Rotavirus Infection Alters Peripheral T-Cell Homeostasis in Children with Acute Diarrhea

Yuhuan Wang, Penelope H. Dennehy, Harry L. Keyserling, Kevin Tang, Jon R. Gentsch, Roger I. Glass, and Baoming Jiang

Division of Viral Diseases and Scientific Resources Program, Centers for Disease Control and Prevention, and Department of Pediatrics, Emory University School of Medicine, Atlanta, Georgia; Division of Pediatric Infectious Diseases, Rhode Island Hospital, Providence, Rhode Island; and Fogarty International Center, National Institutes of Health, Bethesda, Maryland

Received 30 August 2006/Accepted 22 January 2007

The patterns of gene expression and the phenotypes of lymphocytes in peripheral blood mononuclear cells (PBMC) from children with diarrheal caused by rotavirus and healthy children were compared by using DNA microarray, quantitative PCR, and flow cytometry. We observed increased expression of a number of genes encoding proinflammatory cytokines and interferon or interferon-stimulated proteins and demonstrated activation of some genes involved in the differentiation, maturation, activation, and survival of B lymphocytes in PBMC of patients with rotavirus infection. In contrast, we observed a consistent pattern of lower mRNA levels for an array of genes involved in the various stages of T-cell development and demonstrated a reduction in total lymphocyte populations and in the proportions of CD4 and CD8 T lymphocytes from PBMC of patients. This decreased frequency of T lymphocytes was transient, since the proportions of T lymphocytes recovered to almost normal levels in convalescent-phase PBMC from most patients. Finally, rotavirus infection induced the activation and expression of the early activation markers CD83 and CD69 on a fraction of CD19 B cells and the remaining CD4 and CD8 T lymphocytes in acute-phase PBMC of patients; the expression of CD83 continued to be elevated and was predominantly exhibited on CD4 T lymphocytes in convalescent-phase PBMC. On the basis of these findings at the molecular, phenotypic, and physiologic levels in acute-phase and convalescent-phase PBMC, we conclude that rotavirus infection induces robust proinflammatory and antiviral responses and B-cell activation but alters peripheral T-cell homeostasis in children.

Rotaviruses, members of a genus of the family Reoviridae, are nonenveloped, triple-layered capsid particles that contain a genome of 11 double-stranded RNA segments (30). Rotaviruses are the single most important cause of severe diarrhea and are responsible for an estimated 611,000 deaths of children worldwide per annum (40). The virus infects the mature villus epithelial cells of the small intestine, and infection often leads to fever, vomiting, and severe dehydrating diarrhea in children. Recent studies have reported antigenemia or viremia in children with diarrhea caused by rotavirus (7) and demonstrated extraintestinal spread and infection of rotavirus in gnotobiotic pigs and mice (4, 14).

Rotavirus infection induces innate and adaptive immune responses, including production of cytokines and virus-specific antibodies (28). Cytokines, which are produced primarily by macrophages and T lymphocytes in the early phase of viral infection, are markers used to indicate the quality and type (Th1 or Th2) of immune response (6). Cytokines also play important roles in the pathogenesis of and protection against acute viral infections and may influence or reflect the clinical outcome of rotavirus disease (3, 29, 35, 45, 48). Antibodies, on the other hand, are induced later in response to rotavirus infection and are a critical component of the host defense mechanisms that protect animals or children from rotavirus infection or diarrhea (28). Despite the presence of many studies that have examined rotavirus pathogenesis and immunity, including the response and function of cytokines and antibodies as mediators for and as correlates of protection against rotavirus infection in animals or humans, little is known about the early overall responses and the development and regulation of lymphocytes in children with acute rotavirus diarrhea.

