Study of Thymus and Thymocytes in Bolivian Preschool Children During Recovery from Severe Protein Energy Malnutrition

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ABSTRACT. Impaired cellular immunity in malnourished children is well known but rarely recovery of immunity was assumed during nutritional rehabilitation. Nutritional Acquired Immune Deficiency Syndrome (NAIDS) was directly or indirectly responsible for high morbi-mortality in preschool children. In the CRIN (Centro de Reha-bilitación Inmuno-Nutricional), 45 children aged 6 to 55 months, hospitalized with severe malnutrition, were studied during 9 weeks. Anthropometric measurements and echography of the thymic left lobule were carried out weekly and T cell subsets (CD3, CD1a) were measured monthly. Weight for height was 79% of NCHS (National Center for Health Statistic) median upon admission and still 90% at

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5 weeks. The immature lymphocyte level was 29% upon admission, 16% after 5 weeks and 10% after 9 weeks (discharge); at the same time the thymic area measured respectively 39 mm^2, 193 mm^2 and 349 mm^2. Nutritional recovery was faster than Immune recovery. "Apparently" healthy children which had recovered after 5 weeks were still immune depressed and we must consider them as high risk children. These observations indicate that cellular immunity should be evaluated regularly during malnutrition rehabilitation to avoid the frequent failure of nutritional rehabilitation programs. A noninvasive method like thymic echography enables to evaluate indirectly immune recovery.

KEYWORDS: Thymus; Lymphocytes; Protein-energy malnutrition; Preschool children

INTRODUCTION

One and a half century before the discovery of the immunological role of the thymus by Miller,[1] thymic atrophy in malnourished patients was first described by Menckel in 1810,[2] and Simon in 1845 who called the thymus: "a very sensitive barometer of malnutrition."[3]

Observations on the relationship between nutrition and infectious diseases[4] and impaired immunocompetence in malnutrition,[5] introduced the concept of synergic action between: Malnutrition, decreased immunocompetence and increased infections on morbi-mortality in preschool children.[6] This Immune dysfunction secondary to malnutrition or NAIDS (Nutritionally Acquired Immune Deficiency Syndrome)[2] is directly or indirectly responsible for the death of one child every 2 seconds in the whole world.[7] These NAIDS children show impaired cellular immunity[8,9] with thymic involution[10] or nutritional thymectomy.[11]

Before the discovery and general laboratory use of monoclonal antibodies (MAb), T and B lymphocyte subpopulations were estimated with Rosette formation (SRBC), Complement receptor and specific mitogen responses.[12,13,14] The increase of non-T/non-B cells or "null cells" in PEM[15] corresponds to "a deficiency of thymic inductive capacity which may result in impaired differentiation and maturation of T lymphocytes."[16]

Previous investigation[10] provided direct evidence that nutritional thymic involution is accompanied by an altered content of thymulin and that "this functional change is probably one of the main causes of the deficiency in cell-mediated immunity."

Clearly, severely malnourished children are immunodepressed too.[5] Rapid repletion of weight deficits reduces hospitalization time[17] according to clinical criteria but some authors[18] emphasized that the relationship of weight for height as indicator of recovery does not coincide with the recovery of other anthropometric or physiological parameters. Parent et al.[19] observed that "biochemical parameters were back to normal within 20 days while the return to normal of the immunological indices was more protracted."

We observed the same gap between anthropometric and thymic recovery. This gap explains many early discharges based on apparent recovery of the children whereas "correct" discharge should consider anthropometric and immunological recovery. To save these NAIDS children it is not only necessary to cure clinical or nutritional aspects of PEM but also to restore their immunological function.[19,20,21]

To reach this objective, we created the CRIN (Centro Rehabilitación Inmuno-Nutricional), Bolivia's first center able to restore both, nutritional and immunological aspects of PEM, in Cochabamba (alt. 2600 m), Bolivia.

PATIENTS AND METHODS

From severely malnourished children hospitalized in the Hospital Materno-Infantil "German Urquidi," Cochabamba, between May 1989 and December 1991 and treated for respiratory and/or intestinal infections, forty-five children were selected and admitted to the CRIN, with the consent of the hospital ethical committee. These children are those for which parental consent had been obtained for a 2 months follow-up study in the CRIN.

