subjects in this study, but thymulin activity was also corrected by the addition of zinc in vitro. It is likely that some of the T cell dysfunctions observed as a result of zinc deficiency in humans may indeed be due to decreased thymulin activity. In the experimental human model, we observed changes in lymphocyte subpopulation and decreased IL 2 activity during the zinc-depletion phase that were corrected by zinc supplementation. Our studies thus provide suggestive but not conclusive evidence that the changes in lymphocyte subpopulation and IL 2 activity may be related to thymulin activity.

Changes in lymphocyte subpopulation in zinc-deficient SCA subjects were also observed in this study. An absolute lymphocytosis in SCA subjects as observed here may be related to hyperactive marrow and repeated infections that are common in this disease. We have previously reported similar changes in lymphocyte subpopulation in SCA subjects (21). In this study, we documented an increase in T4+ cells and T4+/T8+ ratio and a significant decrease in T101−, sIg− cells in SCA subjects after zinc supplementation. Our results are consistent with the hypothesis that a majority of T101−, sIg− cells probably represented immature T cells and that an in-

Table II. Lymphocyte Subpopulations in Experimental Human Model

Subjects	Total lymphocytes	T cells (T101+)	B cells (sIg+)	T101− (sIg−)	T helper (T4+)	T suppressor (T8+)	T4/T8 ratios
			No. of cells/mm³ of blood (±1 SD)				
A.							
Baseline	2,956	2,263	455	238	1,138	1,116	1.02
Zn depletion	3,313	2,460	425	428	926	1,279	0.72
Zn repletion	3,079	2,238	527	314	1,465	1,246	1.18
B.							
Baseline	2,063	1,609	263	191	827	604	1.37
Zn depletion	2,089	1,517	230	334	798	752	1.06
Zn repletion	1,831	1,406	240	203	851	616	1.39

The results are averages of two determinations at baseline, two determinations during the fifth and sixth months of the Zn depletion phase, and two determinations during Zn repletion in the eighth and ninth months of the study.

*Table III. Lymphocyte Subpopulations in Adult SCA Patients**

	Total lymphocytes	T cells (T101+)	B cells (sIg+)	T101− (sIg−)	T helper (T4+)	T suppressor (T8+)	T4/T8 ratios
			No. of cells/mm³ of blood (±1 SD)				
SCA							
1. Before	2,754	1,052	772	759	674	919	0.73
After	3,023	1,254	1,149	604	1,254	665	1.88
2. Before	4,207	2,545	883	778	1,703	1,346	1.26
After	5,779	3,900	1,271	577	3,409	1,618	2.10
3. Before	4,083	2,653	816	612	1,449	1,163	1.20
After	4,772	2,958	1,264	524	2,529	1,097	2.30
4. Before	4,230	2,538	782	909	1,226	1,099	1.10
After	6,818	5,011	988	818	3,818	1,804	2.04
Mean±SD							
Before	3,819±713	2,197±756	813±50	765±122	1,263±438	1,132±176	1.07±0.24
After	5,098±1,616	3,281±1,591	1,168±132	631±129	2,753±1,135	1,296±516	2.08±0.17
Paired *t* test							
P	0.088	0.13	0.006	0.01	0.04	0.49	0.0008
Controls (n = 21)	2,779±819	2,122±684	332±160	372±149	1,428±472	980±367	1.54±0.40

* The results are averages of two determinations before and two determinations after zinc supplementation.

crease in thymulin activity after zinc supplementation resulted in an increase in the mature T cells.

Zinc deficiency in human subjects must be viewed to exist in a spectrum. We have, on the one hand, extreme examples of severe zinc deficiency characterized by bullous-pustular dermatitis, diarrhea, alopecia, mental disturbances, neuro-sensory changes, and intercurrent infections due to cell-mediated immune disorders. Such a state of zinc deficiency has been observed in patients with acrodermatitis enteropathica, after total parenteral nutrition without zinc supplement, and after penicillamine therapy (22, 23).

