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Immature Circulating Lymphocytes In Severely Malnourished Guatemalan Children

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Summary: The percentage of peripheral blood lymphocytes forming rosettes with sheep erythrocytes (E-rosettes) was determined in 33 severely malnourished Guatemalan children, and in two groups of clinically well but mildly growth retarded children from the same environment. Mean E-rosettes in the acutely ill patients was lower than the value observed in the mildly malnourished children, although there was considerable overlap between groups. These data differ from previously published studies of severely malnourished children from other parts of the world in that not all patients had decreased values for E-rosettes, in contrast to the uniform depression reported by others. As all patients were clinically similar, the results suggest that there may be specific nutrient defects associated with protein–energy malnutrition that particularly affect immune function. In addition, in vitro incubation of lymphocytes from the acutely malnourished children with the thymic factor, thymosin fraction 5, increased the percentage of E-rosettes in a dose-dependent fashion. These data suggest that immature, thymosin-responsive T cells are present in circulation. It is possible that in vivo thymosin administration may be beneficial for malnourished individuals.
Key Words: Lymphocytes—Malnourishment—Guatemala—Thymosin administration.

Severe protein–energy malnutrition (PEM) is associated with immune system defects (1–3). These acquired immunodeficiencies may impair host responses to infectious diseases in undernourished individuals, resulting in longer lasting and/or more severe illnesses. Functional lesions of the immune system are thus central to the synergistic cyclical interaction of nutrition and infection leading to overt PEM. Defects in cell-mediated immunity are most commonly and consistently described and are manifested by abnormalities of in vivo delayed-type hypersensitivity skin test responses, changes in populations of circulating T lymphocytes, and depressed responses to mitogenic lectins (4).

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Because of the marked atrophy of the thymus gland, the cellular depletion of T-dependent regions of spleen and lymph nodes in severe PEM, and the importance of the thymus in development and maturation of T cells, it is possible that thymic abnormalities underlie the observed defects in T-cell-mediated immunity (1–3). The thymus influences maturation of T cells by direct cell-to-cell contact and/or production of soluble factors (hormones) (5–7). Addition of thymic hormones to human T cells in vitro can induce the expression of surface markers such as the sheep erythrocyte adherence sites (receptor) that mediate the E-rosette phenomenon (8–10). Whereas little response is observed in subjects who have normal numbers of T cells, in some diseases in which the number and percentage of circulating T cells are reduced, a short incubation in vitro with thymic factors increases the number of cells that form E-rosettes and improves

in vitro T-cell function (8,11–13). These observations suggest that the deficiency of mature T lymphocytes in such patients may be, in a pathophysiological sense, problems of maturational arrest or retardation.

In the present study, peripheral blood lymphocytes from 33 severely malnourished Guatemalan children were exposed in vitro to the partially purified bovine thymic factor, thymosin fraction 5. The results confirm previous reports from Africa (14) and Asia (15) that thymic factors increase E-rosettes during the acute phase of severe PEM. In addition, our data demonstrate that only some but not all clinically similar PEM patients have a reduction in the percentage of circulating T cells, that the response to thymosin in vitro is inversely related to the percentage of E-rosettes in the absence of thymosin addition, and that the effect is directly related to the dose of thymosin.

MATERIALS AND METHODS

Patients

Thirty-three Guatemalan children with acute PEM were studied within 4 days of admission to the hospital. Thirty-two were between 3 and 19 months of age (mean, 9 months), and one was 30 months old. All had diarrhea on admission or a history of antecedent diarrhea. Weight-for-age was less than 70% of the Institute of Nutrition of Central America and Panama (INCAP) growth standards for Central America, indicating severe PEM, while 31 children were edematous, consistent with severe protein deprivation (kwashiorkor). Due to the variable degree of edema present, however, accurate assessment and comparison of subjects were not possible. Nevertheless, all patients were clinically similar and severely ill.

With parental permission, a 10-ml sample of peripheral blood was obtained in preservative-free heparin (20 U/ml), placed on ice, and transported within 30 min to the laboratory at INCAP for the investigation described below. In addition, during the course of other INCAP studies, 5 ml of blood was obtained from 13 children (10–18 months of age) from the coastal lowlands, and from 28 children (9–18 months old) living in the highlands. These children were all clinically well at the time, but exhibited mild retardation in growth (deficit in height-for-age of less than 15%). These samples were placed on ice and transported to INCAP within 2 h.

