Thymulin (Zn-FTS) activity in protein-energy malnutrition: new evidence for interaction between malnutrition and infection on thymic function[1-3]

Salimata Wade, PhD; Gérard Parent, MD; Fanny Bleiberg-Daniel, PhD; Bernard Maire, PhD; Mouhamadou Fall, MD; Dominique Schneider, BSc; Béatrice Le Moullac, BSc; and Mireille Dardenne, MD

ABSTRACT The combined effects of protein-energy malnutrition (PEM) and infection on thymic function evaluated by specific plasma thymulin activity were studied in Senegalese children: 29 hospitalized in Dakar for severe malnutrition and various diseases; 9 infected without sign of severe PEM, living in Dakar; 13 apparently healthy, uninfected, living in Dakar; and 7 apparently healthy, uninfected, living in Paris. Most of the free-living children in Dakar suffered from mild to moderate PEM. The specific thymulin activity (total plasma activity minus the activity recorded after adsorption of the plasma with a monoclonal antithymulin antibody) was almost undetectable in the infected children and was normal only in the children living in Paris. Such activity might be decreased by moderate and severe PEM and severe malnutrition may not be the only underlying cause of depressed level of thymulin in malnourished children from the Third World. Concurrent infections are important factors. *Am J Clin Nutr* 1988;47: 305–11.

KEY WORDS Protein-energy malnutrition, thymulin (Zn-FTS) activity, lymphocyte subpopulations, infections

Introduction

Malnutrition and infectious diseases are the two major health problems of developing countries. Since the report of Scrimshaw et al in 1968 (1), malnutrition is widely recognized to be in synergy with infection to create a vicious cycle in children. The high frequency of infections in children with protein-energy malnutrition (PEM) has long suggested an impairment of their cell-mediated immune responses (2–6). Severe thymic atrophy is a striking and consistent finding in undernourished children; involution of the thymus gland in such children has led to the term *nutritional thymectomy*. However much remains to be learned about the underlying mechanisms. Many studies indicated that T-lymphocyte division and proliferation are severely restricted (2–6) but very little information is available regarding the epithelial structure of the thymus. Mugerwa (7) observed well-preserved thymic epithelial cells in children with kwashiorkor. In contrast, Jambon et al (8) in a postmortem study examined the thymuses of children with various degrees of malnutrition and reported marked modification in the severely malnourished children, who were nearly completely depleted of thymic reticuloepithelial cells. Evidence of thymic epithelium abnormalities in malnourished mice also was reported by Mittal et al (9, 10).

In vitro studies with peripheral T-lymphocytes from children with severe malnutrition indicated that thymic hormones such as thymopoïetin as well as thymosin fraction V increased the percentage of T-cell rosettes (11, 12). Concentration of thymic hormones may be altered but the few reports in the literature were conflicting. Thymulin levels also were studied in malnutrition. Thymulin (or Zn-FTS) is a basic nonapeptide with a molecular weight of ~1000, 10 times lower than that of thymosin but 10–1000 times more active. It is secreted exclusively by the

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thymic epithelium; its activity, which is age dependent, was found in the serum of various species (13). The strict thymic dependency of thymulin was confirmed by many studies (14–17). Thymulin is known to promote most T-cell functions (18).

In the absence of an available direct biochemical assay, the determination of blood thymulin activity is achieved by a bioassay (rosette assay) developed by Dardenne and Bach (19). Using this rosette inhibition assay, Chandra found reduced levels of thymulin in children with severe malnutrition (20) and in small-for-gestational-age infants (21). The decreased activity was restored to normal after 6–8 wk of nutritional therapy (22). In contrast, we found no significant effect in moderately or severely malnourished and infected children (23). As it is well established that during antigen-induced T-cell activation, an *allogeneic* factor (AF) is produced with a thymulin-like activity on the rosette assay (24, 25), we proposed differences in the rate of concomitant infections to explain the discrepancy between our results and those of Chandra.