While some studies of mice demonstrated that both B and T lymphocytes are important in protective immunity (16, 31), others demonstrated a T-cell-independent activation of B lymphocytes in the early phase of rotavirus infection and documented that virus could be cleared in the absence of T or B lymphocytes or antibodies (8, 17). Studies of gnotobiotic piglets and monkeys have shown that immunoglobulin A (IgA) in the gut and IgG in serum mediated protection against rotavirus diarrhea or infection (53, 56). These studies, while furthering our understanding of the immune response, have not clearly defined the early development, regulation and activation, or phenotypes of B and T lymphocytes or T-lymphocyte subsets in response to rotavirus infection. Moreover, none of the animal models are ideal, and they may not accurately predict the immunity and pathogenesis of rotavirus disease in humans. Neither small nor large animal models (e.g., mice or monkeys) develop disease when infected with rotavirus and therefore are not of great use in the study of innate immunity and disease initiation or progression. Mice differ from humans in both innate and adaptive immune responses, and they lack some important genes that have critical functions in immunity and disease processes in humans (37). Gnotobiotic piglets are the disease model that has the best potential to further our under-
standing of pathogenic mechanisms and host responses to rotavirus, but it is not clear whether piglets can reliably give us complete and relevant insights into the early responses to infection in children.

In the present study, we directly examined the early responses to rotavirus infection by comparing the global patterns of gene expression and phenotypes of lymphocytes in peripheral blood mononuclear cells (PBMC) from children hospitalized with acute diarrhea caused by rotavirus and from healthy children by using high-density oligonucleotide expression arrays, quantitative PCR, and flow cytometry. We demonstrated that rotavirus induced robust inflammatory responses and B-cell activation but altered peripheral T-cell homeostasis, as demonstrated by a decrease in total lymphocyte numbers and by a moderate to severe reduction in the proportions of CD4 and CD8 T lymphocytes in acute-phase PBMC from children. These findings in early response events during acute rotavirus infection will help provide insights into our understanding of innate and adaptive immunity and the pathogenic mechanisms of disease.

MATERIALS AND METHODS

Subjects and specimen collection. From March 2001 to March 2004, we collected blood and fecal specimens from children less than 3 years of age who were admitted for treatment of acute rotavirus gastroenteritis at two pediatric hospitals, Children’s Healthcare of Atlanta in Atlanta, Georgia, and Hasbro Children’s Hospital in Providence, Rhode Island. Aside from the gastroenteritis, the children were generally in good health, and they had not received rotavirus vaccines and had no prior history of rotavirus diarrhea. All patients had symptoms typical of rotavirus infection (fever, vomiting, diarrhea, or dehydration), and all were confirmed positive by the testing of fecal specimens by using enzyme immunoassay and PCR. Composite illness severity scores were calculated on the basis of fever, vomiting, diarrhea, and dehydration (15). A blood sample was drawn at the time of hospital admission from some children, and complete blood counts were determined using a Coulter LH 750 hematometry analyzer (Beckman Coulter, Fullerton, CA). Written informed consent was obtained from all parents or guardians, and children in the two hospitals were then enrolled using the same eligibility criteria. Prior to patient enrollment in the study, complete blood counts were determined from blood specimens of eight patients at the time of hospital admission. All but one of the patients had normal or near-normal cell counts, and the values obtained were used as baseline controls in the study. All patients had total lymphocyte counts of 1.6 × 10^9/μl, with percentages of stained PBMC of patients and controls analyzed by flow cytometry. Significant differences between the percentages of stained PBMC of patients and controls were analyzed by t test.

Complete blood counts. From the controls and patients enrolled, we selected two cohorts of healthy children and children with acute diarrhea caused by rotavirus who had no infection in the proceeding months and had an adequate amount of blood specimens for the microarray (8 controls and 10 patients) and flow cytometry (6 controls and 7 patients) experiments (Table 1). Prior to patient enrollment in the study, complete blood counts for three of the five white blood cell types (neutrophils, lymphocytes, monocytes, basophils, and eosinophils) were determined from blood specimens of eight patients at the time of hospital admission. All but one of the patients had normal segment neutrophil counts, whereas one had slightly lower monocyte counts. In contrast, five of seven patients (71%) with acute rotavirus diarrhea (i.e., <7 days after the onset of illness) had total lymphocyte counts of ≤ 2.9 × 10^9/μl, the lower limit of the normal cell count range in healthy children.

Gene expression analysis. Levels of gene expression in PBMC from 10 patients with rotavirus infection were deter-
mined and expressed relative to those of the 8 controls. Of the 12,626 gene sequences (10,000 genes) examined, mRNA lev-

12,626 gene sequences (mined and expressed relative to those of the 8 controls. Of the 3906 WANG ET AL. J. VIROL.

