Peripheral Blood Lymphocyte Subsets in Infants with Diarrhea with and without *Giardia Lamblia* Infection

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ABSTRACT. The aim of the present study was to define the cellular immune response during gastrointestinal *Giardia lamblia* infection in young children. The level of lymphocyte subsets was determined in the peripheral blood of infants with *G. lamblia*-associated diarrhea or acute gastroenteritis and from control infants without diarrhea. The proportion of peripheral blood lymphocytes (PBL) expressing the CD8 marker (suppressor cytotoxic T cells) and the CD57 marker (natural killer cells and subset of CD8+ T cells) was highest in infants with acute gastroenteritis, lower in infants without diarrhea, and lowest among those with *G. lamblia*-associated diarrhea. The level of CD4+ PBL (helper T cells) did not differ significantly among the three groups of children tested. The level of memory, or helper-inducer, CD4+CD29+ PBL was increased markedly in acute gastroenteritis as compared with their level among the other two groups, whereas naive or virgin CD4+CD45RA+ PBL had the reciprocal distribution among the three groups of infants. In contrast to acute gastroenteritis from other causes, *G. lamblia*-associated diarrhea did not elicit changes in lymphocyte subsets. (*Pediatr Res* 33: 15–18, 1993)

Abbreviations

NK, natural killer
PBL, peripheral blood lymphocyte
PE, phycoerythrin

however, yielded inconclusive results. Whereas in some studies of acute giardiasis an anti-*Giardia* IgM response was noted (5, 6), in others, an increase in specific IgA but not IgM or IgG antibodies was observed (7). Diminished cellular cytotoxicity for *G. lamblia* has been observed in a patient with symptomatic giardiasis resistant to drug therapy (8). Other studies have failed to detect significant differences in the proportion of B and T lymphocytes in patients with acute giardiasis, persistent giardiasis, asymptomatic carriers, and controls (5).

Distinct lymphocyte subsets can be identified by their reactivity with MAb (9, 10). Helper T lymphocytes can be detected by their reactivity with CD4 MAb, whereas suppressor cytotoxic T lymphocytes can be identified by their reactivity with MAb against CD8. The CD57 marker (Leu7) is characteristic for human NK cells (11) and for a subset of suppressor-cytotoxic T cells (12). It has recently been shown that CD4+ T cells can be further subdivided into two subsets—that of the CD29+CD4+ “memory” or “primed” subset and that of the CD45RA+CD4+ subset of “naive” cells (13, 14). The level of memory CD4+ T cells is low among infants and young children and increases with age (15).

In the present study, MAb were used to define the cellular immune response during *G. lamblia*-associated diarrhea in infants. The levels of various subsets of lymphocytes were determined in the peripheral blood of infants suffering from acute diarrhea associated with *G. lamblia* infection. In parallel, PBL from infants with acute gastroenteritis and control infants without diarrhea were tested.

MATERIALS AND METHODS

Subjects studied. The children included in the study, all below the age of 3 y, were seen at a pediatric outpatient clinic in a rural area during a 12-mo period. Each child was weighed unclothed and measured during the visit at the clinic. The weight and height of the children were expressed as the percentage of the 50th percentile for weight or height for age.

During the study period, 421 children with acute diarrhea were examined in the clinic. Acute diarrhea was defined as the passage of two or more abnormally formed stools per 24 h for a period of less than 14 d. The stools of all infants who presented with acute diarrhea were examined for trophozoites or cysts of *G. lamblia*. Wet mounts of each stool sample were prepared in 0.9% NaCl and examined by an experienced parasitology technician by light microscopy for 10 min. As stool samples were examined within 2 to 5 h of being obtained, preservatives were not used.

In 19 children (4.5%) *G. lamblia* was found in the stools. Blood specimens were available for testing from eight of these children. Twelve children with diarrhea but without *G. lamblia*

Giardia lamblia is an intestinal parasite implicated as a cause of disease ranging from acute gastroenteritis to chronic diarrhea, malabsorption, and failure to thrive (1). Still, most individuals infected with *Giardia* are apparently asymptomatic. Children with asymptomatic *Giardia* infection show no disadvantage in terms of growth achievement and may even tend to suffer less from gastrointestinal and respiratory illnesses compared with uninfected children (2). The marked susceptibility of patients with immune deficiency states to chronic and recurrent giardiasis suggests that immune mechanisms play a predominant role in determining the pathogenicity of *G. lamblia* (3, 4). Studies on specific anti-*Giardia* Ig levels during *Giardia* infection have,

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were also included for comparison. The stools of all infants with diarrhea were examined for parasites other than *G. lamblia*, including cryptosporidium, and for bacterial pathogens (pathogenic *Escherichia coli*, *Shigella*, *Salmonella*, and *Campylobacter*). The stools were also assayed by ELISA for the presence of rotavirus using a commercially available diagnostic kit (Eldan Technologies Co., Jerusalem, Israel).

