Flow Cytometry Study of Lymphocyte Subsets in Malnourished and Well-Nourished Children with Bacterial Infections

Oralia Nájera,1* Cristina González,2 Guadalupe Toledo,3 Laura López,4 and Rocío Ortíz2

Departamento de Atención a la Salud, Universidad Autónoma Metropolitana—Xochimilco,1 and Hospital Materno-Pediátrico,3 Xochimilco, and Departamento de Ciencias de la Salud, Universidad Autónoma Metropolitana—Iztapalapa,1 and Secretaría de Salud del Gobierno del Distrito Federal,4 Iztapalapa, México

Received 19 November 2003/Returned for modification 6 January 2004/Accepted 9 March 2004

Protein-energy malnutrition is the primary cause of immune deficiency in children across the world. It has been related to changes in peripheral T-lymphocyte subsets. The aim of the present study was to evaluate the effects of infection and malnutrition on the proportion of peripheral-lymphocyte subsets in well-nourished non-bacterium-infected (WN), well-nourished bacterium-infected (WNI), and malnourished bacterium-infected (MNI) children by flow cytometry. A prospectively monitored cohort of 15 MNI, 12 WNI, and 17 WN children was studied. All the children were 3 years old or younger and had only bacterial infections. Results showed a significant decrease in the proportion of T CD3⁺ (P < 0.05 for relative and P < 0.05 for absolute values), CD4⁺ (P < 0.01 for relative and absolute values), and CD8⁺ (P < 0.05 for relative values) lymphocyte subsets in WNI children compared to the results seen with WN children. Additionally, B lymphocytes in MNI children showed significant lower values (CD20⁺ P < 0.02 for relative and P < 0.05 for absolute values) in relation to the results seen with WNI children. These results suggest that the decreased proportions of T-lymphocyte subsets observed in WNI children were associated with infection diseases and that the incapacity to increase the proportion of B lymphocyte was associated with malnutrition. This low proportion of B lymphocytes may be associated with the mechanisms involved in the immunodeficiency of malnourished children.

MATERIALS AND METHODS

Subjects. Three groups of children were studied.

(i) Group I. WN children. This group consisted of 17 children (11 boys and 6 girls). Their ages ranged from 8 to 29 months. All the children had normal weight and height according to the established values for Mexican children without infectious diseases and associated immunological or hematological disorders (25). The children were outpatients at the hospitals managed by the city government (Xochimilco and Iztapalapa Maternal-Pediatric DDF Hospitals).

(ii) Group II. WNI children. This group consisted of nine boys and three girls aged from 8 to 24 months. All were suffering from severe bacterial infection: four children showed respiratory infection, three showed gastrointestinal infections, and five showed mixed gastrointestinal and respiratory infections. The children had adequate weight and height according to their ages. All the children were hospitalized.

(iii) Group III. MNI children. This group consisted of eight boys and seven girls aged from 8 to 29 months. All presented with malnutrition: 5 presented with second-degree malnutrition (weight/height ratio deficit > 25%), 10 presented with third-degree malnutrition, 3 presented with kwashiorkor, and 7 presented with marasmus (weight/height ratio deficit > 40%). The clinical signs and symptoms of malnutrition, as well as weight and height deficits, were used to determine the severity of malnutrition according to the established values for Mexican children (25). They had been admitted to the hospital because six children had gastrointestinal infections, four had respiratory infections, and five had both gastrointestinal and respiratory infections.

Bacterial infections in WNI and MNI children were diagnosed rigorously on the basis of clinical data and laboratory routine tests. The children that were referred with clinical suspicion of tuberculosis, viral infection, allergic diseases, or cardiac diseases were excluded from the study. The study was approved by the
Medical Ethics Committee of the General Direction of Medical Services of the Mexico City Government (DDF).

**Cell preparation and staining.** Whole-blood samples were collected in blood collection tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, N.J.) with sodium heparin anticoagulant and were processed on the day of collection. Cell viability was determined with double fluorescein diacetate and ethidium bromide staining (31). More than 95% of the cells were viable. Lymphocyte subsets were determined using the lysing whole-blood method (14) with minor modifications. Commercially conjugated antibodies to fluorescein isothiocyanate (FITC), phycoerythrin (PE), and peridinin chlorophyll protein (PerCP) dyes (immunocytometry system, Becton Dickinson, San Jose, Calif.) were used, including (i) isotype control; (ii) CD45 FITC-CD14 PE; (iii) CD3 FITC-CD16 plus CD56 PE-CD20 PerCP, (iv) CD4 FITC-CD8 PE, and (v) CD3 FITC CD3 HLA-DR PE. For this purpose a sample of 100 µl of whole blood was incubated for 20 min at room temperature in the dark with 20 µl of the combined antibodies. After incubation, the cells were washed followed by lysis of erythrocytes with 2 µl of fluorescence-activated cell sorter lysing solution (Becton Dickinson); after a second washing, the cells were fixed in 1% paraformaldehyde.

