Immune Response of Children Who Develop Persistent Diarrhea following Rotavirus Infection

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A prospective study was conducted with Bangladeshi children with rotavirus (RV) diarrhea to assess whether nutritional and clinical parameters, RV serotypes, levels of interleukin-10 (IL-10), tumor necrosis factor alpha (TNF-α), and gamma interferon (IFN-γ), and RV-specific antibody titers in plasma and stool were associated with the development of persistent diarrhea. Children with watery diarrhea for 6 to 8 days, selected from the Dhaka Hospital of the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B), were enrolled in the study and monitored until diarrhea improved. Children were classified as having acute diarrhea (AD) if diarrhea resolved within 14 days of onset and as having persistent diarrhea (PD) if diarrhea persisted for more than 14 days after onset. Uninfected, control children (n = 13) from the Nutrition Follow-Up Unit of ICDDR,B were also enrolled. Of the 149 children with diarrhea enrolled, 29 had diarrhea with RV alone, of which 19 had AD and 10 developed PD. Samples of stool and blood were collected from all children on enrollment. Stool samples were collected again from children when they developed PD. Of the 10 children who had an initial RV infection and then developed PD, only one had persistent RV infection. Plasma levels of IL-10 and TNF-α were higher in children with diarrhea compared to uninfected controls but were similar in children with AD and PD. Plasma IFN-γ levels were higher in children who developed PD than in those with AD (P = 0.008) or uninfected controls (P = 0.001). In stools, the levels of TNF-α, the only cytokine detected, were similar in the three groups of children. RV-specific immunoglobulin G (IgG) titers in plasma were higher in uninfected children than in those with AD (P < 0.001) or PD (P = 0.024) but titers were similar in children with AD and PD. RV-specific IgA titers in plasma and stool were similar in the three groups of children. From all observed parameters, only elevated plasma IFN-γ levels were associated with subsequent development of PD. However, a larger sample size is necessary to substantiate this observation.

Rotavirus (RV) infection is the most common cause of hospitalization due to diarrhea in Bangladeshi children below 5 years of age (31) and is associated with considerable morbidity and mortality. Persistent diarrhea (PD), which is defined as diarrhea lasting for 14 days or more, is responsible for 30 to 50% of deaths due to diarrheal illness in developing countries (13). Although RV infection is not considered a significant risk factor for PD (7, 23), it does cause PD (19) especially in immunodeficient children (28). The cause(s) of PD is not well understood; however, one of the risk factors for PD, identified for the development of PD (4), the nutritional status and dietary intake have shown that antibodies may be associated with protection but antibodies do not consistently confer protection from infection or illness (6, 8, 12, 14, 18, 24, 33).

This study was aimed at assessing whether children with RV infection have altered levels of IFN-γ, IL-10, tumor necrosis factor alpha (TNF-α), and RV-specific antibodies in plasma and stool, before the onset of PD. These cytokines were selected on the basis of their possible antiviral activities (27) and effects on immunoglobulin A (IgA) secretion (25). Cytokine levels and RV-specific antibody titers were compared in three groups of children as follows: (i) children with RV infection for 6 to 8 days who recovered within 14 days of onset of diarrhea, (ii) children with RV infection for 6 to 8 days in whom diarrhea persisted for more than 14 days after onset, and (iii) uninfected, control children. Also, since malnutrition and cell-mediated immunity have been shown to be independent risk factors for the development of PD (4), the nutritional status and the general immune responses of the three groups of children were compared.

MATERIALS AND METHODS

Patient population. Children, 7 to 24 months of age, attending the Clinical Research and Service Centre of the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B), Dhaka, Bangladesh, with complaints of watery diarrhea for 6 to 8 days were initially enrolled. These children were hospitalized until diarrhea improved. If diarrhea improved within 14 days of onset, children were classified as having acute diarrhea (AD), but if diarrhea persisted beyond 14 days, children were classified as having PD. Improvement in diarrhea recovery from RV infection. The role of RV-specific immunoglobulins (Ig) in protection from RV infection is controversial. Various studies of natural RV infection or of volunteers challenged with RV have shown that antibodies may be associated with protection but antibodies do not consistently confer protection from infection or illness (6, 8, 12, 14, 18, 24, 33).