3906 WANG ET AL. J. VIROL.

ized differential expression of genes with function in B-cell development. We detected elevated expression of a group of

Differential expression of genes related to B-cell development and activation. We found that rotavirus infection in-
duced differential expression of genes with function in B-cell development. We detected elevated expression of a group of
genets that occur prior to adaptive immunity are thought to represent an early host defense against viral infection and also to mod-
ulate the adaptive immune response (45).

Elevated expression of genes with functions in early activation of lymphocytes. Rotavirus infection induced elevated expression of genes encoding costimulatory molecules, CD69 and CD83, in acute-phase PBMC from 8 of 10 children (Fig. 1B). Expression of these molecules indicates activation, matu-
rature, and involvement of PBMC, including T and B lympho-
cyes, in early events of immune response, such as antigen presentation and regulation in the development of cellular immunity. Of note, the expression of CD69 and CD83 remained elevated in convalescent-phase PBMC from four of the five children whose blood specimens were available for testing (data not shown). One patient did not have elevated expression of these costimulating molecules in acute- and convalescent-phase PBMC.

Activation of genes with function in inflammatory and antiviral activities. Rotavirus infection activates genes coding for proinflammatory cytokines (tumor necrosis factor alpha [TNF-α], prointerleukin-1β, interleukin-1β [IL-1β], and IL-6) and chemokines (IL-8 and GRO-β) (Fig. 1A). TNF-α is a primary response cytokine and a marker for inflammation, whereas IL-1β and IL-6 are pleiotropic. These cytokines are potent signaling molecules that induce the synthesis of acute-phase proteins and mediate the immunopathogenesis of various in-
fectious or inflammatory diseases (12, 35, 48). IL-8 and related GRO-β chemokines are involved in inflammation by primarily activating the chemotaxis of neutrophils (1, 20). Rotavirus infection also up-regulates genes encoding cytokines or proteins, such as IL-1R antagonist, alpha/beta interferon (IFN-α/β) receptor, or IFN-stimulated proteins, that are involved in anti-
flammatory and antiviral activities (Fig. 1A). IFN responses that occur prior to adaptive immunity are thought to represent an early host defense against viral infection and also to mod-
ulate the adaptive immune response (45).

TABLE 1. Demographic and clinical data and complete blood counts in children with rotavirus diarrhea*

<table>
<thead>
<tr>
<th>Group</th>
<th>Subject</th>
<th>Sex</th>
<th>Age (mo)</th>
<th>No. of days after illness onset before blood samples were obtained</th>
<th>Illness severity score</th>
<th>Complete blood count</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>First sample</td>
<td>Second sample</td>
<td></td>
</tr>
<tr>
<td>Patients in microarray study</td>
<td>1</td>
<td>M</td>
<td>6</td>
<td>4</td>
<td>NA</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>F</td>
<td>7</td>
<td>3</td>
<td>29</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>M</td>
<td>9</td>
<td>6</td>
<td>NA</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>F</td>
<td>10</td>
<td>5</td>
<td>30</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>F</td>
<td>12</td>
<td>5</td>
<td>NA</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>M</td>
<td>16</td>
<td>2</td>
<td>NA</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>F</td>
<td>17</td>
<td>11</td>
<td>38</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>F</td>
<td>19</td>
<td>3</td>
<td>NA</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>M</td>
<td>20</td>
<td>4</td>
<td>36</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>M</td>
<td>29</td>
<td>3</td>
<td>34</td>
<td>ND</td>
</tr>
<tr>
<td>Patients in flow cytometry study</td>
<td>1</td>
<td>F</td>
<td>5</td>
<td>1</td>
<td>56</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>F</td>
<td>6</td>
<td>5</td>
<td>34</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>F</td>
<td>7</td>
<td>3</td>
<td>34</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>F</td>
<td>9</td>
<td>4</td>
<td>27</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>M</td>
<td>9</td>
<td>2</td>
<td>32</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>F</td>
<td>10</td>
<td>4</td>
<td>28</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>M</td>
<td>11</td>
<td>ND</td>
<td>ND</td>
<td>12</td>
</tr>
</tbody>
</table>