Isolation of Lymphocytes

Lymphocytes were obtained by conventional Ficoll–Hypaque gradient centrifugation (Ficoll-Paque, Pharmacia, Piscataway, NJ, U.S.A.). The interface cells were harvested, washed three times in Hanks balanced salt solution (HBSS), and resuspended in 5 ml of HBSS. To this, 25 μl of a 1% suspension of washed latex particles (0.8-μ diameter; Difco, Baltimore, MD, U.S.A.) in normal saline, and 1 ml of autologous fresh plasma, were added. The samples were mixed and incubated at 37°C for 30 min. Cells containing phagocytized latex (macrophages) were excluded from lymphocyte counts. An aliquot of the cell suspension was mixed with an equal volume of 0.4% trypan blue, the number of viable lymphocytes was counted, and the suspension was adjusted to 5×10^6 viable lymphocytes per milliliter in HBSS.

Preparation of E-Rosettes

Lymphocytes (5×10^5 cells in 0.1 ml) were added to 0.1 ml of a 1% suspension of sheep erythrocytes (S-RBCs) in saline and 25 μl of heat-inactivated fetal calf serum previously adsorbed with S-RBCs. Samples were mixed, incubated in a water bath at 37°C for 5 min, centrifuged at 500 rev/min for 5 min, and then placed in the refrigerator overnight. The following morning the pellet was gently resuspended by tapping the tube. A drop of the sample was placed in each slide of a hemocytometer chamber with one drop of 0.4% trypan blue. Rosettes were enumerated in duplicate in 200 viable cells. The number of nonrosetting lymphocytes and both large or E-rosettes (three or more attached S-RBCs) and small rosettes (one to two attached S-RBCs) were recorded. For the last two counts, attachment of the S-RBCs was confirmed by observing any motion of the erythrocytes when the slide was gently tapped. The mean value of the duplicate counts was recorded. Only lymphocytes with three or more attached SRBCs were considered to be true E-rosetting cells. These results are referred to as the control, or no thymosin dose level, in the analysis.

Incubation with Thymosin Fraction 5

Thymosin fraction 5 (f-5), lot number C98035 (a gift of Hoffman LaRoche, Nutley, NJ, U.S.A.), was diluted in HBSS to concentrations of 40 or 400 μg/ml. Equal volumes of lymphocyte suspension

and f-5 were mixed and incubated at 37°C for 1 h, providing final f-5 concentrations of 20 and 200 μg/ml (8–10.14). The control tube contained HBSS in place of f-5. Following incubation, E-rosettes were prepared and counted as described above.

Statistical Methods

The percentage of E-rosetting lymphocytes from acute PEM patients and lowland and highland children were compared using one-way analysis of variance (ANOVA) with a linear contrast, comparing acute PEM infants with the combined lowland and highland subject. Significance levels were chosen as $\alpha = 5\%$. The effect of thymosin dosage levels on the distribution of rosetting and nonrosetting cells was analyzed using Friedman's rank sum test; the difference between the number of E-rosettes and the nonrosetting cells and the number of small rosettes was also analyzed. Chi-square (corrected for continuity) was used to compare the percentage of infants (PEM) who had increased numbers of E-rosettes with chance hypothesis (50/50) for the (200 μg/ml) treatment. Linear regression analysis was performed with the BMDP statistical package to evaluate the relationship of the response to f-5 and the initial percentage of E-rosettes in the nonthymosin samples. Proportional analysis of f-5 induced changes in nonrosetting cells. Changes in small rosettes were performed by McNemar's test.

RESULTS

E-Rosettes

The percentage of E-rosettes in the 33 acutely malnourished patients and the two mildly malnourished groups are shown in Fig. 1. The mean number of E-rosette cells for the PEM patients was 86.5 (or 43.3% of 200 cells counted), compared with 108.5 (54.2%) for the lowland children and 100.5 (50.3%) for the highland children. These differences were significant [$F(2,71) = 3.83$, $p < 0.05$]. By use of a linear contrast, the number of E-rosetting cells in the PEM group was found to be significantly smaller than the other two groups [$F(1,71) = 6.91$, $p < 0.025$].

