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Humoral and Cellular Aspects of Intracellular Bacterial Killing in Guatemalan Children with Protein-Calorie Malnutrition

*Gerald T. Keusch, Juan Jose Urrutia, Raul Fernandez, Oscar Guerrero, and Gustavo Castañeda

**Mt. Sinai School of Medicine, New York, New York 10035; and Institute of Nutrition of Central America and Panama, and Roosevelt Hospital, Guatemala City, Guatemala*

Children with protein-calorie malnutrition (PCM) experience infectious diseases so frequently that infection almost becomes the norm, except for brief interludes between episodes (1). The physiological basis for this is not known, although there is reason to suspect that host defense mechanisms may be adversely affected by PCM (2,3). Previous studies from India suggest a major defect in polymorphonuclear (PMN) leukocyte function in malnourished children (4). However, there is little comparative data available from other parts of the world to determine if this is a universal accompaniment of PCM.

This study was undertaken to determine PMN metabolism and bactericidal activity and to assay serum opsonins in Guatemalan children with PCM. Unfractionated whole blood was used for two reasons: (a) to avoid possible artifactual damage to PCM cells during fractionation procedures and (b) to evaluate study methods using whole blood for prospective field studies.

PATIENTS AND METHODS

Children on the pediatric wards of Roosevelt Hospital in Guatemala City were studied. They were classified according to weight for age in comparison to the 50th percentile of INCAP normal standards into a hospital control group (>80% of standard), two malnourished groups (60-80% of standard), and a group (<60% of standard) with more prominent marasmus (Table 1). No child was receiving antibiotics at the time of study. As a control group, eight nonhospitalized children free of clinical illness at the time and living in a village in the Guatemalan highlands where significant malnutrition and growth retardation are prevalent (mean weight/age = 70%) were studied.

Hematological parameters were determined by standard clinical methods.

TABLE 1.

Population	Age (years)	n	Diagnoses
Village control	3.6 ± 0.13 (3.1–3.9)	8	No clinical disease
Hospital control	4.4 ± 1.37 (2.3–11.0)	6	Pneumonia, cervical adenitis, piperazine intoxication, paresis, fever unknown origin, amebic liver abscess
60–80% Wt/age	4.0 ± 1.60 (1.3–11.7)	7	Diarrhea (6) Diarrhea plus ? measles
<60% Wt/age	3.0 ± 0.68 (0.9–5.0)	6	Diarrhea (3), URI (2) Diarrhea plus bronchopneumonia

Mean ± SEM. Range, in parentheses.

Activation of the hexose monophosphate shunt during phagocytosis was studied by the radiometric method of Keusch and Douglas (5). Heparinized whole blood (1 ml) was incubated with 0.25 μCi of 1-^{14}C-glucose and 10^9 latex spherules, 0.81 μm diameter (Bacto-latex) at 37°C. After 60 min incubation with shaking in sealed serum vials, $^{14}CO_2$ release into the air phase was measured without further processing with a radiometric β-radiation detector (Bactec R301, Johnson Laboratories).

Intracellular bactericidal activity of PMNs in whole blood was studied by the method of Keusch et al. (6). *Staphylococcus aureus* (strain 876663) and *Escherichia coli* (strain 286), both resistant to the complement dependent serum bactericidal system, were grown overnight in trypticase soy broth. The organisms were washed three times in phosphate-buffered saline (PBS), pH 7.4, and resuspended in PBS to achieve $5 \times 10^5 - 10^6$ colony-forming units per millimeter. Heparinized whole blood was placed in a sterile polypropylene plastic tube, 13 × 75 mm, and inoculated with 0.1 ml of the bacterial suspension. After thorough mixing, a 0.1 ml portion was removed to 9.9 ml of distilled water to lyse all cells and release intracellular bacteria. Viable bacterial counts were determined by standard pour plate methods. The remainder of the sample was incubated with end-over-end tumbling at 37°C. Additional viable counts on lysed aliquots were obtained at 1, 2, and 3 hours.

