Immunological Assessment and its Predictive Role in Malnourished Infants with Diarrhoea and/or Systemic Infections

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Summary

The immune capacity of 47 marasmic 1–12 months old ill infants with weights below 75 per cent of Brazilian standards and of 12 well-nourished healthy controls was studied, by measuring: the absolute and relative T and B lymphocyte subpopulations, the blastogenic response to phytohaemagglutinin (PHA), expressed as stimulation index (SI), and the delayed hypersensitivity skin test with PHA. Eight (17 per cent) of the malnourished infants died and results were evaluated with respect to ultimate outcome. In addition a discriminant analysis was retrospectively applied to the immunological data of the two groups of malnourished infants.

Both groups of malnourished infants presented significantly lower values than the control group for all measurements, although not very evident for B lymphocytes. The immunodepression was more intense for the infants who died than for those who survived, as shown by significantly lower values for the SI, the skin test, and by the discriminant analysis. The discriminant analysis led to a linear predictive model which accurately classified 85 per cent of the survivors and 100 per cent of those who died. It was concluded that immunological parameters may be useful to select prospectively those infants who need a different therapeutic approach because of their high risk of mortality.

Introduction

Malnourished infants with diarrhoea frequently have a protracted clinical course and severe infective episodes often occur. These infants carry a poor prognosis as their mortality rates are very high, possibly due to their depressed immunocompetence. Immunodeficiency, mainly in the cell-mediated immunity secondary to severe malnutrition, has been well documented and extensively reviewed.[1,2] However, mostly children over 9–12 months of age have been studied and very few infants of lower age have been included in some studies.[3–6] In the comparatively fewer studies of marasmic infants in their first year of life, immunodeficiency was not convincingly demonstrated.[7–9] In our experience[10] and of others in Latin America,[11] however, the greatest mortality in infancy beyond the neonatal period occurs mostly in malnourished infants of the marasmic type (marasmus and underweight infants), under 12 months of age but mainly under 6 months, with the above cited clinical course. We therefore studied some aspects of the immunological status of malnourished infants admitted during their first year of life with diarrhoea, septicaemia, and/or bacterial meningitis to the pediatric ward of the Faculty of Medicine of Botucatu in São Paulo, Brazil.

Having demonstrated immunodeficiency for the malnourished infants, in the second part of the study we calculated a linear predictive model[12,13] from the obtained data. The objective was early detection of infants who because of their high risk of mortality need a special therapeutic approach.

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Clinical materials and methods

Subjects

We studied 12 control infants (Group 1–G1) and 47 hospitalized malnourished infants aged 1–12 months. The malnourished infants were separated into two groups according to clinical outcome: those who sur-

vived and were discharged from the ward alive (Group 2–G2) and those who died during the hospitalization (Group 3–G3). Criteria for their inclusion in the study were: at least second degree active malnutrition according to Gomez's weight for age criterion,[14] viz., weight below 75 per cent of Brazilian standards[15] and loss of subcutaneous fat; no oedema, hair, or skin changes as seen in kwashiorkor; at least one of the following diagnoses—acute or protracted diarrhoea, septicaemia, or bacterial meningitis—and no disseminated intravascular coagulation at the time of admission to the ward. The control infants were well-nourished (weight for age above 90 per cent of Brazilian standards),[15] with birth weights above 2500 g, without infection or diarrhoea for at least 1 month and without previous recurrent or severe infections. Permission of the parents was obtained before inclusion in the study.

Immunological methods

The immunological evaluation was carried out after rehydration of the infants and within the first 5 days of hospitalization (the majority within the first 3 days) when no clinical signs of nutritional improvement were noticeable. Absolute and relative numbers of T and B lymphocytes, the *in vitro* blastogenic response of lymphocytes to phytohaemagglutinin (PHA) in the presence of homologous serum, and the delayed hypersensitivity skin test with PHA were measured.

The lymphocyte suspension was prepared by the Ficoll–Hypaque density gradient method[16] and adjusted to 3×10^6 cells/ml (Ficoll–Pharmacia Fine Chem., Uppsala; Hypaque–Winthrop Prod. Inc., New York). T and B lymphocyte subpopulations were identified, respectively, by rosette formation with sheep erythrocytes (E) or with human erythrocytes sensitized with antibody and complement (HEAC).[17] Briefly, for T lymphocytes, 0.1 ml of 0.5 per cent (vol/vol) suspension of washed E in saline was mixed with equal volumes (in triplicate) of the lymphocyte suspension, centrifuged at 200 g for 5 min and incubated for 1 h at 4°C. The percentage of rosette-forming cells was determined microscopically in a haemocytometer after addition of one drop of methylene blue (0.33 per cent in saline). At least 300 lymphocytes were counted and only cells possessing three or more adhering erythrocytes were scored as rosette-forming cells.