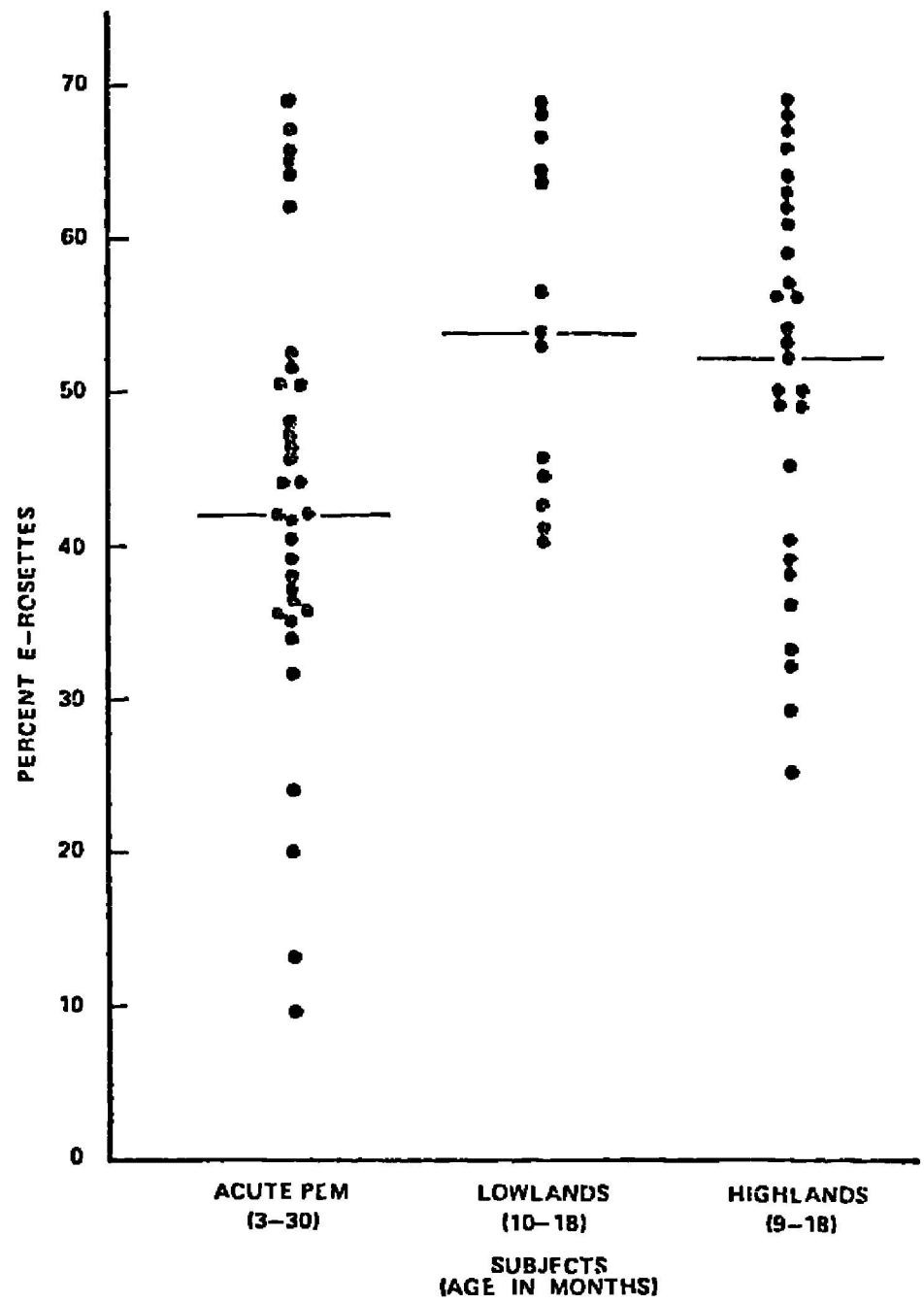

FIG. 1. Percentage of peripheral blood lymphocytes forming E-rosettes with sheep erythrocytes in 33 children with acute protein–energy malnutrition (PEM), and in 13 clinically well infants with mild malnutrition and growth retardation from the coastal lowlands, and in 28 infants from the highlands of Guatemala.