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was defined as a decrease in stool frequency to ≤5 in 24 h and/or change of stool consistency from liquid or loose stools to soft stools. Uninfected, control children were enrolled from the Nutrition Follow-Up Unit of ICCDR,B. This unit is an outpatient medical unit for nutritional surveillance of 10,000 children of 6 months of age and without any apparent infection for at least 1 month.

On enrollment, freshly collected stools from all children were examined microscopically, cultured for enteric bacteria (34), and assessed for the presence of RV by an enzyme-linked immunosorbent assay (ELISA) as described previously (28). Briefly, RV strains were first electrophoretotyped by polyacrylamide gel electrophoresis (PAGE), and PAGE-positive samples were then typed. Typing was done initially by a monoclonal antibody-based ELISA for G types 1 to 4, followed by reverse transcriptase-PCR (RT-PCR) for G types 1 to 4 and for P types (32). Presence of enterotoxigenic (ETEC), enteropathogenic (EPEC), and enterohaggregative (EAE) Escherichia coli was determined by using specific DNA probes as described previously (1, 16). From children who developed PD, second samples of stool were collected at 15 to 18 days after the onset of diarrhea and examined for enteropathogens including RV and diarrheagenic E. coli as described for the first sample. The study was approved by the Ethical Review Committee of ICCDR,B.

For the analysis, among 149 diarrheal children enrolled, only those with RV infection alone on enrollment without copathogens were included. Children were clinically evaluated by their medical history, daily physical examination, and laboratory investigations, which included determination of total and differential counts of leukocytes and serum electrolyte levels as indicated. All children were managed with appropriate fluid replacement therapy. Some children received antibiotics in the hospital if they had concurrent infections such as respiratory tract infection. All samples were collected before antibiotic treatment was started.

Collection and storage of samples. Samples of stool and venous blood (7 ml) were collected from all diarrheal children on enrollment, i.e., at 6 to 8 days after onset of diarrhea. Four milliliters of blood was collected aseptically in sterile heparinized Vacutainer tubes (Becton Dickinson, Rutherford, NJ). 2 ml of blood was collected in EDTA-containing Vacutainer tubes (Becton Dickinson) and 1 ml of blood was collected in sterile glass vials. Fresh blood collected in heparinized Vacutainer tubes was separated on Ficoll-Hypaque (Pharmaca, Uppsala, Sweden). After centrifugation at 500 × g for 25 min, peripheral blood mononuclear cells (PBMs), which formed a band at the interface, were collected, washed, and counted. Separation of plasma (for estimation of cytokines) and serum from fresh blood collected in EDTA-containing Vacutainer tubes and glass vials, respectively, was done by centrifugation for 10 min. Plasma and serum were stored in aliquots at −70 and −20°C, respectively, until assayed. Neat stool was stored at −20°C for RV typing. Stool extracts, for the estimation of cytokines, were prepared and stored as described before (2).

Assay of immune response. The proliferative response of PBMs was assessed on resting cells by measuring spontaneous DNA synthesis as described previously (3). Results were expressed as counts per minute.

Delayed-type hypersensitivity (DTH) responses were measured by skin tests with 3-Henyl-2-phenyl-2-propanol (HPA) (Aventis, Lyon, France) or Candida albicans (Difco, Detroit, MI), 50 µg/5 µl in PBS in 1 cm2 of skin on the back of children. Antigens were used at a concentration of 1:100 in 0.9% saline. Skin reactions were measured after 48 h and were expressed as mean diameter of induration (mm) ± standard deviation.

Determination of cytokine levels. IL-10 was measured in plasma and fecal extracts with an ELISA kit (Endogen Inc., Boston, Mass.) which was capable of detecting <3 pg of human IL-10 per ml of sample. Assays were done in duplicate. Concentrations were calculated by interpolation from a standard curve and were expressed as picograms per milliliter of plasma or picograms per gram of stool.