A volume of 0.5 to 1.0 mL of blood was drawn and assayed for Hb, hematocrit, white blood cell count, differential, and fluorimetric lymphocyte subpopulation studies. The total lymphocyte number per mm³ was calculated by multiplying the total white blood cell number per mm³ by the proportion of lymphocytes.

This study was approved by the medical ethical committee of the Hadassah University Hospital. Informed consent was obtained from the parents of all children studied.

Detailed analysis was carried out with PBL obtained from the following groups of age-matched infants (Table 1): 1) Eight infants with acute diarrhea and *G. lamblia* in their stools; 2) twelve infants (who were not infected with *G. lamblia*) with acute diarrhea; and 3) nine infants, some of whom suffered from mild upper respiratory infections without diarrhea.

Immunofluorescent assays. Venous blood from children was collected in heparin and analyzed by the whole blood method, in which fluorescent MAb were added to the unseparated blood and the immunofluorescence of the lymphocytes was determined after lysis of the red blood cells (16).

The conjugated MAb used were as follows: FITC-conjugated Leu-3 (CD4), PE-conjugated Leu-2 (CD8), and FITC-conjugated Leu-7 (CD57) (all from Becton-Dickinson, Mountain View, CA). PE-labeled 4B4 (CD29) and PE-labeled 2H4 (CD45RA) were obtained from Coulter Immunology, Hialeah, FL.

Fluorescent MAb were added to 100 μ L of whole blood. The following pairs of MAb were used for two-color flow cytometry: 1) PE-Leu-2:FITC-Leu-3; 2) PE-Leu-2:FITC-Leu-7; 3) PE-4B4:FITC-Leu-3; and 4) PE-2H4:FITC-Leu-3.

To 100 μ L heparinized blood, 5–10 μ L of each of the two fluorescent MAb were added. The mixture of blood and antibodies was kept at room temperature for 30 min, and then the erythrocytes were lysed with 2 mL of a buffered solution of 0.829% NH₄Cl, as described by Hoffman *et al.* (16). After two incubations of the blood with NH₄Cl for 10 min at room temperature, the suspension was centrifuged at 200 $\times$ g for 5 min, and the pellet was washed twice with PBS. Finally, the cells in the washed pellet were resuspended in 0.1% paraformaldehyde and kept at 4°C until they were analyzed by flow cytometry. A FACScan flow cytometer (Becton-Dickinson) was used. The acquisition and analysis of the fluorimetric data were carried out using the Consort 30 program. For each test, at least 10 000 cells were acquired. The total lymphocyte population was gated by the usual procedures according to their typical forward light scatter and right-angle light scatter (17). The immunofluorescent staining was determined by two-color flow cytometry using the contour graph program of Consort 30.

Statistical analysis. Data were analyzed by *t* test.

RESULTS

Clinical observations. Of the 12 children in the present study with *G. lamblia*-negative acute diarrhea, pathogenic *E. coli* (0127, 0142, 0119, 0126) was cultured in four children and rotavirus was found in two. Among the eight children with *Giardia* infection one was concomitantly infected with *E. coli* 0119, and in another child, the dwarf tape worm *Hymenolepis nana* was detected. In the control group without diarrhea, one child was found to have *H. nana* in the stool, and no other pathogens were detected.

The weight and height of the children in the present study were expressed as a percentage of the 50th percentile for age. The weight of children with non-*Giardia* diarrhea was significantly lower than that of children with *Giardia*-positive diarrhea ($p < 0.05$) and nonsignificantly lower than that of children without diarrhea (Table 1). The height of children with diarrhea was not significantly different from that of children in the other groups.

The total lymphocyte number per mm³ was lowest in the group with acute diarrhea, highest in the group without diarrhea, and intermediate in the group of infants with *Giardia*-associated diarrhea (Table 1).