* Complete blood counts were done from blood specimens collected at the time of hospital admission, 1 to 4 days before children were enrolled for the study. Lymph, lymphocytes; Neut/SEG, segment neutrophils; Neut/BAND, band neutrophils; Mono, monocytes; WBC, white blood cells; NA, not available; ND, not determined. The normal cell count ranges are 2.9 x 10^3/μl to 9.4 x 10^3/μl, 1.0 x 10^3/μl to 9.5 x 10^3/μl, 0.0 x 10^3/μl to 0.7 x 10^3/μl, and 0.4 x 10^3/μl to 2.4 x 10^3/μl for lymphocytes, segment neutrophils, band neutrophils, and monocytes, respectively, in healthy children.

on October 23, 2017 by guest http://jvi.asm.org/ Downloaded from
factor regulated by IL-3 (NFIL3), Bfl-1, and FADD-like IL-1β-converting enzyme-inhibitory protein (FLIP) genes. Expression of these early molecules and markers may regulate signaling response, maturation, migration or survival of B lymphocytes (13, 26, 33, 38, 44).

We detected lower levels of expression of genes encoding several negative regulators of B-cell development that determine whether pre-B cells survive or die (Fig. 1D). Antigen receptors CD20, CD21, CD24, CD72, and CD79B influence the survival of B cells via different mechanisms, including the induction of apoptosis by the activation of multiple caspase cascades and mitogen-activated protein kinase, the activation of the alternative complement pathway and the formation of membrane attack complexes, and the regulation or modulation of B-cell receptor signaling (42, 49, 51). Of note, CD21 and CD72 have dual functions and are also involved in the positive regulation of B lymphocytes (23, 42).

Lower levels of mRNA for genes related to T-cell development. Children with rotavirus infection had overall lower levels of mRNA for an array of genes involved in the various stages of T-cell development and activation than did controls (Fig. 1E). These genes encode proteins in the following five categories: (i) CD antigens (CD1C, CD2, CD3D, CD28, CD96, and CD2-binding protein); (ii) TCR; (iii) the Src family kinases and signaling molecules (Lck and Lck substrate, linker for activation of T cells [LAT], and SH2 domain-containing leukocyte protein 76 [SLP-76]); (iv) cytokines and cytokine receptors (IL-16, CD27, IL-17R, IL-27R, and IL-7R); and (v) differentiation and activation proteins RP1, LIGHT, and MAL. These molecules are involved in antigen presentation, differentiation, activation, survival, and homeostasis of T lymphocytes (2, 9, 18, 47, 50).

Of note, we detected elevated expression of several transcription factor genes, the AREB6 and IL-2 enhancer binding factor genes, that are involved in the expression of IL-2 by T lymphocytes (data not shown). AREB6 negatively modulates and inhibits IL-2 expression after T-cell activation (25), whereas IL-2 enhancer binding factor has been described to have a positive role in regulating IL-2 gene expression (39).

Genes involved in other immune functions. In our study, rotavirus infection resulted in the differential expression of a number of genes with other immune functions in children with acute diarrhea. These included elevated expression of genes involved in the modulation of inflammatory and immune responses or in the degradation of targeted viral or short-lived proteins by the ubiquitin system (data not shown). Among the genes that were down-regulated were those encoding lymphocyte antigens, lymphotoxin β, chemokine ligands and receptors as well as other signaling molecules, and a group of adaptor proteins (e.g., docking protein 1, GRB2, and Src-like adaptor protein [SLAP]).

Confirmation of gene expression by quantitative PCR. To confirm the patterns of expression observed in these microarray experiments, we performed expression analysis of selected genes by quantitative PCR. We demonstrated and confirmed expression patterns for a number of genes, including the IL-6, IL-7R, and RGS1 genes in PBMC of children with diarrhea caused by rotavirus (Table 2).