This study was designed to further investigate the effect of malnutrition-infection interaction on thymulin activity and to examine the production of AF in groups of Senegalese children with various degrees of malnutrition and infection.

Subjects and methods

Subjects

A total of fifty-eight Senegalese children aged 4–30 mo (mean age 18 mo) were investigated. The children were selected according to a careful clinical examination and were grouped as follows:

Twenty-nine children were hospitalized in Dakar (Senegal, West Africa) for various diseases including malnutrition; most of these subjects were suffering mainly from respiratory diseases, gastrointestinal infections, and measles (group M).

Twenty-two age-matched children coming for vaccination in a maternal child care center of Dakar were divided into two groups. The first (group I) comprised nine children without clinical signs of PEM but who were diagnosed as suffering from various common infectious diseases (diseases of the lungs, measles, diarrhea, otitis, and colds). The second (group CD) involved 13 apparently healthy children free from any evidence of recognizable clinical diseases.

Seven healthy aged-matched Senegalese children were screened in Paris (France) and served as control subjects (group CP).

The mothers and the medical staff were informed about the object of the study and their full consent was obtained. The study was conducted in accordance with the Senegalese ethical committee for human experimentation.

Ages and weights were recorded and compared with the expected weight for age, weight for height, and height for age from the NCHS standards (26).

General measurements

Blood was withdrawn by venipuncture and collected in cooled (4 °C) heparinized tubes before any nutritional and anti-infectious treatment was started. Plasma was immediately separated and kept frozen at −70 °C until analyzed. An aliquot of

blood was used to determine the hemoglobin levels by the cyanmet-hemoglobin method (Sigma Chemical Co, St Louis, MO) and hematocrit, red blood cell, and white blood cell counts by standard techniques.

To standardize all the measurements and to account for unknown environmental factors, blood was also drawn from a healthy white adult subject living in Dakar more than 1 y. This subject was used as the internal reference. Plasma albumin, prealbumin, transferrin, orosomucoid, and c-reactive protein levels were measured according to the radial immunodiffusion method of Mancini et al (27).

Immune studies

Lymphocytes were isolated from peripheral blood by Ficoll-Hypaque gradient-density centrifugation. Monoclonal anti-T cell antibodies (Ortho Diagnostics Systems Inc, Westwood, MA) were used to evaluate the proportion of various T-lymphocyte subsets by indirect immunofluorescence: the percentage of mature T cells, helper-inducer and cytotoxic-suppressor cells were determined using OKT3, OKT4, and OKT8, respectively (28). sIg-bearing lymphocytes (B-lymphocytes) were quantified by direct immunofluorescence with a fluorescein conjugated Ig-antiserum.

Thymulin activity of the plasma samples was measured by the rosette assay described by Dardenne and Bach (19) and reported in detail elsewhere (23). This test analyzes the conversion of relatively azathioprine (AZ)-resistant and antithy1-serum-resistant rosette-forming cells (RFC) from adult thymectomized mice into thy1 positive RFC that are more sensitive to inhibition by AZ. Briefly spleen rosette-forming cells from adult thymectomized mice have a low sensitivity to inhibition by AZ compared with normal spleen RCF. Thymic peptides or thymulin content of serum correct this abnormality after 60 min in vitro incubation with spleen cells. Normally several dilutions of samples are used. The highest dilution of a serum sample that induces the rosette inhibition by AZ is considered to be the active dilution.

To differenciate thymulin activity from that of AF, each plasma sample was analyzed directly and after specific immuno-adsorption with a monoclonal antithymulin antibody obtained in mice (29). Briefly sera under study were incubated for 30 min at 37 °C with the monoclonal antibody. The residual activity was evaluated in the rosette assay after filtration of the mixture through ultrafiltration membrane (YMT, Micropartition System MPS-1, Amicon, Danvers, MA). All the determinations were carried out in duplicate and the results were expressed as log-2 reciprocal titer of the highest active dilution.