Serum was obtained from blood samples allowed to clot at 4°C for 3 hours. Separated serum was immediately frozen at −70°C until thawed for assay of opsonic activity. No serum was allowed to thaw between initial freezing and assay. Leukocytes were obtained from normal adult donors by dextran sedimentation of heparinized blood, washed twice in Hanks balanced salt solution (HBSS) containing 10 IU/ml of heparin, and resuspended to 10×10^6 phagocytic cells (polymorphonuclear leukocytes plus monocytes) per milliliter. The leukocyte suspension (0.5 ml) was added to 0.4 ml of serial dilu-

tions of test or control serum in HBSS to achieve a final concentration of 0.63, 1.25, 2.5, 5, and 10 percent serum (vol/vol). The study was initiated by addition of 0.1-ml washed diluted *S. aureus* 876663 or *E. coli* 286 (approximately 5×10^7/ml) to achieve a 1:1 bacteria:phagocyte ratio. After mixing, a zero time sample was removed, hypotonically lysed in distilled water and enumerated by the serial dilution-pour plate technique. The cell-serum-bacteria mixtures were tumbled end-over-end for 2 hours when viable bacterial counts were again determined. Controls incorporated in all experiments included a cell control (no serum) and a serum control (no cells).

RESULTS

Hematology values for the study population are shown in Table 2. Both malnourished groups were anemic compared to the village and hospital con-

TABLE 2. *Hematology values for study population*

Population	n	Hemoglobin	Hematocrit	Total WBC	% Phagocytes[a]
Village control	8	—	37.7 ± 0.50	13503 ± 1586.0	33.0 ± 4.6
Hospital control	6	12.7 ± 0.35	37.3 ± 1.56	7758 ± 753.7[b]	49.2 ± 6.7[c]
60–80% Wt/age	7	7.9 ± 0.73[d]	29.9 ± 1.00[d]	8721 ± 146.5[b]	39.6 ± 4.2
<60% Wt/age	6	8.5 ± 0.68[d]	26.5 ± 1.80[d]	7125 ± 788.3[d]	40.5 ± 5.3

Mean ± SEM.
[a] Polymorphonuclear leukocytes plus monocytes.
[b] Significantly different from village control at $p < 0.01$.
[c] Significantly different from village control at $p < 0.05$.
[d] Significantly different from village control at $p < 0.005$.

trol populations. Leukopenia and neutropenia were not present, and the total number of phagocytic cells per milliliter of blood was similar in all groups.

Metabolic conversion of 1-^{14}C-glucose to $^{14}CO_2$ during phagocytosis is shown in Table 3. Both resting and phagocytizing values were diminished to a similar extent in all three hospitalized groups compared to the village children.

TABLE 3. $^{14}CO_2$ *Production from 1-*^{14}*C-Glucose (nCi/10*^{6} *phagocytes/hr)*

	n	Resting	Phagocytizing
Village control	7	0.23 ± 0.08	2.48 ± 0.21
Hospital control	6	0.10 ± 0.03	1.18 ± 0.24[a]
60–80% Wt/age	7	0.12 ± 0.06	1.52 ± 0.19[a]
<60% Wt/age	6	0.15 ± 0.08	0.81 ± 0.26[a]

Mean ± SEM.
[a] Significantly different from village control at $p < 0.005$.

TABLE 4. *Whole blood bactericidal assay versus S. aureus*

Population	n	Phagocyte/bacteria	Bacterial killing (%)[a]
Village control	8	22.0 ± 2.56	93.8 ± 1.34
Hospital control	6	35.5 ± 5.27[b]	84.6 ± 1.70[b]
60–80% Wt/age	7	29.4 ± 6.30	80.1 ± 3.29[c]
<60% Wt/age	6	27.7 ± 6.23	91.7 ± 2.84

Mean ± SEM.
[a] Percent of inoculum killed after 1 hour of incubation.
[b] Significantly different from village control at $p < 0.05$.
[c] Significantly different from village control at $p < 0.01$.

TABLE 5. *Whole blood bactericidal assay versus E. coli*

Population	n	Phagocyte/bacteria	Bacterial killing (%)[a]
Village control	8	91.0 ± 7.78	93.0 ± 2.10
Hospital control	6	86.2 ± 19.20	84.1 ± 4.20[b]
60–80% Wt/age	7	78.8 ± 14.45	81.9 ± 4.81[b]
<60% Wt/age	6	81.0 ± 15.86	84.5 ± 5.02[b]

Mean ± SEM.
[a] Percent of inoculum killed after 1 hour of incubation.
[b] Significantly different from village control at $p < 0.05$.