Reduction in frequency of T lymphocytes. After expression analysis and confirmation, we determined whether the profiles

FIG. 1. Image profiles of genes with functions in inflammation and immunity. Microarray experiments were conducted and cluster analysis of genes that were differentially expressed in PBMC of healthy children and patients with diarrhea caused by rotavirus were done as described in the text. Each vertical column represents a subject, and each horizontal row represents a gene. Red squares indicate elevated expression, green squares indicate reduced expression, and black squares indicate no significant change in the level of gene expression. The left 8 columns correspond to healthy controls and the right 10 columns correspond to patients with rotavirus diarrhea. Rotavirus infection induced elevated expression of genes involved in inflammation and antiviral activities (A), early activation markers (B), and the differentiation, maturation, activation, and survival of B lymphocytes (C). In contrast, rotavirus infection induced repressed or reduced expression of genes involved in the negative regulation of B lymphocytes (D) and in the proliferation, differentiation, activation, survival, and homeostasis of T lymphocytes (E). OAS, oligoadenylate synthetase; MIP-1α, macrophage inflammatory protein 1α.
of gene expression can reliably reflect or predict immune responses to rotavirus. Because the small volumes of blood specimens and the lack of adequate numbers of PBMC did not allow us to conduct both microarray and flow cytometry analyses for the same subjects, we examined the phenotypes of B and T lymphocytes in PBMC from a separate cohort of six healthy controls (two girls and four boys), ranging in age from 2 to 7 months, and seven children with rotavirus diarrhea (Table 1) with flow cytometry using PE- or FITC-labeled monoclonal antibodies specific to surface markers CD4, CD8, CD19, or αβ TCR (Fig. 2). The frequency of CD19 B lymphocytes in PBMC of children with diarrhea ranged from 14.3% to 41.1% (mean, 27.7%) (Fig. 2A). In convalescent-phase PBMC, the proportions of CD19 B lymphocytes in six patients remained elevated. One patient had a lower proportion of CD19 B cells but a high frequency of CD4 T lymphocytes in convalescent-phase PBMC.

We determined the phenotypes of T lymphocytes in PBMC from three controls and five rotavirus patients because they had adequate blood specimen volumes and cell counts for all the experiments. The proportions of T lymphocytes in four of five patients ranged from 10.4% to 26.8% (mean, 20.0%) for CD4 and from 9.0% to 22.6% (mean, 17.0%) for αβ CD4 (Fig. 2B and C). The proportions of T lymphocytes in three controls ranged from 38.6% to 60.5% (mean, 50.9%) for CD4 and from 36.7% to 53.7% (mean, 46.8%) for αβ CD4, the values that agree with those reported for healthy children (24). The difference between these CD4 lymphocytes for patients and those for controls was significant (P < 0.01). The proportions of CD4 and αβ CD4 T lymphocytes in convalescent-phase PBMC were recovered to levels similar or close to those in healthy children. These increases were significant (P = 0.01).

We observed a similar moderate to severe reduction in the percentages of CD8 T lymphocytes. The proportions of T lymphocytes in three controls ranged from 7.4% to 13.5% (mean, 10.9%) for CD8 and from 6.1% to 10.5% (mean, 8.6%) for αβ CD8. The proportions of CD8 and αβ CD8 T lymphocytes in four infants with acute diarrhea caused by rotavirus were significantly (P < 0.05) lower than those in healthy infants, ranging from 1.6% to 3.8% (mean, 2.8%) and from 1.7% to 3.7% (mean, 2.9%), respectively (Fig. 2D and E). The proportions of CD8 and αβ CD8 T lymphocytes in convalescent-phase PBMC were significantly (P < 0.01) higher than those in acute-phase PBMC of patients, but they were similar to those in the PBMC of healthy children. The fifth patient had a slight decrease in CD4 T cells or no decrease in CD8 T cells in acute-phase PBMC but a severe reduction in convalescent-phase PBMC.

**Activation of T and B lymphocytes.** We sought to examine whether any of the diminished or remaining T lymphocytes in acute-phase PBMC expressed the two early activation markers, CD69 and CD83, in response to rotavirus infection. We analyzed PBMC from six healthy infants and found no activation in T lymphocytes. The percentages of activated T lymphocytes ranged from 0% to 0.5% (mean, 0.3%) for CD4/CD69, from 0.1% to 4.0% (mean, 1.2%) for CD4/CD83, from 0.1% to 0.7% (mean, 0.3%) for CD8/CD69, and from 0% to 0.4% (mean, 0.2%) for CD8/CD83. In contrast, we detected elevated activation percentages for CD4/CD69 (from 2.7% to 10.5% [mean, 5.5%]) and CD4/CD83 (from 10.5% to 25.8% [mean, 16.6%]) T lymphocytes in all seven acute-phase PBMC of patients with diarrhea (Fig. 3A and B). In convalescent-phase PBMC, the percentages of CD4/CD69 T lymphocytes in five patients decreased to levels close to those in control infants,