All of them came from poor homes in Cochabamba suburban areas. Socio-economic characteristics are low income families, crowded living conditions, lack of sanitation and early weaning.

Kwashiorkor, Marasmus and combined PEM diagnoses were based on weight for height,[22] arm/head circumferences ratio[23] and clinical findings such as: presence of edema, loss of subcutaneous tissue and diminished muscle mass.[24]

The children received a diet divided in four steps during two months:

- Initial Phase (1 week): To decrease risk of transitory lactose intolerance,[25] we used a milk-based diet with one half concentration of lactose, distributed in 7 feedings/day and supplying 1.5 to 2.5 g of protein and 120 to 150 kcal/kg body weight/day, according to the PEM pattern.

- Transition Phase (1 week): Gradually and slow increase of protein and energy.
- "Caloric-Protein Bombing" Phase (6 weeks): For rapid weight gain with sufficient energy for protein deposition, we gave 5 g of protein and 200 kcal/kg body weight/day.[26,27]
- Discharge Preparation Phase (1 week): Gradual decrease of protein and energy.

From CRIN admission, each child received a clinical examination daily. Weight, height, arm and head circumferences as well as triceps skinfold thickness were measured weekly according to standardized methods from Jelliffe.[28] Weight for age, height for age, weight for height, arm/head circumferences ratio or Kanawati-MacLaren Index and upper arm bone-muscle area were calculated.[23,29,30]

Weekly, thymus size was assessed by mediastinal echographic examination using an echo camera (ALOKA SSD-210 DXII, Tokyo) with a 5 MHz linear pediatric probe.[31] In order to standardize thymus evolution, we determined the longitudinal echographic section area of the left thymus lobe between the second and fourth rib.

For identification of lymphocyte subpopulations, 3 to 5 ml of blood were collected by venipuncture with Liquemine (Roche, Paris) as anticoagulant on day 0, 35 (week 5) and 63 (week 9). After leucocyte separation with Ficoll-Paque (Pharmacia, Uppsala), cell suspension was adjusted to 10^7 cells/ml, divided in 2 aliquots and incubated 2 hours with RPMI 1640 (Gibco, Gaithersburg, MD) or RPMI + thymulin (Choay, Paris).[32]

Quantification of lymphocytes CD3 and CD1a[31] was performed using OKT3 and OKT6 Monoclonal antibodies (Ortho Diagnostic Systems–France, Roissy) with FITC-GAM (Fluorescein Isothiocyanate Conjugate-Goat Anti Mouse). Counting was performed on at least 200 cells under a UV fluorescence episcopic microscope.

RESULTS

Clinical Evaluation and Anthropometric Measurements

Twenty-five girls and 20 boys were studied aged 6 to 55 months (mean age =16 months). Kwashiorkor, Marasmus and Marasmic-Kwashiorkor or mixed PEM were found respectively in 38, 24, and 38 percent of the children. Table 1 shows the descriptive statistics for anthropometry on admission (week 0), in the middle of the hospitalization time (week 5) and

on discharge (week 9). Because of the high prevalence of edematous PEM forms (Kwashiorkor and mixed PEM), the weight loss was hidden. This leads to a relatively high percentage of NCHS reference median for weight for height at the time of admission, and for this reason, we also used the Kanawati-MacLaren Index.

All anthropometric parameters increased significantly during the first five weeks. If we only had considered anthropometric parameters, especially weight for height, the children would have been discharged at that time (5 weeks).

Evaluation of the Immunological Status

To evaluate the immunological status we used two methods: a non-invasive thymic echography and the determination of peripheral T lymphocyte subsets. Table 2 presents the estimated area of the left thymic lobe between the second and fourth rib. Compared to normal thymic values established in Cochabamba,[33] we can consider that admission values reflected a "nutritional thymectomy"[11] and that thymic recovery was effective after 9 weeks. Although cardiac movements modified thymic size and induced a high variability of the thymic values, the kinetic of thymic recovery showed respectively a five fold and nine fold increase after 5 and 9 weeks.