IFN-γ was measured with an ELISA as described earlier (26), and the detection limit for IFN-γ was 110 pg/ml. Briefly, flat-bottomed 96-well microtiter plates (Nunc, Roskilde, Denmark) were coated with the recombinant human IFN-γ (Chromogenix AB, Mölndal, Sweden) at a concentration of 2 µg/ml. After washing and blocking, undiluted samples and standard recombinant IFN-γ (R&D, Abingdon, United Kingdom) were added at different concentrations from 6,000 to 15 pg/ml in PBS containing 0.1% bovine serum albumin (BSA). Standards and samples were added in duplicate, and the mixtures were incubated overnight at 4°C. After washing, biotinylated MAb to human IFN-γ (Chromogenix AB) diluted 1:5,000 in 1% PBS-PBST was added to each well, and the mixtures were incubated at room temperature for 1 h. The substrate ortho-phenylenediamine dihydrochloride (OPD) (Sigma) at a concentration of 1 mg/ml in 0.1 M citrate buffer (pH 4.5) was added, and the reaction was stopped with 2 M H2SO4. The optical density was then measured at 450 nm in a spectrophotometer (Titertek Multiskan Plus). Positive and negative control samples were included in all assays and consisted of pooled supernatants from PBMs collected from healthy individuals and stimulated with phytohemagglutinin (Murex Diagnostics Ltd., Dartford, United Kingdom) for positive control) or from unstimulated PBMs (for negative control) of known IFN-γ concentration. Concentrations were calculated by interpolation from a standard curve and were expressed as picograms per milliliter of plasma or picograms per gram of stool.

Statistical analysis. Comparisons among the three groups of children were done with the Kruskal-Wallis test (for nonparametric data) or one-way analysis of variance (for parametric data). Comparisons between two groups were done by using the Mann-Whitney U test (for nonparametric data) or the t test (for parametric data). For comparisons between proportions, the chi-square statistic was used. Multiple regression analysis was carried out to determine the effects of age, nutritional status, sex, and concomitant infection on plasma levels of IFN-γ. Data were considered to be normally distributed if the Shapiro-Wilk test indicated normality. Data were analyzed by using the Statistical Package for Social Sciences (version 7.5 for Windows; SPSS Inc., Chicago, Ill.).

RESULTS

Patient characteristics. A total of 149 children with watery diarrhea were enrolled, of whom 108 had AD and 41 developed PD. Of these children, 29 were included who had diarrhea due to RV alone, without other enteropathogens. Of the 29 children with RV infection, 19 had AD and 10 developed PD. The clinical characteristics of the children are shown in Table 1. Children who developed PD were younger than uninfected children (P = 0.008) but were similar in age to children with AD. The frequency of passage of stool and vomiting on enrollment (Table 1) and the extent of dehydration (data not shown) were similar in children with AD and in those who developed PD. From children who developed PD, stool samples were collected again approximately 1 week later, i.e., when they developed PD, and tested for the presence of enteropathogens. Out of these 10 children with PD, RV was detected in 1, EPEC alone was detected in 1, EPEC with Aeromonas sp. was detected in 1, ETEC was detected in 2, EAEc was de-
TABLE 1. Clinical features and nutritional status of children on enrollment

