In vitro lymphocyte-differentiating effects of thymulin (Zn-FTS) on lymphocyte subpopulations of severely malnourished children[1-3]

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ABSTRACT This work investigates how thymic dysfunction contributes to the depression of cell-mediated immunity in protein-energy malnutrition (PEM). In Bolivian children hospitalized for severe PEM, the size of the thymus was measured by echography, and the lymphocyte subpopulations were detected by using monoclonal antibodies. These data were compared with those obtained from healthy control subjects. Regardless of the clinical form of PEM, our results show a high degree of T lymphocyte immaturity in severely malnourished children, which correlates with a severe involution of the thymus. Before in vitro incubation with thymulin, this significant increase in the percentage of circulating immature T lymphocytes was concomitant with a decrease in mature T lymphocytes and a slight increase in cytotoxic T subpopulations. After in vitro incubation with thymulin, immature T lymphocytes decreased and mature T lymphocytes increased. *Am J Clin Nutr* 1994;60:274–8.

KEY WORDS Protein-energy malnutrition, immune deficiency, thymulin

Introduction

It is well established that severe forms of protein-energy malnutrition (PEM) lead to an immune deficiency that increases susceptibility to infections, the severity of which is strongly related to the high mortality observed in malnourished subjects, particularly in the young (1, 2).

Although each compartment of the immune system may be affected, numerous reports underline that cell-mediated immunity (CMI) is the most seriously affected. In vivo the main disorders are delayed-cutaneous-hypersensitivity test responses, depressed responses to T-specific mitogens, and modifications in circulating T lymphocyte populations (3–6). Given the importance of the thymus in the maturation and functional differentiation of T lymphocytes (7) and its role as the key organ of immunity (8), it is likely that the marked atrophy of the thymus encountered in severe PEM (9–12) causes the CMI defects. The thymus is the site of T cell differentiation and maturation by direct cell to cell contacts at the level of the epithelium network, and/or through secretion of lymphocyte-differentiating hormones by epithelial cells (12–14).

Despite evidence of maturation and differentiation disorders of peripheral T lymphocytes (4–6), rare histological studies prove a direct thymic epithelium dysfunction in PEM (11, 14). Mittal et al (15, 16) showed that lipid destruction of secreting vacuoles in epithelial thymus cells are concomitant with low serum thymulin bioactivity in malnourished mice. Jambon et al (17) showed a necrotic involution of thymulin-secreting epithelial structures and a drastic reduction in thymulin concentration in the remaining epithelial cells in the atrophied thymus of children who had died of malnutrition.

It is possible that the degenerative process might have a similar effect on other lymphocyte-differentiation hormones secreted by the thymus epithelium. Savino and Dardenne (18) showed that thymopoietin and thymosin α-1 are produced in humans by the same epithelial cells as those producing thymulin. Moreover, it has been shown that thymopoietin or crude thymosin fraction 5 (mainly composed of thymosin α-1) can improve percentages of E rosette-forming cells within peripheral mononucleated cells isolated from severely malnourished children (19–21). Chandra (22) observed clearly decreased serum thymic hormone activity in malnourished children, whereas in cases of supervening infections, Maire et al (23) and Wade et al (24) reported a thymulin-like activity with a classical test based on in vitro maturation of splenic lymphocytes in thymectomized mice (25).

T lymphocytes made active by infections produce an allogenic factor causing the same differentiating activity on immature murine T lymphocytes as that of thymulin (26). This fact may explain these discrepancies. It also suggests a possible beneficial effect of such a substance in malnourished children suffering intercurrent infections.

Thus, despite constant echographic observations of severe thymus atrophy in critically malnourished Bolivian children, with or without supervening infection, it appeared important to define the impact of association of the two clinical states on lymphocyte differentiation.

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[2] Supported by the Commission Des Communautés Européennes [CEE contrat TSD-M-384-F (S), ORSTOM and IBBA].

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Received November 2, 1992.

Accepted for publication February 28, 1994.

2 7 MARS 1995

To attain this objective, we studied young children hospitalized for severe PEM and presenting common intercurrent infections and we monitored peripheral B, mature T, and immature T lymphocytes. We also tested modifications in the distribution of these lymphocytes after a short incubation with an active dose of thymulin (27). We chose thymulin, first discovered in human blood (28) and initially named FTS (Facteur Thymique Sérique), for various reasons: its hormonal quality is one of the best documented (29); it promotes most T lymphocyte functions and acts in early as well as in late stages of the T lymphocyte-differentiating process (30); and it improves CMI and immunoglobulin A (IgA) production in children suffering from severe immune deficiencies (31).