Lymphocyte subsets. Table 2 shows differences among the three groups of infants in the proportion of CD4+ (helper T cells), CD8+ (suppressor/cytotoxic T cells), and CD57+ (NK cells and a subset of CD8 cells). The proportion of CD4+ lymphocytes (Table 2) and their absolute number (not shown) were higher among infants with *G. lamblia* infection than among control infants without diarrhea and lowest among infants with non-*Giardia* diarrhea. However, none of these differences was statistically significant.

The population of CD8+ cells in each group showed a pattern opposite to that of the distribution of CD4+ cells. The proportion of CD8+ cells was highest among infants with non-*Giardia* diarrhea, lower in control infants without diarrhea, and lowest among infants with *G. lamblia* infection. The proportion of CD8+ T cells was significantly higher among infants with non-*Giardia* diarrhea than among infants with *G. lamblia* infection ($p < 0.05$). The children with non-*Giardia*-associated diarrhea showed a decrease in CD4+ helper T cells with a concomitant increase in CD8+ suppressor-cytotoxic cells. Thus, the mean CD4:CD8 ratio was lower in this group of infants than in both the *Giardia*-associated diarrhea ($p < 0.05$) and the control group. It is noteworthy that in infants with *G. lamblia*-associated diarrhea, the CD4:CD8 ratio was in fact in the normal range. Similar to the population of CD8+ cells, the proportion of CD57+ (Leu-7+) cells was highest among infants with acute gastroenteritis and lowest among infants with *G. lamblia*-associated diarrhea, although these differences were not statistically significant (Table 2).

Subsets of CD4+ lymphocytes. The most striking observation in the present study was the finding of a substantial difference in the level of subsets of CD4+ lymphocytes among the three groups of children studied. The proportion of memory-helper T cells, CD4+CD29+ double-positive lymphocytes, and their percentage

Table 1. Clinical data concerning infants studied*

Clinical parameter	Infants with non- <i>Giardia</i> diarrhea	Infants without diarrhea	Infants with <i>Giardia</i> -associated diarrhea
No. of infants	12	8	8
Age (mo)	13.5 $\pm$ 6.9	14.5 $\pm$ 5.1	16.4 $\pm$ 6.2
Weight (% of 50th percentile)	90.7 $\pm$ 9.7	99.4 $\pm$ 11.0	100.5 $\pm$ 7.6†
Height (% of 50th percentile)	97.7 $\pm$ 3.5	100.3 $\pm$ 2.0	100.3 $\pm$ 2.3
Duration of diarrhea (d)	4.6 $\pm$ 3.8		5.5 $\pm$ 4.5
No. of lymphocytes per mm ³	2982 $\pm$ 818	4870 $\pm$ 2679	3465 $\pm$ 1442

* Values are mean $\pm$ SD.

† Weight was significantly higher in infants with *Giardia*-associated diarrhea than in infants with non-*Giardia* diarrhea ($p < 0.05$).

Table 2. *T lymphocytes and NK cells among various groups of infants**

Lymphocyte subset	Infants with non- <i>Giardia</i> diarrhea	Infants without diarrhea	Infants with <i>Giardia</i> -associated diarrhea
CD4+	36.05 ± 6.82 (12)	40.39 ± 7.77 (9)	41.55 ± 7.92 (8)
CD8+	29.92 ± 7.36 (11)	23.58 ± 9.33 (9)	21.66 ± 5.43 (8)†
CD4/CD8 ratio	1.33 ± 0.43 (11)	1.56 ± 0.45 (9)	1.98 ± 0.42 (8)†
CD57+ (Leu-7+)	16.05 ± 9.92 (11)	10.11 ± 9.15 (9)	8.81 ± 5.88 (8)

* Values are mean ± SD. Numbers in parentheses are the number of infants tested.

† The proportion of CD8+ PBL and the CD4:CD8 ratio in infants with *Giardia*-associated diarrhea were significantly different from those in infants with non-*Giardia* diarrhea ($p < 0.05$).

of the total population of helper T cells (CD4+ cells) was 1½- to 2-fold higher among infants with non-*Giardia* diarrhea as compared with their proportion among either infants without diarrhea or infants with *G. lamblia* infection (Table 3). Although the total number of CD4+ lymphocytes was lowest in infants with acute non-*Giardia* diarrhea, the total number of cells of the CD29+CD4+ subset (615 ± 61 cells per mm³) was found to be significantly higher than that among infants with *Giardia*-associated diarrhea (332 ± 54 cells per mm³) ($p < 0.02$). The absolute number of CD29+CD4+ lymphocytes was intermediate in the group of infants without diarrhea (476 ± 126 cells/mm³).