Statistical analysis

Differences in mean values between the groups were determined by analysis of variance. Specific tests such as the Duncan and the Tukey tests were also used (30).

Results

Anthropometric and hematologic measurements of the children are shown in **Table 1**. The average age was similar in the four groups. According to the classification proposed by Waterlow (31), nearly all the children in group M were wasted: most of them exhibited weight-for-height values < 80% of the NCHS standards except three who were edemic and 1 who suffered from measles. Eighteen children from this group were wasted and stunted (height for age < 95% of the reference). For the indices weight/age

TABLE 1
Description of the subjects (Senegalese children) grouped according to their clinical examination

Groups*	Age	Sex ratio	Weight† for age	Weight† for height	Height† for age	Red blood cells	Hemato-crit	Hemo-globin	Leucocyte counts
	mo	*M:F*	%	%	%	$10^{12}/L$		*g/L*	$10^9/L$
M (n = 29)	17.3 ± 1.3‡	17:12	58 ± 2.2§	67 ± 1.9§	92 ± 1.1§	3.73 ± 0.18‖	28 ± 1.2‖	85 ± 3.8§	14.7 ± 1.7§
I (n = 9)	16.5 ± 1.6	6:3	90 ± 3.2§	93 ± 2.2§	97 ± 0.9	3.96 ± 0.18	32 ± 1.1	99 ± 5.6	14.1 ± 1.7§
CD (n = 13)	15.3 ± 1.5	6:7	96 ± 3.2‖	99 ± 2.5	99 ± 1.0	4.23 ± 0.16	31 ± 1.4	90 ± 5.1‖	8.9 ± 0.6
CP (n = 7)	18.1 ± 2.7	5:2	110 ± 1.9	105 ± 1.6	104 ± 0.9	4.28 ± 0.11	33 ± 1.0	113 ± 3.4	9.9 ± 1.7

* Group M = children hospitalized for severe malnutrition; group I = children diagnosed as suffering from common infectious diseases without signs of either kwashiorkor or marasmus; group CD = apparently healthy children living in Dakar; group CP = apparently healthy children living in Paris.
† % of the 50th percentile of the NCHS values.
‡ Mean ± SEM.
§ $p < 0.01$ vs the group CP.
‖ $p < 0.05$ vs the group CP.

and weight/height, no significant differences were found between groups I and CD; for these measurements, the Tukey's test separated the four groups into three (M-I, CD, and CP) and two (M and I-CD-CP) distinct categories, respectively. Three children in group I and two in group CD were mildly wasted (weight for height 80–90% of the reference) and no child in either group was stunted.

Anthropometric measurements of group CP were within the normal range. Physical development was better achieved in this group than in group CD.

Most of the children, excluding group CP were anemic; however, anemia was more pronounced in children from groups M and CD than in those from group I. Mean values for red blood cell counts were significantly less in group M than in groups I, CD, or CP while the white blood cell counts were significantly higher only in groups M and I.

The various degrees of malnutrition and infection are reflected in the values obtained for plasma proteins. Table 2 summarizes the measures for the four groups. Depressed levels of albumin, prealbumin, and transferrin and increased levels of orosomucoid and C-reactive protein (CRP) were found in group M. There was no difference

in the mean plasma albumin level between groups CD and CP. However, the prealbumin level was significantly lower in group CD than in group CP. Albumin and prealbumin concentrations were significantly less in group I compared with groups CD or CP. The plasma transferrin level was decreased only in group M; it was increased in group CD.

As observed in group M, increased values of orosomucoid and CRP were also apparent in group I. In the clinically uninfected children (CD and CP) CRP was undetectable. The mean orosomucoid concentration was significantly lower in group CP than in group CD ($p < 0.05$).

Plasma protein concentrations of the subject used as the internal reference are reported in Table 3. All the measurements were within the normal range for age.