We also explored the thymus by using new noninvasive ultrasonographic methods (32, 33). In addition, a cutaneous test of delayed hypersensitivity against tuberculin was made in the malnourished children previously vaccinated with bacille Calmette-Guerin (BCG).

TABLE 1
Clinical and nutritional indexes of the children studied

	Malnourished group (n = 23 M, 19 F)	Well-nourished control group (n = 6 M, 9 F)
Nutritional diagnosis		
Marasmus	13	0
Kwashiorkor	16	0
Marasmic-kwashiorkor	13	0
Fever	17	0
Edema	29	0
Dehydration	42	0
Pulmonary infection	8	0
Age (mo)	16.9 ± 0.8[1]	21.9 ± 2.4
Weight for height (% of NCHS)	73.3 ± 1.6	105.1 ± 3.6
Weight for age (% of NCHS)	63.4 ± 1.8	96.9 ± 4.0
Height for age (% of NCHS)	92.4 ± 0.9	96.2 ± 0.9
Arm circumference for age (%)	68.2 ± 1.3	94.9 ± 2.3
Cranial circumference (cm)	43.8 ± 0.3	45.9 ± 0.5
Triceps skinfold thickness (mm)	4.9 ± 0.9	9.1 ± 0.5

[1] $\bar{x}$ ± SEM.

Subjects and methods

Subjects

With the consent of medical authorities previously informed of the research protocol, we studied two groups of Bolivian children recruited from the Pediatric Departments of the Materno-Infantil Hospital G Urquidi in Cochabamba, the third largest city of Bolivia.

The malnourished group consisted of 42 children of both sexes, 11–28 mo old (17.0 ± 0.8 mo, $\bar{x}$ ± SEM), hospitalized for < 1 wk for severe PEM. On admission, 13 presented with marasmus, 16 with kwashiorkor, and 13 with marasmic-kwashiorkor, according to the Wellcome classification (34). All were affected by common and classical intercurrent infections. Though sometimes severely malnourished, children who were first admitted for diseases, which in themselves could depress immunity (such as hypothyroid goiter and severe anemia), or who were referred to infectious wards on clinical suspicion of tuberculosis, acute Chagas disease (35), or acquired immunodeficiency syndrome (AIDS) (36), were excluded from the study.

The control group, recruited from healthy children in routine consultation in the same hospitals, included 15 well-nourished children of both sexes, 11–35 mo old (21.9 ± 2.4 mo), who had never shown any sign of malnutrition or severe infection. Clinical and nutritional characteristics of both groups are shown in **Table 1.**

Methods

Hemograms were available only for the malnourished group. The hospital's laboratories did not perform these tests in healthy subjects, so hemoglobinemia was determined by the cyanmethemoglobin method (Sigma Chemical Co, St Louis).

A delayed test of cutaneous hypersensitivity against tuberculin was performed on previously BCG-vaccinated children of the malnourished group by using the MONOTEST (Institut Mérieux, Lyon, France) on the inner side of the left arm. The induration diameters were measured 48 h later.

We used a portable echograph (ALOKA SSD, Tokyo) provided with a 5-MHz longitudinal pediatric probe to visualize the thymus gland, according to the technique previously reported for thymus pathology cases (32, 33). To determine an index of the thymus mass we calculated the area of the longitudinal echographic section of the left lobe of the thymus, between the superior edge of the second rib and the inferior edge of fourth rib, placing the probe on the left edge of the sternum. The accuracy of length or width measurements was ± 1 mm.