Values of T-lymphocyte subpopulations are presented in the same table. Monoclonal antibody OKT3 marks all T-lymphocytes (CD3+). We observed a low level of CD3 lymphocytes in NAIDS children immediately after admission, compared to "normal" children values: 67% and 63% according to Chandra[34] and Wade.[35] Similar values were reached only at discharge.

Monoclonal antibody OKT6 marks thymocytes (CD1a) or immature lymphocytes when they are present in peripheral blood. We observed a very high level of immature lymphocytes compared to normal levels: 8% (Parent et al. unpublished data). This result is in accordance with observations made by Keusch et al.[36] with 42% of non-rosetting cells also considered as immature cells.

To alleviate deficiency of the thymic function in severe forms of PEM, thymic hormone therapy was considered.[10] The first step based on a personal communication by Jambon was to try the *in vitro* effect of thymulin on lymphocyte subpopulations.

We observed a level effect of thymulin on lymphocytes CD3 but each incubation with thymulin reduced the percentage of immature lymphocytes (CD1) to half of its initial value.

TABLE 1. Evolution of anthropometrical parameters during 9 weeks of CRIN hospitalization (mean ± SD).

Hospitalization time	Week 0		Week 5		Week 9
number of children	45		44		44
age (months)	16.4 ± 8.2		17.6 ± 8.3		18.6 ± 8.3
Weight (kg)	6.5 ± 1.9	**	7.7 ± 2.2	NS	8.4 ± 2.2
Height (cm)	± 69.5 ± 7.6	NS	70.5 ± 7.4	NS	71.6 ± 7.3
Arm circumfer.(cm)	10.8 ± 1.8	***	12.4 ± 1.7	*	13.3 ± 1.8
Weight (height) % med. NCHS	79.1 ± 8.8	***	90.6 ± 9.6	NS	93.2 ± 16.8
Z-score	−2.2		−1.0		−0.5
Arm/head ratio × 1000	252 ± 34	***	284 ± 32	*	302 ± 31
Triceps skinfold (mm)	4.8 ± 2.0	***	6.7 ± 2.0	*	7.6 ± 2.0
Up.arm muscle area (cm^2)	7.0 ± 1.9	***	8.6 ± 2.1	*	9.7 ± 2.4

Student test:
NS. Not Significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

TABLE 2. Evolution of immunological parameters during CRIN hospitalization (mean ± SD).

Hospitalization time	Week 0		Week 5		Week 9
number of children	44		38		37
Thymus area (mm^2) ■	38.9±3.2	***	193.4±18.5	***	348.7±21.1
number of children	45		40		35
OKT3 (%) RPMI	60.2±5.6	***	64.9±4.9	*	67.4±4.1
OKT6 (%) RPMI	28.3±5.3	***	15.8±4.7	***	10.5±4.3
OKT3 (%) RPMI+FTS-Zn	65.9±5.3	NS	67.2±11.5	NS	70.8±3.8
OKT6 (%) RPMI+FTS-Zn	14.5±3.8	***	8.7±2.5	***	5.3±3.1

■ Mean ± SEM.

Student test was given between 2 adjacent values on the same line. NS: Not Significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

DISCUSSION

The children hospitalized in the CRIN who were studied were 6 to 55 months old (mean age = 16 months) with 71 percent between 12 and 24 months and only 3 children older than 2 years.

A high percentage of edematous forms was found among these children (76 percent of PEM), close to the percentage observed by McMurray[37] in Colombia in similar malnourished children groups. Weight loss hidden by edema may explain the relatively high weight for height at time of admission: 79 percent of the NCHS median (equivalent to -2.2 Z-score) with weight for age, respectively, 69%, 60% and 50% of the NCHS reference median for Kwashiorkor, mixed form and Marasmus.

The arm/head circumference ratio is less affected by edema because upper arm edema is less frequent than foot and leg edema. This ratio presents border values to severe PEM according to the threshold established by Kanawati[23] in Lebanese children.

We observed a weight gain of 2 kg in 2 months with our diet (200 Kcal and 5 g protein/kg/day). These results are similar to the ones reported by Olson[26] in Thailand: 3 kg in 3 months, with 4 g protein and 175 Kcal/kg/day. The rate of weight gain per day was 4.9 g/kg/d, similar to the values estimated by Olson[26] and Fjeld.[17]

Comparison to the findings of other authors was difficult because each group used different anthropometric parameters and the time of recovery varied from 30 to 90 days.[17,18,37,38]

If we had considered anthropometric recovery based on weight for height with 90 percent of the NCHS median—or 1 SD as cut-off point—we could have discharged these children after the fifth hospitalization week, but we observed a gap between anthropometric and thymic recovery.