Growth retardation in adolescents, male hypogonadism, skin changes, poor appetite, mental lethargy, delayed wound healing, neurosensory changes, and anergy are some of the manifestations of the moderately zinc-deficient state. The conditions in which this level of zinc deficiency have been noted include nutritional deficiency, as reported from many developing countries, alcoholics with liver disease, malabsorption syndrome, uremics, some subjects with SCA, and other chronically debilitating diseases (22, 23).

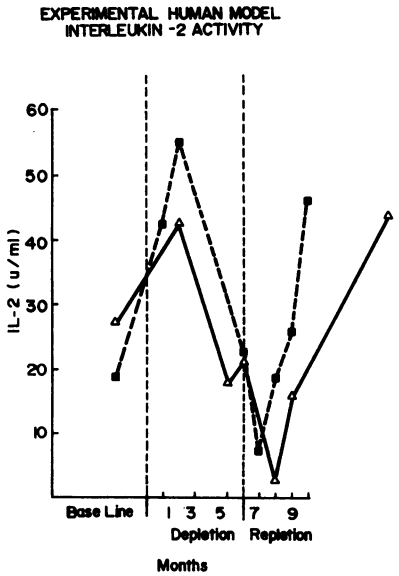

Figure 7. Changes in IL 2 activity as a result of zinc restriction and zinc repletion in the experimental human model subjects are shown here. Each data point represents the averages of two separate determinations. ■, subject 1; *P* = NS. △, subject 2; *P* = NS.

Methods for the assessment of zinc status are currently in transition from somewhat inadequate and nonspecific techniques to what we consider as more specific and accurate indicators of body zinc status. Concentrations of zinc in plasma, urine, and hair have been proposed as potential indicators of body zinc status (24, 25). Currently, plasma zinc appears to be the most widely favored parameter for assessment of human zinc status, and it is decreased in cases of severe and moderate deficiency of zinc. However, it is now well established that several physiologic and pathologic states may affect the zinc levels in the plasma and urine such that reduced plasma or urine zinc levels alone cannot be taken necessarily as an indicator of low body zinc status (24, 25). Zinc in hair and erythrocytes does not reflect acute or recent status of zinc in the body, inasmuch as these tissues are slowly turning over. Furthermore, in the cases of mild or marginal deficiency of zinc in humans, the plasma levels of zinc may remain normal; and, clinically, there may not be any overt evidences of zinc deficiency, thus creating a difficult diagnostic dilemma. In our experimental human model, changes in plasma zinc levels as a result of zinc depletion were observed later than the changes in zinc levels of lymphocytes, granulocytes, and platelets, thus suggesting that plasma zinc is not a sensitive indicator of zinc deficiency in humans.

We have used the assay of zinc in more rapidly turning over cells such as lymphocytes, granulocytes, and platelets as indicators of zinc status in human subjects. We have previously reported encouraging results with respect to zinc levels in granulocytes as an indicator of zinc status (26).

Although the body of an adult 70-kg male contains ~ 2,300 mg zinc, only 10% exchanges with an isotopic dose within 1 wk (27). Approximately 28% of zinc resides in the bone, 62% in the muscles, 1.8% in the liver, and 0.1% in the plasma pool. In an adult animal model, zinc concentrations of muscle and bone do not change as a result of mild or marginal zinc deficiency (28). In cases of mild or marginal deficiency of zinc in humans, one apparently cannot expect a uniform distribution of the deficit over the entire body pool, but that most likely those compartments with high turnover rates (liver and peripheral blood cells such as lymphocytes, granulocytes, and platelets) would suffer disproportionate deficit.

In our experimental human model studies, we created a negative zinc balance of ~ 1 mg/d (29). Although in 6 mo, this amounts to 180 mg of total negative zinc balance, this is still a small fraction of the total body zinc. However, if one were to consider only the 200–400 mg of zinc that is represented by liver zinc and mobile exchangeable pool, a negative balance of 180 mg may be a considerable fraction of the exchangeable pool.

These studies clearly show that at the mild level of zinc restriction used in our experimental diet, a measurable effect on zinc concentration of cells such as lymphocytes, granulocytes, and platelets was observed, thus providing a basis for using cellular zinc levels as indicators of human body zinc status. The levels of cellular zinc in this study are lower in the control subjects compared with previously reported values (18). This is because in previous studies, platelets as contaminants in lymphocytes and granulocytes were not removed adequately, and gave those preparations higher values.