Lymphocytes were isolated from peripheral blood by Ficoll-Hypaque density centrifugation. Five to 10 mL venous blood was obtained by venipuncture by using Liquemine (Roche, Neuilly-sur-Seine, France) as an anticoagulant and then centrifuged within the following hour at 400 × g for 30 min at 4 °C. The plasma was rapidly discarded and the buffy coat was collected and suspended in 7 mL Hanks-Wallace medium (Gibco, Cergy-Pontoise, France). The reconstituted leukocyte suspension was then softly layered on 5 mL Ficoll-Hypaque (density = 1.077 g/L; Pharmacia, Saint-Quentin-Yvelines, France) and centrifuged for 30 min at 300 × g at 4 °C. The mononucleated cells deposited at the interface were then removed, washed, and centrifuged for 5 min at 400 × g, first in Hanks Wallace medium containing 5% bovine serum albumin (BSA, Gibco), then in RPMI 1640 medium (Gibco) containing 5% BSA. They were finally suspended at a concentration of 10^{10} cells/L in the last medium. To avoid the introduction of exogenous thymulin, we used purified BSA. Cell viability was evaluated by the trypan-blue exclusion test and was > 90%.

Thymulin was provided by the Institut Choay, Paris (37). To one volume of the previous cell suspension an equal volume of thymulin solution (5 μg/L) in RPMI 1640 was added and two volumes of RPMI 1640 containing 5% BSA. The mixture was then incubated for 2 h with gentle agitation every 10 min. A control incubation, with pure RPMI 1640 only, was made under the same experimental conditions. The thymulin concentration, time incubation, and temperature chosen were those that produced optimum in vitro T lymphocyte maturation in immunodeficient children (27).

Immediately after the preceding incubations, cell suspensions of thymulin (+) and thymulin (−) were incubated with five monoclonal antibodies specific for the following human circulating lymphocyte-differentiation stages (Ortho Diagnostics Sys-

TABLE 2
Lymphocyte subpopulations by type of severe protein-energy malnutrition[1]

Lymphocyte subpopulation	Well nourished (n = 15)	Marasmus (n = 13)	Kwashiorkor (n = 16)	Marasmus-kwashiorkor (n = 13)
	%	%	%	%
CD3	61.7 ± 1.3^{a}	49.6 ± 2.4^{b}	52.8 ± 1.2^{b}	50.9 ± 1.6^{b}
CD4	41.7 ± 1.3^{a}	37.1 ± 4.0^{ab}	37.8 ± 1.1^{b}	37.1 ± 2.0^{ab}
CD1a	7.8 ± 0.8^{a}	24.2 ± 2.5^{b}	27.7 ± 1.1^{b}	30.9 ± 2.2^{b}
CD8	27.3 ± 1.3^{a}	29.5 ± 1.1^{a}	29.9 ± 1.7^{ab}	34.4 ± 1.9^{b}
CD4/CD8	1.53 ± 0.08^{a}	1.26 ± 0.13^{ab}	1.26 ± 0.08^{b}	1.08 ± 0.06^{b}
CD21	31.8 ± 0.9^{a}	33.7 ± 1.8^{ab}	32.6 ± 2.1^{ab}	36.0 ± 1.7^{b}

[1] $\bar{x} \pm$ SEM. Values with different superscript letters are significantly different, $P < 0.001$.

tems Inc, Westwood, MA), according to a standard procedure (38): CD21: identification of B lymphocytes; CD3: identification of mature T lymphocytes; CD4: identification of helper-inducer T lymphocytes; CD1a: identification of immature T lymphocytes or cortical thymocytes; and CD8: identification of cytotoxic-suppressor T lymphocytes. Two hundred microliters of cell suspension was incubated with 5 μL monoclonal antibody in an ice-water bath for 30 min with gentle agitation every 10 min. The cell mixture was washed and centrifuged for 10 min at 300 $\times$ g and 4 °C with 2 mL phosphate-buffered saline (PBS, 0.01 mol/L), pH 7.2. The procedure was repeated twice. Each time the supernate was removed with a Pasteur pipette and $\approx$100 μL of medium was left in the tube. Then 100 μL of diluted (1:20) fluorescein-conjugated immunoglobulin-antiserum was added to each tube and the washing procedure was repeated. Finally, marked cells were resuspended and stored in 1 mL of 1% purified formaldehyde solution in PBS at 4 °C. For counts under ultraviolet (UV) fluorescence episcopic microscopy ($\times$400) cells were resuspended in PBS containing 30% (vol:vol) glycerol and 0.2% (wt:vol) sodium azide. Cell count was done with $\geq$ 200 cells, and the results were expressed as a percentage of fluorescent cells. We performed single labeling of lymphocytes because of the limited amount of blood available and certain material constraints.

Statistics

The results are expressed as mean $\pm$ SEM. Comparisons between two means were made by Student's t test. Multiple comparisons of means were made by variance analysis (F).