The HEAC assay was carried out as follows: a suspension of washed human erythrocytes (HE) was adjusted to 2.5 per cent (vol/vol) in saline and incubated for 30 min with an equal volume of a subagglutinating dilution of a rabbit antiserum (A) to HE. 0.1 ml of undiluted fresh mouse serum supplied the complement (C) and was added to 2 ml of the HEA, incubated for an additional 30 min at 37°C and adjusted to 0.5 per cent (vol/vol) in saline. 0.1 ml of HEAC was mixed with an equal volume of the lymphocyte suspension and immediately centrifuged at 200 *g* for 5 min at room temperature. The percentage of rosettes was determined, as described above.

White blood cell counts for calculation of the absolute numbers of lymphocytes and, hence, of T and B lymphocytes were obtained by standard automated clinical laboratory technique.

The *in vitro* lymphocyte transformation response to PHA was studied as described.[18] Briefly, the lymphocyte suspension was adjusted in sterile conditions to a final concentration of 10^6 cells/ml containing 20 per cent homologous serum from a normal donor. Each culture tube contained 2.5 ml of the cell suspension and 0.1 ml (40 µg) of PHA-P (Difco Lab., Detroit). Control tubes were incubated without PHA. After incubation for 72 hours the incorporation of tritiated thymidine (Schwarz Bio Res., New York) into trichloroacetic acid precipitated material was measured for 5 minutes in a Beckman LF-3150T liquid scintillation system (Beckman Instruments Inc., California) and expressed as stimulation index (SI).

$$SI = \frac{\text{counts per minute of PHA-stimulated cells}}{\text{counts per minute of control cells}}$$

The *in vivo* skin reactivity to PHA was studied as follows: 2 µg of purified PHA (Wellcome Reagents Ltd, London) diluted in 0.1 ml of saline were injected intradermally into the volar aspect of the forearm.[19] The induration was measured after 24 hours.[20] Results as the mean of two perpendicular diameters were expressed in millimetres. All immunological measurements were made by one of the authors (CMCM).

Statistical analyses

For comparison among the three groups we applied the Kruskal–Wallis non-parametric test[21] to the immunological and some of the clinical data. For significant results at the 95 per cent confidence level, a contrast between mean of ranks was applied.[22] Further clinical data were submitted to the chi-square or Fisher's exact test.[21] Discriminant analysis[12,23] was performed for comparison between G2 and G3 and to establish a linear predictive model, using the variables B/mm^3, T/mm^3, SI and PHA skin test, previously selected as those with the best discrimination power. The original values of these parameters were converted into square roots (B/mm^3, T/mm^3) and natural logarithms (SI) in order to render them more adequate for the discriminant analysis. The discriminant analysis was also used without the SI, as its use retards the application of the predictive linear model for at least 4 days, because of the necessary time to complete the measurements.

Results

Eight of the 47 malnourished infants (17 per cent)

TABLE 1
Features of control (G) and malnourished infants who survived (G2) or died (G3)

	G1	G2	G3
No. of infants	12	39	8
Male:Female	8:4	18:21	3:5
Median age(d)	194	132	122
(range)	(58–290)	(33–333)	(64–303)
Infants <6 m (%)	5 (41.7)	25 (64.1)	5 (62.5)
Median weaning	97	26	38
age (d) (range)*	(0–183)	(0–183)	(0–122)
Median birth	3295	3100	3000
weight (g) (range)	(2600–3550)	(1300–3950)	(2000–3300)
Birth weight			
<2500 g† (%)	‡	7/29 (24.1)	2/7 (28.6)
Median percentage weight		44.6	44.7
deficit (range) after rehydration	‡	(26.5–62.5)	(28.8–56.6)

* $P<0.02$ (G1 > G2). $P>0.05$ for all other data.
† Data unknown for 11 infants.
‡ No low birth weight and weight deficit, due to inclusion criteria

died within 2 weeks of the immunological evaluation, one of bronchopneumonia, one of meningitis and 6 of overwhelming septicaemia, of whom five suffered disseminated intravascular coagulation (G3). The remaining 39 (83 per cent) were discharged clinically well, although still nutritionally not completely rehabilitated (G2).