<table>
<thead>
<tr>
<th>Study group (ρ)</th>
<th>No. of children (n)</th>
<th>Age (mos)</th>
<th>Frequency of vomiting/24 h</th>
<th>Weight for age</th>
<th>Nutritional status</th>
<th>General immune response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>No. with lower SD</td>
<td>Mean (± SD)</td>
<td>Median (25th to 75th quartiles)</td>
</tr>
<tr>
<td>Uninfected children (13)</td>
<td>7 (53.8)</td>
<td>12.0 (6.5–16.5)</td>
<td>NA</td>
<td>72.7 ± 11.4</td>
<td>46.7 ± 3.2</td>
<td>0.257 to 1.020</td>
</tr>
<tr>
<td>Children with AD (19)</td>
<td>11 (57.9)</td>
<td>10.0 (8.0–12.0)</td>
<td>15.0 (9.0–20.0)</td>
<td>2 (0–5)</td>
<td>4 (21)</td>
<td>73.6 (46.2–115)</td>
</tr>
<tr>
<td>Children who developed PD (10)</td>
<td>5 (50.0)</td>
<td>9.0 (7.0–10.3)</td>
<td>13.0 (12.0–22.5)</td>
<td>3 (0–4)</td>
<td>3 (30)</td>
<td>77.1 (40.6–110)</td>
</tr>
</tbody>
</table>

Values are medians (25th to 75th quartiles).

a: National Health and Nutrition Examination Survey.
b: Not applicable.

Comparisons among the three groups were done by using one-way analysis of variance (continuous, parametric data) and the Kruskal-Wallis test (continuous, nonparametric data). Comparisons between two groups were not significant.

General immune response. Spontaneous proliferation was significantly different among the three groups of children (P = 0.013). PBMs from children with AD showed significantly higher spontaneous proliferation (median, 3,785 cpm; 25th to 75th quartiles, 1,627 to 5,767 cpm) than those from uninfected children (median, 984 cpm; 25th to 75th quartiles, 517 to 2,256 cpm; P = 0.005). PBMs from children who developed PD had a proliferative response (median, 2,364 cpm; 25th to 75th quartiles, 1,062 to 3,298 cpm) that was similar to those with AD and those of uninfected children. DTH responses were compared on the basis of the number of children who had a positive response to at least one antigen. The three groups of children were similar with at least one antigen positive in 11 of 12 uninfected children, 13 of 17 children with AD, and in 5 of 7 children who developed PD.

RV-specific antibodies in plasma and stool. Titers of RV-specific IgA in the plasma and stool were similar in the three groups of children (Table 2). RV-specific IgG titers (Table 2) were significantly different among the three groups of children (P = 0.002) with titers being higher in uninfected children than in those with AD (P < 0.001) or those who developed PD (P = 0.024); titers were similar in children with AD and those who developed PD.

Cytokine levels in plasma and stool. On enrollment, plasma levels of IL-10 (Fig. 1A), TNF-α (Fig. 1B), and IFN-γ (Fig. 1C) were significantly different among the three groups of children (P = 0.001, 0.021, and 0.001, respectively). Compared to uninfected children, children with RV infection, whether with AD or PD, had higher plasma levels of IL-10 (P < 0.001 and 0.026, respectively) and TNF-α (P = 0.022 and 0.013, respectively). IL-10 and TNF-α levels in the plasma were similar in children with AD and those who developed PD. IFN-γ levels in the plasma (Fig. 1C) were higher in children who developed PD than in uninfected children (P = 0.001) or those with AD (P = 0.008); there was no difference in levels between children with AD and uninfected controls. In order to assess whether differences in plasma IFN-γ levels were significant due to higher values in two children who developed PD (Fig. 1C), comparisons were repeated without these two outliers. Such comparisons showed that significant differences in IFN-γ levels in the plasma remained when the three groups were compared (P = 0.008) and when children with AD and those who developed PD were compared (median, 1,151 pg/ml; 25th to 75th quartiles, 1,032 to 1,393 pg/ml; P = 0.038).

In the stools, IL-10 and IFN-γ were not detectable in the three groups of children. Although TNF-α was detectable in the stools, levels were similar in the three groups of children (data not shown).
Effects of other factors on IFN-γ levels in the plasma. As other factors such as age, nutritional status, sex, and concomitant infections could have an influence on IFN-γ levels, multiple regression analysis was carried out to assess their effects on plasma IFN-γ levels. None of the variables tested had a significant effect.