Results

The clinical characteristics (on hospital admission) and anthropometric indexes (at the beginning of the study) of the two groups of children are shown and compared in Table 1. The anthropometric indexes of the malnourished group were much lower than the National Center for Health Statistics (NCHS) standards chosen as references. Moreover, every malnourished child presented one or several signs of classical intercurrent infections such as diarrhea with dehydration, fever, pulmonary infections, etc. Although the average age of the control group was slightly higher, only 26% of the children in this group were weaned in comparison with 91% in the malnourished group on admission.

Mean hemoglobin concentration in the malnourished children was 11.2 $\pm$ 0.4 g/L and only two cases presented more severe anemia with hemoglobin < 10 g/L. Red blood cell counts were slightly below expected age level with an average of 3.95 $\pm$ 0.13 $\times$ 10^{12} cells/L. Circulating leukocyte counts showed 39.9 $\pm$ 3.0% lymphocytes and 56.8 $\pm$ 2.9% neutrophils, which are slightly different values than expected at this age (39).

None of the 28 malnourished children previously vaccinated with BCG and who were tested with the MONOTEST presented an induration diameter > 4 mm and in 21 of them (75%) no response was detected.

All severely malnourished children (n = 42) regardless of the clinical form of PEM presented a severe involution of the thymus gland (surface area of 48.1 $\pm$ 4.7 mm^2). This involution was on average about 1/10th that of the control group value: 48.1 $\pm$ 4.7 vs 446.3 $\pm$ 19.3 mm^2 ($\bar{x} \pm$ SEM).

The different lymphocyte subpopulation counts were not significantly different among the three severe forms of malnutrition, except for immature T lymphocytes (CD1a), which barely reached the 5% limit of significance with F = 3.29 vs 3.25 (**Table 2**). This finding indicated a relatively homogeneous behavior of the lymphocyte system in PEM. Therefore, the three forms of malnutrition were combined for further comparisons with the control group. The malnourished children presented the following alterations in lymphocyte subsets: *1*) very high percentages of immature T lymphocytes (CD1a): 27.7% vs 7.6%, $P < 0.001$; *2*) decreased percentages of mature T lymphocytes (CD3): 51.3% vs 61.7%, $P < 0.001$; *3*) slightly increased percentages of suppressor-cytotoxic T lymphocytes (CD8): 31.1% vs 27.3%, $P < 0.05$; *4*) diminished ratios of helper-inducer/suppressor-cytotoxic T lymphocytes (CD4/CD8): 1.2 vs 1.6, $P < 0.05$; *5*) altered percentages of helper-inducer T lymphocytes (CD4): 37.8% vs 41.7%; and *6*) B lymphocytes (CD21): 33.9% vs 31.8% not significantly different from those of the control group.

Incubation with thymulin had no effect on B lymphocytes (**Table 3**). It significantly increased the percentage of CD4 in both groups, and of CD3 and CD8 in the malnourished group only. However the most striking result was a 45% decrease in immature lymphocytes (CD1a) in malnourished children. Although there was a similar relative decrease in the control children, the decrease observed in malnourished children is quantitatively much larger, because of the high initial value in this group.

Discussion

This study confirms a functional decrease in CMI in severely malnourished children having common intercurrent infections, revealed by anergy against tuberculin in those previously vacci-

TABLE 3
In vitro effect of thymulin on lymphocyte subpopulations[1]

Lymphocyte subpopulations	Malnourished children (n = 42)		Control group (n = 15)	
	Without thymulin	With thymulin	Without thymulin	With thymulin
	%		%	
CD3	51.3 ± 1.0	55.6 ± 1.1[2]	61.7 ± 1.3	65.1 ± 1.2
CD4	37.8 ± 1.0	43.4 ± 1.4[2]	41.7 ± 1.3	46.4 ± 1.2[3]
CD1a	27.7 ± 1.0	15.2 ± 0.8[4]	7.6 ± 0.8	3.0 ± 0.3[4]
CD8	31.1 ± 1.0	34.9 ± 0.9[2]	27.3 ± 1.3	29.1 ± 1.1
CD4/CD8	1.22 ± 0.05	1.24 ± 0.06	1.53 ± 0.08	1.59 ± 0.07
CD21	33.9 ± 1.1	34.6 ± 1.4	31.8 ± 0.9	32.2 ± 0.8

[1] $\bar{x}$ ± SEM.
[2–4] Significantly different from without thymulin (within group): [2] $P < 0.01$, [3] $P < 0.05$, [4] $P < 0.001$.

nated with BCG. It also confirms, in vivo, thymus atrophy in severely malnourished children. It clearly shows concomitant immaturity of T lymphocytes in these young patients, findings that are similar to those obtained with other techniques (terminal deoxynucleotidyl transferase activity) in noninfected severely malnourished children (5).