The features and diagnoses of the studied infants are presented in Tables 1 and 2. Sex, age distribution, and birth weight was the same for the three groups ($P>0.05$). Weaning occurred at an older age for the control group than for the survival group ($P<0.02$), but no difference between both groups of malnourished infants was observed. Both groups (2 and 3) were also similar in percentage weight deficit and frequency of diagnoses ($P>0.05$).

Results of the immunological measurements are shown in Fig. 1. Compared to controls, malnourished infants (G2 and G3) presented significantly lower values for all tests that measure cell-mediated immunity (%T, T/mm^3, SI, PHA skin test). For B lymphocytes, however, the differences were not so evident; applying the contrast between mean of ranks for percentage B lymphocytes, the controls differed from G2 ($P<0.05$), but this difference just failed to reach significance at the 5 per cent level for G3 ($0.05<P<0.10$). The same occurred for B/mm^3 when comparing controls to G2 ($0.05<P<0.10$).

Malnourished infants who survived presented significantly higher values for the SI and the PHA skin test than those who died (Fig. 1). Absence of induration (0 mm) 24 hours after PHA intradermal injection was observed in five out of seven (71 per cent) of those infants who ultimately died, but in only two out of thirty-seven (5 per cent) of those who survived (PHA skin test was not available in three infants).

Values for percentage B, percentage T, SI, and the

TABLE 2
Clinical diagnoses of malnourished infants who survived (G2) or died (G3)

	No. (percentage) of infants	
	G2	G3
Acute diarrhoea*	14 (35.9)	1 (12.5)
Protracted diarrhoea*	23 (60.0)	7 (87.5)
Septicaemia		
at admission	7 (17.9)	4 (50.0)
after admission	5 (12.8)	2 (25.0)
Bacterial meningitis		
at admission	5 (12.8)	1 (12.5)
after admission	—	1 (12.5)
Bronchopneumonia		
at admission	12 (30.8)	1 (12.5)
after admission	5 (12.8)	1 (12.5)
Upper respiratory tract infection		
at admission	7 (17.9)	1 (12.5)
after admission	4 (10.3)	—
Skin infections		
after admission	4 (10.3)	—
	39 (100.0)	8 (100,0)

* No infant acquired diarrhoea in the ward. Potential enteropathogens were isolated from six acute and nine protracted diarrhoeic patients (only enteropathogenic *E. coli*, *Salmonella sp.*, *Shigella* sp. and rota-adeno-coronavirus were looked for systematically).
$P>0.05$ for all diagnoses.

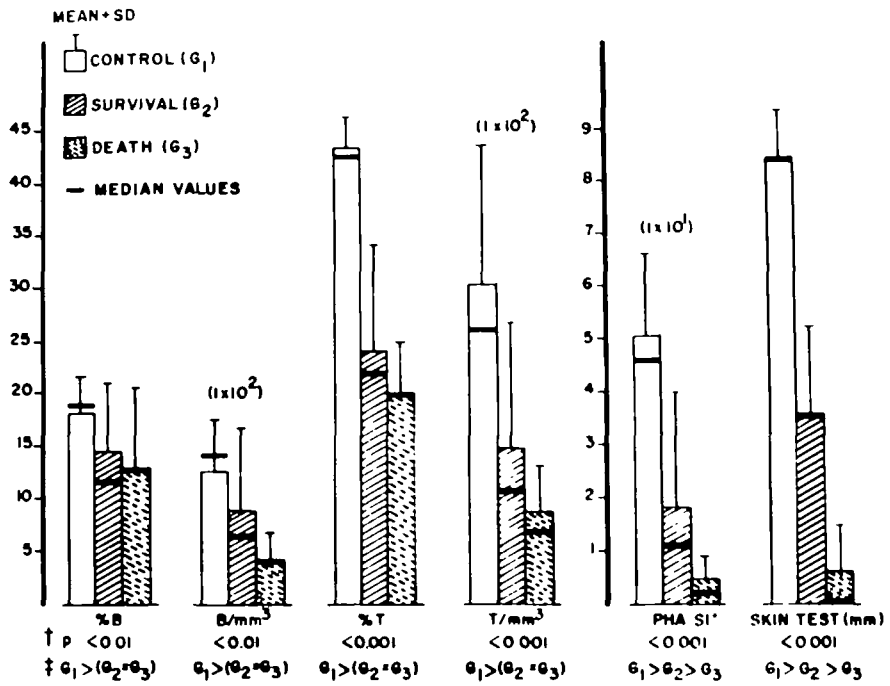

FIG. 1. Immunological parameters of controls and malnourished infants who survived or died.
* PHA SI = Phytohaemagglutinin stimulation index. † Kruskal Wallis test. ‡ Contrast between mean of ranks.
See text for statistics of %B and B/mm³.