**Flow cytometry analysis.** All analyses were performed with a FACScan flow cytometer (Becton Dickinson immunochemistry system) calibrated with Calibrite brand beads and AutoCOMP software. For the standard technique a minimum of 10,000 total events were acquired, with LYSIS II software and LeucoGATE brand reagent (CD45/CD14) used to establish an analysis gate that included at least 95% of lymphocytes and no more than 5% of monocytes in the sample. Markers for determining positive and negative cell results were set by LYSYS II software with conjugated antibodies of irrelevant specificity as negative controls. The lymphocyte gate was determined manually on the basis of forward- and side-scatter characteristics. Data were displayed as two-color dot plots (FL1 versus FL2). All samples were processed within 24 h after staining. The absolute numbers of cells (10⁶/liter) for each lymphocyte subset were calculated by multiplying the corrected relative value by the total lymphocyte count.

**Statistical analysis.** Results are expressed as means ± standard errors and were compared using analysis of variance, nonparametric Wilcoxon test, or t test, as appropriate. Statistical significance was considered to be P < 0.05.

### RESULTS

**Clinical characteristics at hospital admission.** Children with respiratory infection showed fever, coughing, and respiratory distress. Gastrointestinal infection was associated with diarrhea, fever, and several degrees of dehydration. In WN children, the mean hemoglobin concentration was 12.0 ± 2 g/liter; only one patient had anemia (hemoglobin < 10.0 g/liter). In WNI children, the mean hemoglobin concentration was 12.0 ± 0.9 g/liter, with only three cases of anemia. In MNI children, anemia was more severe; the mean hemoglobin concentration for those children was 8.0 ± 1.3 g/liter, and only two children presented a normal hemoglobin concentration.

Total leukocyte counts were as expected for the age group, with an average of 9.0 × 10⁶ ± 1.9 ± 10⁶ cells/liter for WN children, 9.0 × 10⁶ ± 1.1 × 10⁶ cells/liter for WNI children, and 10.0 × 10⁶ ± 2.2 ± 10⁶ cell/liter for MNI children. Percentages of lymphocytes and neutrophils for WN children were 60.0% ± 12.6% and 40.0% ± 11.8%, respectively, and for WNI children were 52.0% ± 10.0% and 45.0% ± 5.0%, respectively; and finally, percentages observed for MNI children were 45.0% ± 13.2% lymphocytes and 59.0% ± 12.1% neutrophils.

**Leukocyte analysis.** The distribution of the relative and absolute values of lymphocytes, granulocytes, and monocytes found in the study groups is shown in Table 1. Lower percentages of total lymphocytes in WNI and MNI children (47.8% ± 4.3 and 47.1% ± 3.7%, respectively) compared to the results seen with WN children (57.3% ± 3.5%) were observed. Statistical analysis showed significant differences between only MNI and WN children (P < 0.04 for relative and P < 0.03 for absolute values). A higher level of granulocytes was found in MNI children (43.5% ± 3.6%) in relation to the results seen with WN children (32.3% ± 3.2%; P < 0.02 for relative and P < 0.02 for absolute values). When monocyte levels were compared, statistical differences were not found (for WN children, 5.0% ± 0.6% for MNI children, 5.9% ± 0.6%; and for WN children, 4.4% ± 0.5%).

The monocyte count in MNI children with respiratory infection (n = 4) was 3.7% ± 1.1%, while in children with mixed infections (n = 5) the count was 8.4% ± 1.0% (P < 0.05; data not shown). The analysis of results for WNI and MNI children showed no differences with respect to either infection type or malnutrition degree (data not shown).