The distribution of naive helper T cells coexpressing the CD45RA and CD4 markers, and their proportion of the total CD4+ population, showed an inverse relationship to that of the memory helper T lymphocyte subset among the various groups of infants. The level of naive helper T cells was significantly lower among infants with non-*Giardia* diarrhea than among infants without diarrhea ($p < 0.05$) or infants with *G. lamblia* infection ($p < 0.05$). Thus, in infants with non-*Giardia*-associated diarrhea, the decrease in helper T cells is due to a decrease only in the level of naive T-helper cells, whereas the level of memory or primed T-helper cells was significantly increased.

DISCUSSION

Mab to distinct differentiation antigens on human lymphocytes allow discriminating analysis of changes in lymphocyte subsets (9, 10). In addition to the identification of B cells, total T cells, helper T cells, suppressor-cytotoxic T cells, and NK cells, it has become possible to identify subsets within some of these populations of lymphocytes. The population of CD4+ helper T cells can now be further divided into two distinct subsets. One CD4+ subset, characterized by the coexpression of the CD45RA marker, originally designated as the suppressor-inducer subset (18) was recently shown to constitute the subset of naive or virgin CD4+ T cells (13, 14). The other subset of CD4+ cells, characterized by the coexpression of the C29 marker, was originally considered the helper-inducer subset (19) but was later shown to constitute the population of memory and activated CD4+ T cells (10, 11, 20). The relative proportion of the two CD4+ T cell subsets changes with age. In cord blood (20) and among PBL of infants, the proportion of CD29+ CD4+ cells is low, whereas that of the CD45RA+ CD4+ subset is high (15). *In vitro* activa-

tion by various mitogens can result in a transition of naive T cells to primed or memory T cells with a concomitant switch of their cell surface antigenic profile (20). It was, therefore, of interest to determine whether similar dynamic changes can occur *in vivo* during an acute infectious episode.

The present study demonstrates that gastrointestinal infection in children under the age of 3 y is indeed associated with significant changes in lymphocyte subset composition. In infants with acute diarrhea not associated with *G. lamblia* infection, the proportion of suppressor-cytotoxic lymphocytes (CD8+ cells) was increased with a significant concomitant decrease of the CD4:CD8 ratio. The level of CD57+ lymphocytes, characteristic for NK cells and for a subset of CD8+ T lymphocytes, was also increased in infants with acute gastroenteritis. Increases in the proportion of CD8+ (21) and Leu-7+ (CD57+) (12, 22) lymphocytes have previously been found to be associated with various viral infections. In contrast to these marked changes, in infants infected with *G. lamblia*, the level of CD4+, CD8+, and CD57+ lymphocytes and the CD4:CD8 ratio were normal. Normal values were also found in infants without diarrhea, some of whom had mild upper respiratory infections. Additionally, the level of primed or memory CD4+ cells (CD29+CD4+ cells), which is low among healthy infants, was strikingly increased in infants with acute gastroenteritis. In parallel, the subset of naive CD4+ T cells (CD45RA+CD4+ cells) was markedly decreased. Not only was the relative proportion of primed CD4+ cells increased in infants with diarrhea, but also their absolute number. This is particularly striking in view of the fact that the absolute number of CD4+ lymphocytes was decreased in these infants. Because the age of the children in the three groups was similar (Table 1), the difference in the level of subsets of CD4+ lymphocytes among them could not be attributed to age differences. Thus, non-*Giardia*-associated infectious diarrhea constitutes a potent antigenic stimulus responsible for a marked increase in the primed or memory T-helper cell subpopulation. Follow-up studies are required to determine whether these changes are transient or whether infectious diarrhea in children imparts a permanent adult-like subset pattern.