PHA skin test below the lower limit of the 95 per cent inclusion interval of the controls were considered abnormally low.[24] For the calculation of the lower limit for B/mm³ and T/mm³ we used the values for lymphocytes/mm³ referred by Stransky.[25] Taking these limits into account, all malnourished infants presented low values for at least one mesurement and 89 per cent presented three or more abnormal parameters. All infants who died had four or more abnormal measurements, whereas this occurred in only 62 per cent of those who survived (Table 3).

The discriminant analyses, with and without SI, revealed significant differences between G2 and G3 ($P<0.001$) and led to linear predictive models which, retrospectively applied to the individual infant of each group, accurately classified most infants (Table 4). The probabilities associated with the individual classification of the discriminant analysis are shown in Table 5. Most infants had a high probability associated with the correct classification and a low probability associated with the wrong prediction.

TABLE 3
Distribution of abnormal immunological measurements in malnourished infants who survived (G2) or died (G3)

No. of abnormal measurements	No. (percentage) of infants*	
	G2	G3
1	2 (5.5)	0 (0.0)
2	3 (8.1)	0 (0.0)
3	9 (24.3)	0 (0.0)
4	14 (37.8)	2 (28.6)
5	8 (21.6)	2 (28.6)
6	1 (2.7)	3 (42.8)
	37 (100.0)	7 (100.0)

* Three infants, two G2 and one G3, excluded (PHA skin test not available).

Discussion

Our study has shown that 1–12 months old infants with malnutrition of the marasmic type, diarrhoea, and/or parenteral infections present an important immunodepression. Two reasons at least could explain our results which conflict with those previously reported for the same type of malnutrition and age group:[7–9] the immunological investigation in the first days after admission to the ward, when no subclinical nutritional rehabilitation had yet occurred[1,26] and the association or the greater severity of the associated infections in our study.

TABLE 4

Discriminant analyses between malnourished infants who survived (G2) or died (G3) using either three or four immunological parameters

Linear predictive model	*P* value	Classification method	Predictive accuracy
$F(x) = 0.0094B^{1/2} - 0.0033T^{1/2} + 1.0989ST + 0.4662 ln SI$*	<0.001	$F(x) > 5.312 \rightarrow G2$	For G2 = 84.6%
		$F(x) < 5.312 \rightarrow G3$	For G3 = 100.0%
$F(x) = 0.0098B^{1/2} - 0.0019T^{1/2} + 1.2130ST$*	<0.001	$F(x) > 4.871 \rightarrow G2$	For G2 = 82.0%
		$F(x) < 4.871 \rightarrow G3$	For G3 = 87.5%

Example of calculation of the linear predictive model for a hypothetical malnourished infant with the following immunological values:

B/mm³ = 450	$F(x) = 0.0094.(450)^{1/2} - 0.0033.(680)^{1/2} + 1.0989.(0.0) + 0.4662.ln(4.0)$
T/mm³ = 680	
ST = 0.0 mm	$F(x) = 2.554$
SI = 4.0	$F(x) < 5.312 \rightarrow G3$

Conclusion: This infant will be classified as belonging to the death group.

* B = B/mm³; T = T/mm³; ST = skin test; *ln* = natural logarithm; SI = stimulation index

It is well known that infections can depress the immune responses.[1,7,27] It is therefore difficult in our study to determine the proportion of immunodepression actually due to malnutrition and that due to enteral and/or parenteral infection(s). Nevertheless, the vicious circle malnutrition—infection and the synergistic interaction between them must be kept in mind always when one deals with these infants. We have deliberately chosen infants with potentially or currently severe parenteral infections because of their high risk of death. Twenty of our infants were admitted with only diarrhoea, or diarrhoea and mild upper respiratory infection, and nine of them developed bronchopneumonia, sepsis and/or meningitis during hospitalization, one of which died. This clearly shows that all our infants must be considered a high risk.

Also low birth weight for gestational age can depress cellular immunity for at least up to 5 years of life[1,28] and, although no data about gestational age were available in our study, this factor could be one additional cause to explain the immunodepression of the infants born with weights below 2500 g. However, birth weight, as well as age, sex, age of weaning, and percentage weight deficit failed to reveal any significant difference in our study, between the infants who died and those who survived.