Table 4 shows the distribution of lymphocytes from the peripheral blood into B cells, mature T cells, helper-inducer cells, cytotoxic-suppressor cells and the ratio of helper-inducer to cytotoxic-suppressor cells. As it was necessary that the immunofluorescence subpopulation counts be done by the same technician, these counts were

TABLE 2
Plasma protein levels

Groups*	Albumin	Prealbumin	Orosomucoid	C-reactive protein†	Transferrin
	g/L	*mg/L*	*g/L*	*mg/L*	*g/L*
M (n = 29)	19.2 ± 1.3‡	63 ± 5.6‡	2.91 ± 0.13‡	6–155 Undetectable in four subjects	1.7 ± 0.17‡
I (n = 9)	31.6 ± 2.0‡	106 ± 9.1	1.45 ± 0.18‡	13–139 Undetectable in four subjects	3.5 ± 0.23
CD (n = 13)	35.0 ± 1.1	149 ± 7.1‡	0.87 ± 0.06§	Undetectable	4.5 ± 0.17‡
CP (n = 7)	37.9 ± 1.1	194 ± 9.9	0.57 ± 0.04	Undetectable	3.2 ± 0.27

* Group M = children hospitalized for severe malnutrition; group I = children diagnosed as suffering from common infectious diseases without signs of either kwashiorkor or marasmus; group CD = apparently healthy children living in Dakar; group CP = apparently healthy children living in Paris. Mean ± SEM.
† Minimum and maximum values are reported. The minimum value detectable in the assay was 4 mg/L.
‡ $p < 0.01$ vs the group CP.
§ $p < 0.05$ vs the group CP.

TABLE 3
Biochemical and immunological measurements of the subject used as the internal reference

Red blood cells	Hematocrit	Hemoglobin	Leucocyte counts	Albumin	Prealbumin	Transferrin	Orosomucoid
$10^{12}/L$		g/L	$10^9/L$	g/L	mg/L	g/L	g/L
4.2	0.43	150	4.3	46.3	324	2.4	0.51

C-reactive protein	Total rosette activity	Allogeneic factor activity	sIg-bearing B-cells	Mature T cells*	Helper-inducer cells (a)	Cytotoxic-suppressor cells (b)	Ratio a:b
mg/L	Log-2	Log-2	%	%	%	%	
0	5	3	8	88	42	26	1.6

* The values (%) for normal control subjects (n = 27) in our laboratory for mature T cells, helper cells, and cytotoxic-suppressor cells are 68.4 ± 1.7, 41.2 ± 1.5, and 26.8 ± 1.21, respectively.

carried out only in children living in Dakar (groups M, I, and CD). Wide variations were observed within the groups, so that individual and mean values were shown. There were no significant differences in the mean values among the three groups for the percentages of B and T lymphocytes or their subsets as well as for the ratio of helper to cytotoxic cells. Tukey's test was unable to separate the groups into several categories. However, within each group anomalies of helper-to-cytotoxic ratio were observed: this ratio was either markedly depressed (range 0.1–0.8) or increased (> 2). The B- and T-cell counts of the subject used as the internal reference were within the normal range (Table 3).

Results of the rosette assay are reported in **Figures 1 and 2** and are expressed as log-2 reciprocal titers. No significant differences were observed in the total plasma thymulin-like activity among groups M, I, and CD and among groups I, CD, and CP; this activity was significantly decreased in group M only when compared with group CP ($p < 0.03$). A significant residual thymulin-like activity (AF), measured after adsorption of the plasma samples with monoclonal antithymulin antibodies, was noticed in groups M, I, and CD compared with group CP; it was also observed in the subject used as the internal reference (Table 3). In contrast, the specific thymulin activity (Fig 2) was markedly reduced in groups M and I and most of these children exhibited undetectable activities. Thymulin was present in the plasma of children from group CD but

the level was low compared with that of children from group CP.