<table>
<thead>
<tr>
<th>Subject</th>
<th>Change in expression (n-fold)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Array</td>
</tr>
<tr>
<td>1</td>
<td>33.6</td>
</tr>
<tr>
<td>2</td>
<td>1.3</td>
</tr>
<tr>
<td>3</td>
<td>30.2</td>
</tr>
<tr>
<td>4</td>
<td>500.0</td>
</tr>
<tr>
<td>5</td>
<td>7.2</td>
</tr>
<tr>
<td>6</td>
<td>19.7</td>
</tr>
<tr>
<td>7</td>
<td>-1.1</td>
</tr>
<tr>
<td>8</td>
<td>39.0</td>
</tr>
<tr>
<td>9</td>
<td>-1.1</td>
</tr>
<tr>
<td>10</td>
<td>8.1</td>
</tr>
</tbody>
</table>

Overall patternb: 7/10 7/9 9/10 6/9 9/10 8/9

**TABLE 2. Comparison of gene expressions by microarray and quantitative PCR**

* Microarray analysis and quantitative PCR were performed as described in the text. Data shown in the table are values of changes (n-fold) in gene expression in PBMC from patients with rotavirus infection relative to those of healthy controls. For quantitative PCR, each sample was tested in triplicate and the average was used to calculate the change. ND, not determined.

b Pattern of differential gene expression in number of patients observed per number of patients examined.
The percentages of CD19/CD83 B lymphocytes (from 7.4% to 31.9% [mean, 14.6%]) and CD19/CD69 (from 1.3% to 7.2% [mean, 3.3%]) and CD19/CD83 T lymphocytes, ranging from 4.4% to 16.1% [mean, 7.8%]), were detected in six acute-phase PBMC (Fig. 3C and D). The percentages of activated CD8/CD69 T lymphocytes in five patients returned to levels close to those in healthy controls 3 weeks later, whereas the percentages of activated CD8/CD83 T lymphocytes, though declining in most patients, remained elevated in convalescent-phase PBMC. The seventh patient had no or small changes in the percentages of CD8/CD69 and CD8/CD83 T lymphocytes in acute- and convalescent-phase PBMC when compared to those of healthy controls.

CD69 and CD83 are early activation markers for rotavirus infection on B lymphocytes as well. The percentages of B lymphocytes in six healthy infants ranged from 0% to 0.4% (mean, 0.2%) for CD19/CD69 and from 0.1% to 6.5% (mean, 1.7%) for CD19/CD83 B lymphocytes. All seven infants with acute diarrhea caused by rotavirus had elevated levels of CD19/CD69 (from 1.3% to 7.2% [mean, 3.3%]) and CD19/CD83 (from 7.4% to 31.9% [mean, 14.6%]) B lymphocytes (Fig. 3E and F). The percentages of CD19/CD69 B lymphocytes in convalescent-phase PBMC from six patients returned to levels similar to those in control children. While the percentages of CD19/CD83 B cells in the convalescent-phase PBMC of six subjects decreased, the percentages of CD19/CD83 B cells in the convalescent-phase PBMC of two subjects remained highly elevated. One patient had slightly increased levels of CD19/CD83 B cells 3 weeks later.

**DISCUSSION**

Despite three decades of study on rotavirus diarrhea, we still do not completely understand the basic mechanisms of pathogenesis of and immunity to this disease. In this study, we examined broad and comprehensive early host responses to rotavirus infection in PBMC of children with acute diarrhea compared to those of healthy controls and demonstrated that rotavirus induced strong inflammatory and immune responses and detected markers or molecules that might be involved in the initiation and progression of disease and in the regulation, proliferation, differentiation, activation, and homeostasis of lymphocytes.