Because nutrition regulates cellular immune responsiveness (23, 24), the question arises whether the above-mentioned observations can be attributed to differences in the nutritional status of the three groups of infants. The mean weight of infants with non-*Giardia*-associated diarrhea was lower than that of the other

Table 3. *Lymphocyte subsets among various groups of infants**

Lymphocyte phenotype	Infants with non- <i>Giardia</i> diarrhea	Infants without diarrhea	Infants with <i>Giardia</i> -associated diarrhea
CD4+CD29+†	21.10 ± 5.37 (12)	13.47 ± 9.51 (9)‡	11.47 ± 6.85 (7)§
CD4+CD29+/CD4	58.53 ± 4.05 (12)	33.87 ± 23.07 (9)¶	26.33 ± 11.88 (7)¶
CD4+CD45RA+†	9.76 ± 3.88 (11)	22.52 ± 15.53 (8)‡	17.58 ± 9.25 (8)‡
CD4+CD45RA+/CD4	24.59 ± 7.50 (11)	47.09 ± 23.42 (8)‡	38.91 ± 20.25 (8)

* Values are mean ± SD. Numbers in parentheses are the number of infants tested.

† Proportion of PBL coexpressing the two antigenic markers.

‡ $p < 0.05$ vs infants with non-*Giardia* diarrhea.

§ $p < 0.01$ vs infants with non-*Giardia* diarrhea.

|| Proportion of CD4+ PBL coexpressing two antigenic markers.

¶ $p < 0.001$ vs infants with non-*Giardia* diarrhea.

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- infants studied. None of the children suffered from clinically overt dehydration, but as the infants' weights upon recovery were not available, the differences in weight could partly reflect mild dehydration. The lower weight of infants with non-*Giardia* diarrhea could also reflect some degree of malnutrition. In children over the age of 36 mo, malnutrition has been shown to result in impaired immune function as assessed by skin test reactivity (24). No such correlation was, however, found among children 12–35 mo of age (24). Skin tests to commonly encountered recall-antigens evaluate memory immune responsiveness and are dependent on memory T cells. Our study indicates that children with infectious diarrhea exhibit two seemingly conflicting processes—a decrease in the absolute number of CD4+ cells, paralleled by an increase in the absolute number of primed CD4+ cells. Thus, despite the lower mean weight of this group of infants, they were the ones that showed the most remarkable increase of activated memory T cells.
- Children with asymptomatic *Giardia* infection may have an advantage over uninfected children in their nutritional status and in freedom from other illnesses (2). In the present study, lymphocyte subsets of infants with diarrhea infected with *G. lamblia* resembled those observed in control infants without diarrhea. In particular, infection with *G. lamblia*, as opposed to other causes of acute gastroenteritis, was not accompanied by an increase in memory CD4+ cells. This phenomenon may be due to the fact that this parasite remains confined to the intestinal lumen without invading the intestinal mucosa. It may thereby fail to elicit either a systemic inflammatory response or alterations in lymphocyte subsets in the peripheral blood. The small bowel morphologic changes observed during *G. lamblia* infection are believed to be determined, at least in part, by T cell-dependent activity (25). Indeed, it is the gut-associated lymphoid tissue that plays a predominant role in the host defense against intestinal pathogens. T cells expressing mainly the CD4 antigen are present in the lamina propria, whereas T cells expressing the CD8 antigen and NK cells compose the majority of the intraepithelial lymphocytes (26, 27). To what degree the local intestinal immune response and changes in intestinal lymphocyte subsets are in fact mirrored by those detected in the peripheral blood remains to be determined. In AIDS patients, the decrease in the proportion of helper T cells and increase in cytotoxic-suppressor T cells observed in the peripheral blood is similarly noted when studying the lymphocyte population of their intestinal mucosa (28). Further studies are therefore needed to elucidate the changes in intestinal mucosal lymphocyte subsets occurring during *G. lamblia* infection and to correlate them with lymphocyte subset distribution in the circulation.
- It could be argued that, in the absence of more extensive viral studies, the mere presence of *G. lamblia* trophozoites and cysts in the stool may not be sufficient proof that the parasite is the cause of the diarrheal disease. If indeed the diarrhea in *Giardia*-positive cases was caused by another concomitant pathogen, then our findings may suggest that *G. lamblia* infection imparts an inhibitory effect on the cellular immune response, abrogating the expected increase in the level of helper-inducer or memory CD4+ cells as observed among the acute gastroenteritis group.
- Our observation that, in contrast to the dramatic changes in PBL subsets in infants with acute gastroenteritis, *G. lamblia*-associated diarrhea is not accompanied by similar changes is intriguing and warrants further study of the immune response elicited by these pathogens.
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