Effect of f-5 Dosage Levels on E-Rosettes

Figure 2 shows the change in the number of E-rosettes following incubation with f-5 as a function of the initial percentage of E-rosettes in the samples without thymosin. The effect of f-5 was inversely related to the initial percentage of E-rosettes in the control (nonthymosin) sample ($r = -0.58$ and -0.56 for the 20- and 200-μg/ml dose levels of f-5, respectively; $p < 0.01$ in both cases). The mean values for the number of E-rosettes, small rosettes, and nonrosetting cells are shown in Table 1. There was a steady increase in the number of E-rosettes as the amount of added f-5 was increased, and a corresponding decrease in both small rosettes and nonrosetting cells. As these three results are constrained to add to the 200 cells counted in each experiment, the effect of f-5 dosage levels was analyzed using the difference between the number of nonrosetting cells and E-rosetting cells for each child (Table 2). The increase in this value as dosage level increased, from 3.3 without thymosin to 23.3 at the 200 μg/ml dosage level, was significant [$F(2, 64) = 12.29$, $p < 0.005$].

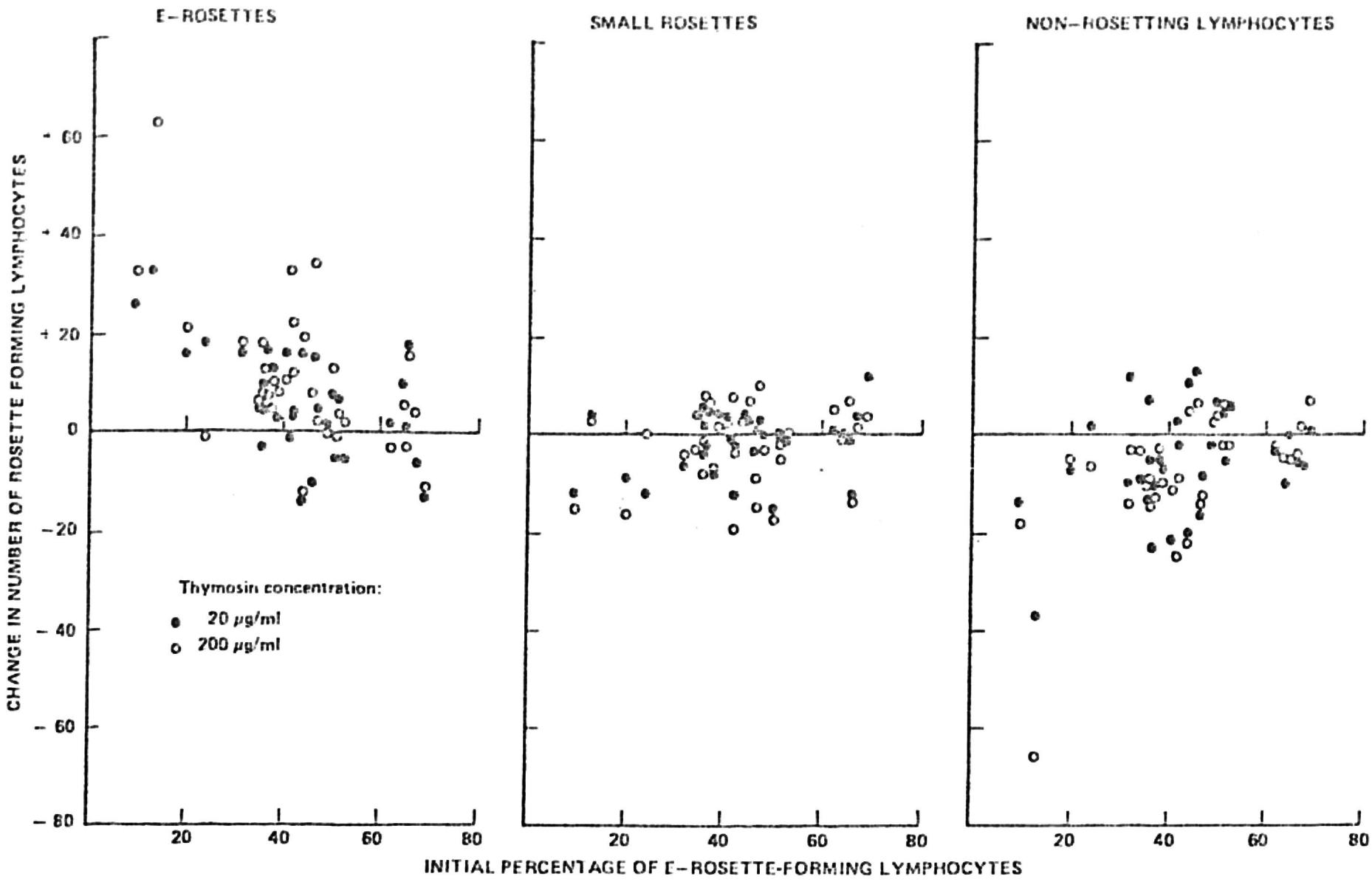

FIG. 2. Effect of in vitro incubation with thymosin fraction 5 (closed circles, 20 μg/ml; open circles, 200 μg/ml) on the number of E-rosettes as a function of the initial percentage of E-rosette-forming cells in control (no thymosin samples). Above the horizontal line indicates an increase in E-rosettes and below the line is a decrease in E-rosette in the thymosin-containing sample.

This parametric result was checked by a distribution-free statistic, Friedman's rank sum test, for each category of lymphocyte (nonrosetting cells, small rosettes, and E-rosettes). The change in both nonrosetting cells and E-rosettes as f-5 dosage increased was significant [$S' = 14.91$, χ^2 (2d.f) = 14.97, $p < 0.01$; 18.94, χ^2 (2d.f) = 18.94, $p < 0.01$], however, the change in small rosettes was not [$S' = 2.31$, χ^2 (2d.f) = 2.31, $p < 0.35$].

Finally, the proportion among the 33 PEM patients showing an increase in E-rosettes when their cells were exposed in vitro to 200 μg/ml of f-5 (27/33 or 82%) was significantly greater than those who did not (6/33, or 18%) [χ^2 (1d.f) = 9.82, $p < 0.005$]. Small rosetting cells either increased or decreased after incubation with f-5 in 31 subjects. The proportion among this group with an increase (14/31) or decrease (17/31) in small rosettes was equal [χ^2 (1d.f) = 2.5, $p > 0.05$], but in either case was associated with a decrease in nonrosetting cells in 24/31 samples (Table 3).