**Lymphocyte subsets.** Significant differences in lymphocyte subsets were observed between WNI and WN children (Table 2). WNI children presented lower mean percentages and lower absolute values than WN children with respect to levels of lymphocyte CD3⁺ (51.6% ± 2.9% versus 61.8% ± 2.4%; P < 0.05 for relative and P < 0.03 for absolute values), CD4⁺,
(28.3% ± 2.2% versus 36.4% ± 1.8%; \( P < 0.01 \) for relative and \( P < 0.01 \) for absolute values), CD8\(^+\) (24.0% ± 1.6% versus 30.2% ± 1.5%; \( P < 0.03 \)), and T HLA-DR\(^+\) (6.0% ± 1.9% versus 11.4% ± 1.3%; \( P < 0.05 \)). For B lymphocytes (CD20\(^+\)) the tendency was different; WNI children showed higher percentages (31.9% ± 2.3%) than WN children (23.8% ± 1.9%; \( P < 0.03 \)). The results for MNI and WN children with respect to lymphocyte subsets showed significant differences only for CD4\(^+\) subsets (30.3% ± 2.0%; \( P < 0.03 \) for relative and \( P < 0.01 \) for absolute values).

An important aim of this study was to compare T-lymphocyte subsets for WNI and MNI children. However, the statistical analysis of T-lymphocyte subsets for the two groups of children did not result in significant differences. In contrast, the proportion of B lymphocytes (CD20\(^+\)) in MNI children was lower than that seen in WN children (22.8% ± 2.1% versus 31.9% ± 2.3%, respectively; \( P < 0.02 \) for relative and \( P < 0.05 \) for absolute values).

The proportions of NK lymphocytes and the CD4/CD8 ratio were similar among the three study groups. Nonstatistical differences were observed.

The results with respect to infection type did not show statistical differences between WNI and MNI children (data not shown) with respect to T-lymphocyte subpopulations. Higher values of CD20\(^+\) cells were related to gastrointestinal infection in WNI children (\( n = 3 \)) versus the results seen with MNI children (\( n = 6 \)), but the differences were not significant (36.8% ± 4.8% versus 27.1% ± 3.4%). Respiratory infection (WNI, \( n = 4 \); MNI, \( n = 4 \)) had the same trend with respect to CD20\(^+\) cell results, without significant differences (29.8% ± 7.2% versus 15.1% ± 7.1%).

The lymphocyte subsets for the group of children suffering from malnutrition showed higher levels of CD3\(^+\) cells in cases of marasmus (65.8% ± 3.9%, \( n = 7 \)) in relation to the results seen with children with second-degree cases of malnutrition (48.6% ± 4.6% \( P < 0.04 \), \( n = 5 \); data not shown).

**DISCUSSION**

Malnourished children are more susceptible to infections than well-nourished children; in consequence, they have been considered immunodeficient. In the present study, lymphocyte subpopulation changes were contrasted between WNI and MNI children. In addition, we studied a group of WN children as a control group. Changes observed in lymphocyte subsets might reflect two facts. First, the comparison of the results seen with WN and WNI children reveals several changes that may be associated with the infection process. Second, differences observed between WNI and MNI children show changes related to nutritional condition.

Data revealed that the mean percentages of CD3\(^+\), CD4\(^+\), and CD8\(^+\) cells decreased in WNI children in relation to the results seen with WN children. When the same lymphocyte subsets were evaluated in MNI and WN children, only a lower percentage of CD4\(^+\) lymphocytes was observed. Previous studies have revealed a reduction of peripheral T cell levels in MNI children in contrast to the results seen with WN children, with a specific decrease in CD4\(^+\) cell and an increase of CD8\(^+\) cell levels (9, 13). Similar results were obtained in a former study of Mexican children; MNI children presented a lower proportion of CD11\(^+\) and CD4\(^+\) T cells compared to WN children (3).

The authors found a higher percentage of CD8\(^+\) lymphocytes in two children. Another study conducted with MNI and WN children showed a higher proportion of immature T lymphocytes (CD1a) and decreases in both the (mature) T CD3\(^+\) cell level and the CD4/CD8 ratio; additionally, the authors reported a slight increase in the proportion of T CD8\(^+\) cells (21).

In the present study, we found significant differences between WN and MNI children only in the proportion of T CD4\(^+\) lymphocytes; the comparison between WNI and MNI children with respect to T-lymphocyte levels showed nonsignificant differences. In contrast, we found a clear association between infection in WNI children and a decrease in T CD3\(^+\) lymphocyte subset levels.