The immunodepression was not very evident for B lymphocytes. But only quantitative aspects (%B, B/mm³) have been studied, and these aspects have usually been reported as normal or only slightly altered in malnourished children.[1,2,8,29] More than merely the quantification of B immunocytes, their response to new antigens with proliferation and secretion of antibody has functional significance. B cell activation is dependent on T helper cells which promote antibody secretion, whereas T suppressor cells inhibit it. In fact, it has been shown that malnourished children present an altered relation of T helper: T cytotoxic/suppressor cells (helper cells being decreased to a greater extent than suppressor cells) and a decreased ability of T helper cells to provide help to B cells in antibody synthesis.[30]

The wards in developing countries are often overcrowded with malnourished diarrhoeic infants and it is certainly not necessary to submit them all to the same critical care therapy. However, because of the high mortality rates in this setting it would be particularly useful to have at hand predictive prognostic indices which allow a rational selection of patients with a high risk of death. Besides, alternative or additional measures[1,31,32] should be sought for these infants. Increased risk of death in children has been associated with higher degrees of malnutrition,[33] but prognostic indices including immunological parameters have, to our knowledge, been published only for mainly surgical adult patients.[13,34–36] Heyworth *et al.* 1975,[37] although not applying a prognostic index, described that the inhibition of *in vitro* PHA-induced lymphocyte transformation was maximal when using plasma from malnourished children who subsequently died.

When the discriminant analysis was used without the SI it did not classify correctly only two infants, one of each group, in addition to the six already

TABLE 5

Probabilities associated with the individual classification of the discriminant analysis when the stimulation index (SI) was included in the predictive linear model

Group into which the infants who survived (G2) were classified	Probability associated to the classification	Group into which the infants who died (G3) were classified	Probability associated to the classification
G2	0.95*	G3	0.97*
G2	0.95*	G3	0.91*
G2	0.99*	G3	0.58‡
G2	0.95*	G3	0.98*
G2	0.99*	G3	0.69‡
G2	0 90*	G3	0.93*
G2	0.91*	G3	0.90*
G2	0.99*	G3	0.95*
G3	0.64†		
G2	0.68‡		
G3	0.95§		
G2	0.96*		
G3	0.66†		
G2	0.86*		
G2	0 98*		
G2	0.94*		
G2	0 71‡		
G2	0.99*		
G2	0.86*		
G2	0.98*		
G2	0.67‡		
G2	0.90*		
G2	0.62‡		
G3	0.72†		
G2	0.77		
G2	0.94*		
G2	0.96*		
G2	0.62‡		
G2	0.97*		
G2	0.99*		
G2	0.61‡¶		
G3	0 75†		
G2	0.71‡		
G2	0.99*		
G3	0.72†		
G2	0.99*		
G2	0.95*		
G2	0.98*		
G2	0.97*		

* Correct classification, with a high probability: typical classification.

† Incorrect classification, but with a low probability.

‡ Correct classification, with a low probability: atypical classification.

§ Survived, although with a very bad initial prognosis (high probability associated to classification into G3).

¶ Without the SI, the PLM classified this infant in the other group.

wrongly classified when the SI had been included in the analysis. We must consider, however, that these two infants had been classified correctly previously (SI included), but with a low probability. In future prospective studies, necessary to validate the method, the discriminant analysis should be used initially when results of B/mm³, T/mm³, and PHA skin test are available (after 24 hours) and reapplied when the SI is available, in order to reclassify the infants if necessary. It is noteworthy that when the SI was included in the predictive linear model all infants who ultimately died were rightly classified and only 6 out of 39 survivors were classified as meeting a fatal outcome.

It must be kept in mind that the PHA skin test with no response at all (0 mm) was highly suspicious of a fatal outcome, as it occurred in a high proportion (71 per cent) of the infants who died, but only in 5 per cent of those who survived. It seems therefore reasonable to apply the PHA skin test as the only immunological test at places where it is not possible to carry out detailed immunological investigations. It is not expensive, it is easy to carry out and can be read in 24 hours thereby enabling quick decisions. Other simple measurements, for instance, serum albumin, the triceps skin-fold, and arm circumference could be used along with the PHA skin test in order to develop a new predictive model.

In conclusion, marasmic infants with diarrhoea and/or parenteral infections presented a variable degree of immunodepression, more intense in those who subsequently died. Due to its simplicity the delayed-hypersensitivity PHA skin test alone could possibly identify most of the infants with a high risk of mortality.

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