TABLE 1. *Effect of thymosin fraction 5 on E-rosette forming lymphocytes in 33 malnourished Guatemalan children*

Rosette classification	Number of cells counted (mean ± SEM)		
	Thymosin dose		
	None	20 μg/ml	200 μg/ml
E-Rosettes	86.7 ± 5.1 (43%)	93.2 ± 4.2 (47%)	98.2 ± 4.4 (49%)
Small rosettes	29.9 ± 1.3 (15%)	28.7 ± 1.2 (14%)	26.8 ± 1.3 (13%)
Nonrosetting cells	83.4 ± 4.6 (42%)	78.1 ± 4.1 (39%)	74.9 ± 3.9 (37%)
Total cells counted	200	200	200

DISCUSSION

In this study, the mean percentage of mature circulating lymphocytes, assessed by the E-rosette technique, was significantly lower in acute PEM

TABLE 2. *Difference between the number of E-rosetting cells and nonrosetting cells by thymosin dosage level*[a]

No thymosin	20 μg/ml	200 μg/ml
3.3 (9.6)	15.2 (8.2)	23.3 (8.2)

[a] Mean response (SEM): E-rosetting cells minus nonrosetting cells (n = 33).

TABLE 3. *Proportional analysis of thymosin-induced changes in small-rosetting and nonrosetting cells*

Change in small rosettes	Change in nonrosetting cells		
	Increase	Decrease	Total
Increase	3	11	14
Decrease	4	13	17
Total	7	24	31

patients compared to clinically well children from the same ecosystem with mild growth retardation. Of the 33 acutely malnourished Guatemalan infants studied, only 13 (39%) had a marked depression, defined as less than 40% E-rosetting lymphocytes in the peripheral blood, compared to 7 of 41 (17%) better-nourished children. The lowered percentage of E-rosettes in the PEM patients is consistent with data from developing countries around the world (1–3,14–19). However, our data show that clinically severe PEM patients do not necessarily have marked depression in this measure of immune status. In fact, there was considerable overlap in percentage of E-rosettes between acute PEM patients and mildly malnourished but clinically well children. Although we cannot explain the differences between our results and other studies, it should be noted that in our assay for E-rosettes great care was taken to count only viable lymphocytes determined by trypan blue exclusion and to exclude latex-ingesting macrophages from the nonrosetting cell count. Previous studies have not been as rigorous in enumeration of true E-rosettes.