Children with acute diarrhea mount a robust and predominantly proinflammatory response to rotavirus, as evidenced by the elevated expression of genes for major cytokines. TNF-α, IFN-α, IL-1β, IL-6, and IL-8 are recognized mediators of inflammation or disease through their specific receptors and signal transduction pathways (3, 32, 35). Our gene array results were in agreement with the profiles of cytokines in acute-phase sera of patients with rotavirus diarrhea, in whom some cytokines were found to act as possible mediators for rotavirus disease (5, 29). On the other hand, children could counter proinflammatory cytokine-induced inflammation or disease processes by producing anti-inflammatory cytokines. For example, TNF-induced zinc-finger protein A20 and IL-1R antagonist induced after rotavirus infection could neutralize the effect of elevated TNF-α and IL-1β and inhibit inflammation. The balance between the two opposing IL-1 molecules has been documented to influence the development and severity of many inflammatory diseases (3). Moreover, activation of IFNs and IFN-stimulated genes (ISGs) in the early days of rotavirus infection might have contributed to virus clearance and rapid recovery from disease as well before the induction of virus-specific antibodies (29). IFNs exert their antiviral and other biological functions through the expression of more than 1,000 ISGs, including the 2-5′ oligoadenylate synthetase gene, which was found activated in this study and could mediate host defense by RNA degradation via a multi-enzyme pathway (45). The functions of the four other ISGs identified in this study are not known. A previous study demonstrated that the ISG15 protein has immunomodulatory effects by augmenting the proliferation of natural killer cells and inducing production of IFN-γ from T cells (11). IFN-α has been tested and found effective on October 23, 2017 by guest http://jvi.asm.org/ Downloaded from
phenotypes. While complete blood counts showed a decrease in the number of total lymphocytes, the phenotypic study clearly demonstrated moderate to severe reductions in the percentages of CD4 and CD8 T lymphocytes in acute-phase PBMC from most patients. Taken together, these findings at molecular, phenotypic, and physiologic levels indicate that T-cell lymphopenia and altered homeostasis of peripheral T lymphocytes are a common manifestation in children with acute rotavirus infection. This altered peripheral homeostasis is temporal, since the proportions of T lymphocytes recovered to almost normal levels in convalescent-phase PBMC. The exact mechanisms for this change in peripheral T-cell homeostasis are not known and are likely to be multifactorial events. Rotavirus infection could induce repressed expression of key markers, receptors, or signaling molecules and thus impair survival and homeostasis of T lymphocytes. For example, CD28, a membrane glycoprotein expressed on virtually all peripheral blood T cells of newborns, is essential for the proliferation and survival of CD4+ T cells, the production of IL-2, and the development of the Th2 type response (52). Reduced expression or lack of this molecule could lead to a reduction or even deficiency of T lymphocytes in rotavirus-infected children. Similarly, reduced expression or lack of IL-7R, a master regulator of survival and homeostasis of peripheral T cells, might have rendered IL-7 incapable of enhancing and expanding T-cell populations in response to rotavirus infection (18).

In addition, this alteration in peripheral T-cell populations may be a direct result of the response to acute rotavirus infection, as naïve T lymphocytes leave the circulation to secondary lymphoid tissues where they are activated, proliferate, and differentiate into CD4+ and CD8+ effector T lymphocytes. Activated effector T cells that express the intestinal homing receptor α4β7 reenter the circulation and home to infected tissues in the small intestine, where they play an important role in antibody and cell-mediated responses and protection against infection (43). Studies of mice have demonstrated that after lymphocytic choriomeningitis virus is cleared, up to 99% of the activated T lymphocytes undergo apoptosis and die, leaving an expanded population of virus-specific memory T cells (22). Whether this is also the case for rotavirus infection needs to be investigated. However, despite an apparent alteration in peripheral T-cell homeostasis, we detected significant percentages of the remaining CD4+ and CD8+ T lymphocytes that expressed CD83 in acute-phase PBMC from the majority of patients examined. A smaller percentage of CD4+ T lymphocytes also expressed CD69. These activated T lymphocytes in circulation may produce cytokines that could contribute to inflammation and recovery from disease in children. In mice immunized with a chimeric VP6 protein, αβ CD4+ T lymphocytes acted as effectors of protection against rotavirus infection (36).