The elevated percentages of immature T lymphocytes (CD1a) in the peripheral blood of severely malnourished children having intercurrent infections tend to confirm the responsibility of thymus dysfunction in T lymphocyte immaturity. It may be postulated that thymus atrophy leads to a lack of epithelial functions responsible for the chemotaxis and differentiation of cortical thymocytes (CD1a). This would lead to the finding of greater numbers of these cells in the peripheral blood.

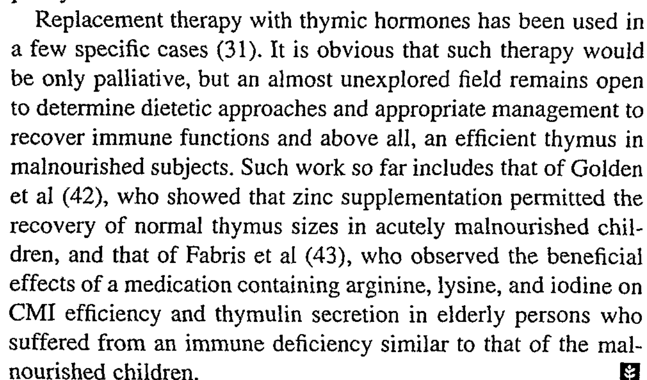
Nevertheless, it must be pointed out that this immaturity was functionally reflected by a decrease in the percentage of mature effector T lymphocytes (CD3), and concomitant with a slight increase in the percentage of suppressor-cytotoxic T lymphocytes (CD8) that decreases the CD4/CD8 lymphocyte ratios. This may be a consequence of the intercurrent infections that would deviate the lymphocyte-differentiating alterations towards immunosuppression and aggravate thymus atrophy, as suggested by Dourov (40). According to Linder (41) the expansion of suppressor-cytotoxic T lymphocytes (CD8) that occurs in many secondary immunodeficiency syndromes may damage the thymus epithelium.

This study investigates the maturation and differentiation effects of thymulin on the main lymphocyte subpopulations in severely malnourished children with common intercurrent infections. The main finding is the spectacular effect of thymulin on immature T lymphocytes (CD1a), which decrease to almost half their previous amount within 2 h. This effect results in the appearance of new mature effector T lymphocytes (CD3) and in a parallel increase in T helper-inducer (CD4) and T suppressor-cytotoxic (CD8) antigen carrying cells.

Note that in both patients and control subjects, thymulin maturating activity concerns only the T lymphocyte lineage, although only modestly in the control group. This suggests that in severely malnourished children with common intercurrent infections thymulin acts in the same way as in healthy children.

Finally, our observations concur with previous reports by Jackson and Zaman (19) and Olusi et al (20) and more recently by Keusch et al (6), who showed the beneficial effects of thymopoietin and thymosin fraction 5 on in vitro differentiation of E rosette-forming cells from severely malnourished children.

These lymphocyte-differentiating hormones are produced by the same thymus epithelial cells as those producing thymulin (14, 18). Previous observations have shown severe necrosis of thymulin-producing epithelial cells in children who had died of severe malnutrition (17). So, our results suggest that recovery of the epithelial secretions of the thymus could correct the T lymphocyte deficit of maturation observed in PEM.



Replacement therapy with thymic hormones has been used in a few specific cases (31). It is obvious that such therapy would be only palliative, but an almost unexplored field remains open to determine dietetic approaches and appropriate management to recover immune functions and above all, an efficient thymus in malnourished subjects. Such work so far includes that of Golden et al (42), who showed that zinc supplementation permitted the recovery of normal thymus sizes in acutely malnourished children, and that of Fabris et al (43), who observed the beneficial effects of a medication containing arginine, lysine, and iodine on CMI efficiency and thymulin secretion in elderly persons who suffered from an immune deficiency similar to that of the malnourished children.

We thank M Dardenne for critically reading the manuscript.

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