Bactericidal activity versus *S. aureus* and *E. coli* are shown in Tables 4 and 5. With the exception of a significant ($p < 0.05$) increase in the phagocyte: bacteria ratio in the hospital control group versus *S. aureus*, the cell:bacteria ratio was similar for all other groups. All three hospitalized groups showed significant impairment of bactericidal activity versus *E. coli* after 1 hour of incubation. Both the hospital control and 60–80% weight-for-age groups showed similar diminished killing of *S. aureus* at 1 hour. Greater than 98% of the initial inoculum were killed at 2 and 3 hours of incubation by all blood samples, and there were no significant differences between groups.

Serum opsonic activity is presented in Table 6. Because of the limited number of samples, data from the 60–80% and <60% weight-for-age groups

TABLE 6. *Ratio of viable counts at 2 hours*

	Malnourished/control serum	
Serum (%)	*S. aureus*	*E. coli*
10	1.10	1.27
5	1.59	3.44
2.5	1.93	3.21
1.25	1.53	2.80
0.63	1.48	2.04

are combined. There was no difference between sera from malnourished children and control when the concentration of serum in the assay was 10%. At serum concentrations below 10%, significantly diminished opsonic activity was observed in the malnourished group.

DISCUSSION

This study demonstrates significant abnormalities in leukocyte metabolism during phagocytosis of latex spherules, intracellular bactericidal activity versus *S. aureus* and *E. coli* and serum opsonic titers in children with malnutrition. Although defects in these three parameters of antibacterial host defense mechanisms can be associated with enhanced susceptibility to infection, it is not clear that this is true in the population studied. The defect in phagocytosis-associated hexose monophosphate shunt activity was quantitative and far different from the results obtained with chronic granulomatous disease (CGD) studied by the same technique (5). While both resting and phagocytizing values were reduced, $^{11}CO_2$ production significantly increased during phagocytosis (5.4- and 12.7-fold in the marasmic-kwashiorkor and kwashiorkor groups, respectively). Similar increment in CO_2 production was observed in the better nourished hospital controls as well, and we have previously seen clinically healthy heterozygote mothers of CGD children whose leukocytes do not stimulate more than four- to five-fold during phagocytosis of latex (7). The magnitude of the relative increase in $^{11}CO_2$ production by phagocytizing samples for the kwashiorkor group is similar to that observed by Douglas and Schopfer in kwashiorkor children in the Ivory Coast studied by the same technique (8).

The observed defect in bactericidal activity was also quantitative and observed only after 1 hour of incubation. Similar to the metabolic studies, hospital controls showed a defect of comparable magnitude to the malnourished children. Heterozygote mothers of CGD children may have a quantitatively greater defect than that observed here when studied by the same whole blood bactericidal method (6), again without clinical manifestations of excessive infections. Douglas and Schopfer (8) have reported a modest decrease in bactericidal activity of isolated kwashiorkor cells at 60 and 120 min of incubation, in contrast to this study in which normal bacterial killing was observed at 120 and 180 min. In both studies, however, kwashiorkor leukocytes were able to reduce bacterial counts in contrast to the CGD-like defect reported by Selvaraj and Bhat in India (4). It is possible that the differences might be related to cellular damage during isolation and washing procedures or to the increased number of bacterial colony-forming units per leukocyte in the assays utilizing isolated white blood cell preparations.

Although opsonic activity of serum from malnourished children was less than control at serum concentrations less than 10% (vol/vol) this cannot account for diminished bacterial killing in whole blood, where the serum

concentration obviously greatly exceeded 10%. Douglas and Schopfer found no serum opsonic defect in kwashiorkor children with an assay that used 8% serum (8). Seth and Chandra (9) were also unable to detect abnormalities in opsonic activity using plasma at a concentration of 10%. However, opsonization might become rate limiting in extravascular sites of infection where opsonic factors (particularly complement components) might be present in concentrations considerably lower than in serum. Diminished bactericidal activity of whole blood, however, must be in part due to a functional abnormality of the leukocytes themselves. This concept is supported by the metabolic abnormalities of the malnourished cells.

It is possible that the severe clinical problem of infection in malnourished individuals may be the result of a summation of small individual defects in multiple key cellular and humoral functions. The cellular abnormalities observed may not, however, be related to malnutrition *per se*, but instead actually be a consequence of infection or stress, since similar abnormalities were present in hospital controls. Solberg and Hellum (10) have previously reported that infection-related granulocytic dysfunction may adversely influence the outcome of the infectious process. No data is provided regarding nutritional status in these patients, hence coexistent malnutrition cannot be excluded in their patients. Prospective field studies may be required to establish whether malnutrition in fact results in leukocyte dysfunction, which in turn predisposes to infection.

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