In contrast, we demonstrated elevated levels of expression for genes involved in the growth, differentiation, activation, and survival of B lymphocytes in children with acute diarrhea caused by rotavirus. In addition, several antigen receptor genes involved in the negative regulation of B lymphocytes were found to be down-regulated, and these down-regulations could theoretically lead to positive selection and development of B lymphocytes. Our gene expression results were in agreement with the observation that the percentages of CD19+ B lymphocytes in acute-phase PBMC of patients were similar to or higher than those in healthy controls, and these B lymphocytes exhibited the early activation marker CD83 and, to a lesser extent, CD69. Our results concurred with a number of published studies of mice and humans. Rotavirus induced massive B-cell activation in the early days of acute infection, as evidenced by increased expression of CD69, but did not activate T lymphocytes (8). Children with acute rotavirus diarrhea had high frequencies of IgM-secreting B cells and a robust IgM response (27, 55). These rapid early B-cell and antibody responses could be attributed to direct interactions between rotavirus capsid proteins VP6 and VP7 and naïve B cells, as demonstrated in a recent study (41). Our observations of B-cell activation and T-cell lymphopenia in acute-phase PBMC further support the independent homeostatic regulation of peripheral B- and T-cell populations during immune responses (22). Of note, since antigen receptors CD21 and CD72 are also described as the positive regulators of B lymphocytes, further studies are needed to determine whether negative or positive regulations or both are actually utilized.

This study is the first to demonstrate that rotavirus infection activates the costimulatory and regulatory molecule CD83, one of the best maturation and activation markers for lymphocytes and dendritic cells. CD83 has been described to play an important role in the induction, regulation, or modulation of immune responses, including the development of cellular immunity and the generation of CD4+ T cells (19, 46). We demonstrated elevated levels of CD83 expression in total mRNA and on activated B and T lymphocytes, particularly on CD4+ T lymphocytes, in both acute- and convalescent-phase PBMC. The significance of these findings is not known, but these effector or memory lymphocytes could mount a spontaneous and strong immune response in a subsequent rotavirus infection. Future studies will need to investigate the biology and function of these activated lymphocytes that express CD83 in children with rotavirus infection.

Our study has a number of limitations and implications. First, because we had only a small volume of one acute-phase blood sample and one convalescent-phase blood sample from each patient with diarrhea, we were not able to determine the kinetics of gene and phenotype expression in response to rotavirus infection. Second, because of difficulty in enrolling subjects and the relatively small sample size, we could not reliably assess the effect of other parameters, such as age, on early responses. Third, we examined patterns of gene expression and phenotypes of lymphocytes in total PBMC. The number of PBMC in each sample was not large enough to allow us to determine patterns of gene expression in a specific type of leukocytes or examine both gene and phenotype expressions in the same subjects. In addition, we determined phenotypes in B cells and T lymphocyte subsets only. Further studies are needed to examine early responses of B and T lymphocytes in lymphoid tissues of the small intestine, the primary site of rotavirus infection, and the phenotypes and roles of other leukocytes, such as NK cells, monocytes, and dendritic cells, in innate and adaptive immunity. Apparent reductions in the numbers and percentages of peripheral T lymphocytes in children with natural rotavirus infection suggests that live oral or parenteral vaccines should be administered to stimulate the expression of the best effector phenotype in B lymphocytes and...
consequently result in maximum production of antibodies with high affinity, titer, and neutralizing activity. Moreover, this novel approach to the identification of early immunity and disease markers may also have potential to predict efficacy or possible adverse events of live attenuated rotavirus vaccines. Last, we demonstrated differential expression of a number of genes encoding proteins or markers that are involved in inflammation, immune regulation, or antiviral activity, but we were not able to determine specific pathways that might mediate these processes or functions. More studies are needed to clearly define molecular mechanisms of disease and further our understanding of immune regulation and response to rotavirus in children.

ACKNOWLEDGMENTS

We thank Joseph Bresee for help with the institutional review board protocol, Sara Nelson, Caminade Moscatiello, and Larry Westerman for collecting and processing blood and fecal specimens, Chi-Cheng Luo and Robin Scarborough for help in microarray experiments and data analysis, Lia Haynes for advice on flow cytometry, and Claudia Cummins, J. M., G. S. Krakowka, and C. G. Thompson.

REFERENCES