Until now, the majority of reported cases of thymic atrophy with PEM were postmortem studies[10,11,39,40] whereas the X-ray diagnosis of thymic hypertrophy was well known.[41] The first qualitative estimation of the thymus size by X-ray in case of PEM was performed by Golden[42] who established 3 degrees, because radiologic visibility of the thymus is only a shadow corresponding to the mediastinal mass,[43] and "its variability is so great that up to now there are no accepted radiological or anatomical standards."[44] Recent techniques like computerized tomography[45] and ultrasonography[43,46,47] can enable to identify and to measure the thymus gland without confusion.

Ricard[48] was the first to investigate ultrasonographic evaluation and quantitative estimation of the thymus in malnourished children in Senegal. Previous investigation on normal thymic values in "healthy" preschool

children in Cochabamba gave an estimated area of 350 mm^2 for the left thymic lobe between the second and fourth rib.[33]

In the CRIN children, the same area was 39 mm^2 at time of admission and reached the normal value only after 9 weeks of hospitalization. In spite of the high variability of the thymus shape due to cardiac movements, the thymic ultrasonography can evaluate the recovery of the thymus gland without confusion because size variation was 1 to 9 fold between admission and discharge.

Before the general use of Monoclonal Antibodies (MAb), T cells and B cells were detected with Rosettes-E and Rosettes-EAC, respectively, but the SRBC receptor corresponds to CD2 and identifies mature and immature lymphocytes in blood. On the other hand, MAb OKT3 (CD3) identifies only mature T lymphocytes and MAb OKT6 (CD1a) immature T cells, therefore it is difficult to compare studies with CD2 to others with CD3, CD4 or CD1.

In malnourished children, a decrease of T3 and T4 cells[34,35] and an increase of "null cells"[34] has been observed. For Chandra[49] these "null cells may be immature and incompletely differentiated T lymphocytes" that result from "deficiency of thymic inductive capacity."

For this reason, it was interesting to study *in vitro* hormonal manipulation of the depressed immune system and especially the effect of thymic hormones. As previously described[18,35,50] thymosin and thymopoietin induced a significant increase in the percentage of E-Rosettes (T cells), a decrease of non-rosetting cells and transformation of those cells, supposed to be immature cells, in rosetting cells. These observations showed the sensitivity of peripheral lymphocytes from PEM children to thymosin and thymopoietin and suggested a deficiency of these factors which are produced like thymulin by the thymic epithelium.[51]

A previous study in Senegalese children who died of malnutrition describes thymic atrophy with depleted thymulin content[51] and a transversal study in Bolivian malnourished children showed the *in vitro* lymphodifferentiative effect of thymulin on peripheral lymphocytes.[52] In our study, the incubation of immature T lymphocytes (CD1a) with thymulin or FTS-Zn[31] had a striking effect: the percentage of immature cells was reduced to half of its initial value and its effect was the same at admission as upon discharge. If we considered a CD1a percentage of 10 or less as "normal value," this percentage was reached only at discharge after 9 weeks. After *in vitro* incubation with thymulin, we reached a percentage lower than 10 after 5 weeks, so we think that thymulin therapy can shorten the recovery of the immune system in NAIDS children.

Anthropometric and clinic recovery of severe malnutrition is the first

step but the discharge of immunedepressed children at this time in developing countries can explain the frequent failure of nutritional recovery programs.

We think that the highly significant correlation between thymus size and CD1 level (r = -0.586) during the 2 months of hospitalization, justifies the use of ultrasonography of the thymus gland for indirect evaluation of the immunocompetence level in malnourished children.

We agree Olusi[18] who says that: "it has become important to investigate immunostimulatory treatment that could be used to rapidly restore their depressed cell-mediated immunity" and think that thymic echography can follow nutritional immune recovery with thymic hormones or nutrients like zinc[42] as thymulin cofactor.[32]

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