G and P types of RV strains. Stool samples from 19 children with diarrhea (AD, n = 13; PD, n = 6) were available for typing RV strains. No PAGE pattern was obtained from nine samples, suggestive of a low concentration of virus particles in stool, and these samples were, therefore, not typed. RV strains from seven children with AD and three children who developed PD were typed, and the results are shown in Table 3. The overall frequency of the strain types observed is similar to that found in the community (32), and there were too few strains to ascertain differences between children with AD and those who developed PD.

DISCUSSION

The immune response in PD due to a defined etiology has not been investigated before. For this purpose, children with a 6- to 8-day history of diarrhea from RV infection were studied. The high percentage of PD from an initial RV infection observed here (10 of 29) could be because children who already had prolonged RV infection were selected. Children with a diarrhea duration of 6 to 8 days were chosen on the basis that a reasonable number would develop PD. Enrollment of children at a more acute stage of diarrhea would result in too large a sample size for the study to be manageable.

Of the 10 children who developed PD, only one was found to have persistent RV infection when second samples of stools were tested for enteropathogens at 15 to 18 days after the onset of diarrhea. This data suggests that PD from an initial RV infection is not due to persistence of RV but to an alter-
ation in some host factor(s) which makes the children more susceptible to other infections or which leads to prolonged malabsorption. Other studies have also shown that PD is often due to sequential infection by different organisms rather than by a single organism (10). Our finding that children with AD and those who developed PD cannot be differentiated clinically during the acute stage of illness corroborates previous findings, which showed that clinical characteristics have a low positive predictive value for the development of PD (22).

In contrast to earlier studies (4, 9, 20) we found that the general immune responses of children were not lower in those who developed PD. Comparisons between the present study and previous studies on the role of the immune response in the development of PD is difficult as our study was conducted with children who already had diarrhea for 6 to 8 days while previous studies were conducted with children prior to the development of diarrhea. In this study, children who developed PD were not significantly malnourished, which is in contrast to an earlier study where malnutrition during AD was found to be a risk factor for the development of PD (23). It is possible that when specific etiologies are considered, the overall risk factors are different. Thus, it has been shown that RV infection occurs more commonly in better-nourished children (31) so that PD from RV infection also may not be related to malnutrition.

The role of RV-specific antibodies in protection against RV infection and illness is controversial (6, 8, 12, 14, 18, 24, 33). RV-specific IgG in serum correlates negatively with illness during the acute stage of infection (14), and this corroborates our finding of higher titers of RV-specific IgG in the plasma of uninfected children than in diarrheal children (both AD and PD). The results from this study suggest that RV-specific IgG or IgA has no role in the development of PD following an initial RV infection. However, a larger sample size is required to confirm this.

The role of IL-10 in RV infection has not been investigated, and the significance of our finding that IL-10 levels in the plasma are elevated in the ing acute RV infection, IL-10 and TNF-α can inhibit entry of RV into cultured epithelial cells (5). In contrast, clearance of RV by CD8+ T cells appears not to be dependent on IFN-γ (17). Thus the role of IFN-γ in RV infection is not clear. In the present study, children with AD and uninfected children had similar levels of IFN-γ, while children who developed PD had higher plasma IFN-γ levels than those with AD or uninfected children. The implications of these findings are not clear but they suggest that (i) IFN-γ has no role in protection against prolonged diarrhea following RV infection, (ii) IFN-γ is a correlate of a more serious pathology, and/or (iii) elevated levels of IFN-γ during the acute stage of RV infection are detrimental. IFN-γ can stimulate the immune response such that it leads to lysis of bystander cells, thereby causing more tissue damage (29). Furthermore, products of IFN-γ-induced cell lysis may cause inflammation of tissue, as has been shown for lymphocytic choriomeningitis virus infection (29). As in this study PD following an initial RV infection was not due to persistence of RV, it is possible that another factor, such as enhanced inflammation during the acute stage of the infection, could have led to prolonged diarrhea. However, we have no evidence to support this hypothesis.

In summary, the results of the present study show that during acute RV infection, IL-10 and TNF-α are elevated in the plasma and the development of PD is associated with a higher IFN-γ response. However, a larger sample size is required to confirm these trends.

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