The reasons for the heterogeneity in E-rosettes in clinically severe PEM patients are not clear. Further studies need to examine possible confounding factors responsible for this variability, for example, differences in micronutrient status or the effects of concomitant or preceding infections (4). The clinical significance of the depressed level of E-rosettes in some mildly malnourished children is also uncertain. Prognostic implications of depressed T cells in mild PEM also need to be studied in prospective fashion. It is possible that this immunologic measure will be a better risk predictor for infection-related morbidity and deterioration in nutritional status than is the commonly used measure, i.e., anthropometry.

In vitro incubation with the thymic factor, f-5, increased the number of E-rosettes in a dose-related fashion in patients with low initial values for E-rosettes. In vitro induction of E-rosetting cells with thymic factors in malnourished patients in Bangladesh and Nigeria has been reported by others (14,15). Thus, functional abnormalities of the thymus gland in PEM patients, such as reduced production of thymic hormones, may be responsible for the decrease in circulating mature T cells. Clinical assay of thymic factors in PEM patients have been performed in only two studies. Serum level of Facteur Thymique Serique (FTS) was decreased in PEM patients in one study (20) and unchanged in another (21). However, only nine patients were evaluated in the former study (20) compared to 33 subjects in the latter study (21) in which the samples were also filtered over an Amicon membrane to remove an FTS inhibitor. The relationship, if any, between serum FTS levels and the presence of circulating f-5 responsive cells is not certain. As f-5 serum levels were not measured in the present investigation, we cannot determine whether or not the in vivo level of f-5 would predict the response to f-5 added in vitro. A less likely explanation for increased immature circulating lymphocytes in PEM is the possibility that the observed changes are due to redistribution of lymphocyte subpopulations between the circulation and central or peripheral lymphoid organs. Independent of cause, however, the clinical evidence of depressed cell-mediated immune functions in malnourished hosts (22) indicates that the changes in circulating lymphocyte populations reflect important functional changes in PEM that contribute to morbidity and mortality.

We also considered whether the change from nonrosetting to E-rosetting cells was an all-or-none or a graded phenomenon by counting small rosettes with one to two attached sheep erythrocytes in addition to E-rosettes. Three findings suggest that the conversion to E-rosettes is via an intermediary small rosette stage. First, the numbers of nonrosetting cells and small rosettes were both inversely related to the number of E-rosettes. Second, in the presence of f-5, the increase in E-rosettes was accounted for primarily by decreases in nonrosetting cells and to a lesser extent by a decrease in small rosettes. Third, independent of the direction of change of small-rosettes induced by f-5, a decrease in the nonrosetting cells was seen in 27/31 instances in which a change in this cell compartment was detected. The graded maturation model predicts that an increase in small rosettes due to conversion of nonrosetting cells could be obscured by simultaneous induction of small rosetting T cells

to become E-rosettes. We therefore propose that f-5 induces a progressive maturation from nonrosetting (immature) to small rosetting cells (partially mature), and finally to mature E-rosettes.

These results suggest that PEM results in a maturational arrest of T-lymphocyte development at the thymic level. This is also consistent with previous findings that in vitro incubation of human lymphocytes with f-5 results in a decrease in null cells (23), and that synthetic thymosin α1, a peptide originally isolated from the crude extract, f-5 (24), modulates thymocyte terminal deoxynucleotidyltransferase activity in mouse cells (a marker of thymocyte maturation), and induces the appearance of T-cell surface maturation antigens on circulating mouse lymphocytes, including Thy 1.2 and Lyt 1.2.3 (25,26). Thus, the T-cell defect in PEM may be considered to be, in part, an endocrine abnormality. If this is true, then the implications of this concept for more rapid restoration of T-cell function in malnourished subjects by thymic hormone replacement therapy are obvious. Further studies of the functional consequences of thymosin addition in vitro are warranted as a next step.

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