Discussion

We previously published findings of an absence of variation in thymulin activity (Zn-FTS) in moderately and severely malnourished and infected Senegalese children (23). In this previous study AF was not measured. The present investigation is an attempt to further understand the effects of malnutrition-infection interaction on specific plasma thymulin activity and AF production.

One of the intrinsic difficulties in evaluating the immune response of undernourished children from developing countries is to separate the effects of malnutrition from that of synergistic infections. The complexity of the interaction between dietary inadequacy, diseases, and environmental context makes it difficult to establish whether the impairment observed is a specific result of a nutritional problem or whether it is secondary to the presence of infection and/or environmental stimuli.

This investigation comprises Senegalese children living in their local environment in Dakar and age-matched Senegalese children living in Paris. Four groups of children were defined, first by clinical examination and further by anthropometric and biochemical measurements. The degree of weight-for-age loss, height and weight-for-height retardations, plus the determination of plasma transferrin,

TABLE 4
Distribution of peripheral lymphocytes into several subsets (mean ± SEM)

Groups*	sIg-bearing B cells	Mature T cells	Helper-inducer cells (a)	Cytotoxic-suppressor cells (b)	Ratio a:b
M	15 ± 1.9	52 ± 3.9	24 ± 4.2	23 ± 2.0	1.2 ± 0.29
Range	3–41	14–81	3–58	5–46	0.1–5.3
I	15 ± 2.9	61 ± 7.6	38 ± 7.8	23 ± 3.1	2.0 ± 0.55
Range	6–31	30–87	4–66	11–35	0.1–4.9
CD	21 ± 4.2	63 ± 3.9	38 ± 3.5	28 ± 4.1	1.6 ± 0.23
Range	4–44	50–83	14–49	15–58	0.2–2.6

* Group M = children hospitalized for severe malnutrition; group I = children diagnosed as suffering from common infectious diseases without signs of either kwashiorkor of marasmus; group CD = apparently healthy children living in Dakar.

albumin, and prealbumin levels were used to diagnose PEM. Presence or absence of infection and/or inflammatory states was confirmed by information from the white-blood-cell counts and the measurement of two acute-phase reactant proteins (orosomucoid and CRP). The acute-phase proteins are plasma proteins submitted to a characteristic pattern of variation that occurs in response to different forms of infection, inflammation, or tissue damage (32); they are consistently elevated during these states. A recent study showed that increased levels of orosomucoid and CRP correlate with the presence of infection or inflammatory reaction with a 99% certainty (33). Groups CD and CP did not show elevation in either CRP or orosomucoid. Although the mean orosomucoid concentration was significantly higher in the apparently healthy children living in Dakar than in those living in Paris, the values exhibited were within the normal range (34, 35).

Anemia was common in all children except in the children living in Paris (group CP). In accordance with previous reports (36, 37), the plasma transferrin level was markedly reduced in group M and was high only in group CD.

Among the visceral proteins used as nutritional markers, prealbumin was proposed as the most sensitive for early detection of malnutrition (37–39). Yet, in the presence of superimposed inflammatory (40) or infectious (33) states, a preferential synthesis of acute-phase reactant proteins occurs in the liver at the expense of synthesis of other proteins, in particular prealbumin. Thus, in stressful conditions, lowered plasma prealbumin results from an

FIG 2. Specific plasma thymulin activity (total activity minus allogeneic-factor activity). Individual values were reported. Group M = children hospitalized for severe malnutrition; group I = children diagnosed as suffering from common infectious diseases without signs of either kwashiorkor or marasmus; group CD = apparently healthy children living in Dakar; group CP = apparently healthy children living in Paris.

elevation of acute-phase reactant protein levels. This often makes difficult the evaluation of the specific effect of nutritional deficiency. However, in this study group CD, which could otherwise be regarded as a control group, showed reduced level of prealbumin without elevation in levels of either CRP or orosomucoid. Thus, the decreased plasma prealbumin in group CD was not a reflection of an acute-phase response. Information about the individual values of prealbumin, orosomucoid and CRP suggested that most but not all children in group CD suffered from mild-to-moderate PEM.