In this study, we identified six subjects who appeared well nourished, active, and without any overt signs of zinc deficiency, but were mildly zinc-deficient according to our criteria as set forth on the basis of cellular zinc levels. One of the six subjects was taking diuretics for mild hypertension. Zinc deficiency because of chronic use of diuretics causing hyperzincuria has been reported previously (29). This was a 58-yr-old male who showed the lowest thymulin activity in the serum. Although serum thymulin activity is known to decline with age, note that after zinc supplementation, the serum thymulin activity increased significantly in this subject in spite of his age. Whether or not a decreased thymulin activity in elderly subjects, which is commonly seen, is correctable with zinc supplementation needs to be investigated in the future. The other five subjects were technical personnel and graduate students who were on self-restricted diets, and two were on vigorous exercise programs that may have increased their zinc requirements. It thus appears that a mild zinc deficiency may not be an uncommon problem in the general population of the United States, but at present this problem remains unrecognized.

It has been known for many years that zinc deficiency in experimental animals results in atrophy of thymic and lymphoid tissue (30–33). These changes are associated with a variety of functional abnormalities. For example, young adult mice maintained on diets deficient in zinc for a 28-d period were found to rapidly develop atrophy of the thymus with preferential involution of the cortex, reductions in the absolute numbers of splenocytes and greatly depressed responses to both T cell-dependent and T cell-independent antigens (33). Some of these abnormalities were shown to be related to defects in T helper cell function (31, 32). Zinc deficiency in mice has also been shown to result in impaired in vivo generation of tumor-specific cytoxic T killer cells, reduced natural killer activity, and impaired development of delayed skin test reactivity after cutaneous sensitization (17, 31–33).

Abnormalities of cellular immunity have also been observed in zinc-deficient humans. An extreme example of the effects of zinc deficiency on the human immune system is acrodermatitis enteropathica, a genetic disorder resulting in zinc malabsorption (31, 33). This condition is associated with frequent severe infections with fungi, viruses, and bacteria. Affected subjects have thymic atrophy, anergy, reduced lymphocyte proliferative response to mitogens, a selective decrease in T4 helper cells, and deficient thymic hormone activity. All of these changes are corrected by zinc supplementation. Less severe cellular immune defects have been observed in patients who became zinc-deficient while receiving total parenteral nutrition. These abnormalities, which include lymphopenia, decreased ratios of T helper and T suppressor cells, and decreased natural killer activity were readily corrected by zinc supplementation (33–35).

Low levels of thymulin activity may be seen in other conditions such as autoimmune diseases and primary or secondary immunodeficiencies (2–4). In these conditions, the low thymulin activity is due to the absence of peptide formation by the thymus. As such, zinc supplementation is ineffective either in vivo or in vitro in correcting this problem. In marginal zinc deficiency, our data show that the decreased thymulin activity was not due to inadequate production of the thymulin peptide. Rather, there was a failure to form zinc-thymulin complexes. Evidence thus far suggests that thymulin incorporates zinc before being secreted by thymic epithelial cells (36). This study shows that in zinc-deficient subjects, the available circulating plasma zinc was unable to form proper zinc-thymulin complexes in the plasma. This may be because zinc in the plasma is bound to several macromolecules and only a very small fraction (1–2%) is ultrafiltrable and thus available for formation of complexes with the inactive circulating peptide. The other possible explanation for this observation may be that for the zinc-thymulin complexes to form in vivo, the thymic environment is essential. In vitro, however, inactive thymulin peptides in the plasma were easily activated by addition of zinc under proper experimental conditions.

Our studies show that besides decreased level of zinc in cells, a decrease in serum thymulin activity correctable with addition of zinc in vitro may also prove to be a sensitive indicator of a mild state of zinc deficiency in humans. We have previously reported decreased serum testosterone level, oligospermia, and decreased natural killer cell activity in subjects in whom a mild state of zinc deficiency was induced experimentally by dietary means (29, 35, 38, 39). Inasmuch as one may expect hormonal and immunological changes to occur in human subjects even if the deficiency of zinc is only mild, it is important to recognize this condition so that corrective measures may be undertaken.

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