Changes in lymphocyte subset levels related to infectious diseases have previously been reported. Patients with amebiasis showed an increase in CD8\(^+\) lymphocyte levels and a decrease in total T-lymphocyte levels (20); analogous results were reported for children suffering prolonged diarrhea (16, 33). Another study showed a decrease in CD4\(^+\) lymphocyte levels in patients with fungal infections (5). An opposite trend (consisting of an increase in CD4\(^+\) lymphocyte levels) was reported for patients with meningitis infection, however, whereas *Staphylococcus* infection caused a reduction in the proportion of CD11\(^+\) and CD8\(^+\) lymphocytes (2). In a study of patients with syphilis, a low proportion of CD4\(^+\) cells and a higher proportion of CD8\(^+\) cells in contrast to means observed for the uninfected population were found (23). In the present study, it was not possible to identify the causal infection agent in all patients; however, other reports have established that the most common agents in Mexican children suffering enteropathogenic diseases are *Shigella* spp., *Salmonella* spp., (enteropathogenic) *Escherichia coli*, and *Campylobacter* spp. (19, 22). For respiratory infections the more-common agents are *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Staphylococcus aureus*, and *Moraxella (Brahmamella) catarrhalis* (1, 15).

An important increase in the proportion of (CD20\(^+\)) B lymphocytes was evident in WNI children compared to the results seen with WN children. In contrast, MNI children did not show an increase in the percentage of CD20\(^+\) cells; the values observed were similar to those obtained for WN children, and in relation to WNI children a significant difference was observed. Several researchers have reported no differences between the percentages of B lymphocytes in malnourished versus well-nourished children (4, 7, 24, 30). Results of the present study support these findings. When the proportions of B cells were analyzed in children with severe malnutrition and well-nourished children, however, results showed a significant decrease in cell levels in malnourished children (28). MNI children studied in this work showed significantly lower percentages of B lymphocytes than WNI children. Probably as an effector response to infection, the WNI children were capable of increasing the proportion of B lymphocytes (CD20\(^+\)) in peripheral blood. While the MNI children showed a failure to increase the proportion of B lymphocytes in peripheral blood, this fact could be related to alterations in the immunological humoral response in these children. In this sense, some studies of malnourished children have indicated a decrease of immunoglobulin A secretion in the nasal-pharynx and gastrointestinal secretions (6, 26).
In a previous study Nájera et al. evaluated lymphocyte activation and reported that MNI children did not present changes in the proportion of T-lymphocyte subsets; results also showed a decrease in TCD3⁺ lymphocyte levels in MNI and WNI children which was associated with the infection. In WNI children, an important increase in levels of B lymphocytes in peripheral blood was observed. This phenomenon was not observed in MNI children, who showed a decrease of B lymphocyte levels in relation to WNI children (18). These findings agree with the results of the present study.

Results obtained in the present study may suggest migration of the lymphocyte subsets when infection is present in the appropriate microenvironment; for example, CD3⁺ and CD4⁺ (helper) lymphocytes were susceptible to decrease in peripheral blood. Probably CD4⁺ lymphocytes are retained in lymphoid tissues where mechanisms of specific immunity are carried out in the phase of recognition and activation (17); the proportion of B lymphocytes (antibody producers that are responsible for one of the effector phases) (11) showed a clear increase in the peripheral blood of WNI children. The specific immunity process is much more complex, nevertheless, since a high number of cytokines produced by lymphocytes are involved. Additionally, antigen characteristics can trigger different immunological mechanisms (macrophages or cytotoxic cell action and synthesis of specific antibodies, for example). The evaluation of these immunity-specific mechanisms in malnourished children appears to be necessary; this may show a complete vision of the immune-response capacities in these children.

In summary, the data obtained in the present study showed a significant decrease in the proportion of T CD3⁺, CD4⁺, and CD8⁺ cells in WNI children and a simultaneous increase in the proportion of B lymphocytes (CD20⁺); both processes appear to be related to infection. In MNI children, the T CD4⁺ lymphocyte subset showed the same trend observed in WNI children. However, B lymphocyte (CD20⁺) levels did not present the expected increase. These data indicate an incapacity of MNI children to increase the proportion of B lymphocytes. This fact may be related to a diminished synthesis of several molecules involved in the immunological response, and this may occur as a consequence of a nutritional shortage in malnourished children. Therefore, specific studies related to humoral response in children suffering protein-energy malnutrition must be designed.

ACKNOWLEDGMENTS

We thank Edith Cortés and Leticia Cortés for their skillful technical assistance.

This work was supported by grants from The Autonomous Metropolitan University (Mexico) and the CONACYT and FOMES-SEP program.

REFERENCES