Support for this view comes from our recent experimental studies: in an animal model of pure moderate PEM, the plasma concentration of prealbumin decreases but the acute-phase proteins are not elevated unless the animals are also infected (Wade et al, unpublished observations). Thus, we suggest, in agreement with a previous report (33), that new information can be gained by simultaneous measurements of prealbumin, CRP, and orosomucoid in the evaluation of nutritional status. Children in group I were marginally malnourished and also infected. Their concurrent infections explained the rather low level of prealbumin observed. Anthropometric and biochemical data showed that children in group M were severely malnourished and chronically infected. The children in group CP were healthy, well-nourished, and uninfected.

In accordance with our previous study (23), moderate or severe malnutrition was not associated with changes in total plasma thymulin-like activity. Decreased thymulin level was reported in malnourished children (20, 22) and in anorexia nervosa patients without superimposed infections (41). In these studies thymulin activity was determined by measuring the total activity expressed in the rosette assay. However, activated T cells may secrete an

FIG 1. Total plasma activity on the rosette assay (allogeneic-factor and thymulin activities). Individual values were reported. Group M = children hospitalized for severe malnutrition; group I = children diagnosed as suffering from common infectious diseases without signs of either kwashiorkor or marasmus; group CD = apparently healthy children living in Dakar; group CP = apparently healthy children living in Paris.

AF, which is a peptide of low molecular weight. AF is distinct from thymulin antigenically and by electric charge. Also, it is not inhibited by the specific serum thymulin inhibitors (24). However, in the rosette assay AF and thymulin are indistinguishable. Therefore, presence of AF in the serum of children may be interpreted as an increase of thymulin. We overcame this error by introducing adsorption with monoclonal antithymulin antibody. Hence we could measure specifically the AF level.

This study demonstrated the significant contribution of AF to the total activity measured: specific thymulin activity (total activity minus AF activity) was nearly undetectable in the severely malnourished and infected children as well as in the moderately malnourished and infected children although their total activity was similar to those of the control children (group CP). However, other environmental stimuli may also induce AF production since AF was detectable in few children from group CP and in all children from group CD; these children, although uninfected produced a significant amount of AF, quite similar to that recorded in the subject used as the internal reference. This subject was a healthy adult man from a high socioeconomic class living in Dakar.

Nevertheless, specific thymulin activity was lower in the apparently healthy children living in Dakar than in the healthy children living in Paris. This might be the result of the effect of moderate malnutrition but this needs to be confirmed by screening well-nourished, uninfected children living in the same environment as age-matched uninfected, moderately malnourished children. In addition, although the children in group I were markedly less undernourished than those in group M, their thymulin activity was similarly depressed. The decrease in specific thymulin activity in groups I and CD compared with the group CP did not result from zinc deficiency since the plasma zinc concentrations of the children were normal except for those belonging to group M (not shown).

The percentage of B lymphocytes, T lymphocytes, and the subsets identified by surface markers bore little relation to the plasma specific thymulin activity. It seems likely that many variables may influence the number and distribution of T cells. We reported such discrepancy in anorexia nervosa patients (41) and lack of correlation between T-cell function and phenotype was shown by others (42, 43). These results suggested that, although thymic hormones may promote most T-cell functions in malnourished subjects (11, 12) or animals (44, 45), decreased plasma activity is not necessarily associated with phenotypic modifications.

We previously demonstrated that thymulin activity was significantly reduced in a model of malnutrition without infection: anorexia nervosa (41). This paper shows that the level of specific thymulin activity is depressed in protein-energy-deficient children. Further studies are warranted to develop a direct assay of thymulin level in the serum. Nevertheless our study demonstrated that in protein-energy malnutrition this level is highly influenced by concurrent infections and/or stimulation by local en-

vironmental factors. It also suggested that severe malnutrition is not the only underlying mechanism of depressed level of thymulin in malnourished and infected children.   ☙

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