Early CD69 Expression on Peripheral Blood Lymphocytes from Children with Dengue Hemorrhagic Fever

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Recent reports have demonstrated immune activation in dengue hemorrhagic fever (DHF) by cytokine and soluble receptor detection in blood. The goal of this study was to determine which cell types are activated and likely to be responsible for cytokine production. Whole blood specimens from 51 Thai children presenting within 72 h of fever onset and with detectable plasma dengue viral RNA were studied by flow cytometry. Absolute CD4 T cell, CD8 T cell, NK cell, and γδ T cell counts were decreased in children with DHF compared with those with dengue fever (DF) early in the course of illness. The percent of cells expressing CD69 was increased on CD8 T cells and NK cells in children who developed DHF more than in those with DF. These data directly demonstrate that cellular immune activation is present early in acute dengue and is related to disease severity.

Dengue hemorrhagic fever (DHF) is the most important arthropod-borne viral disease worldwide and causes much morbidity and mortality in tropical and subtropical regions of the world. First documented to occur in Southeast Asia, DHF is an emerging disease that has spread to Central and South America [1]. Dengue fever (DF) is the uncomplicated form of dengue virus infection and typically resolves without long-term sequelae. DHF, the more severe form of the disease, is defined by fever accompanied by plasma leakage and a bleeding diathesis, including thrombocytopenia. In Southeast Asia, where dengue is endemic, DHF affects primarily infants and school-aged children [2].

Epidemiologic studies have demonstrated that DHF is more common in secondary heterologous dengue infections than in primary dengue infections [3, 4]. Prior studies have implicated a role for immunopathogenesis in the development of DHF [5]. Our prior studies found elevated levels of soluble tumor necrosis factor (TNF) receptors, soluble CD8, and soluble interleukin (IL)-2 receptor in children with DHF compared with those with DF early in the course of infection [6, 7]. However, elevations of cytokines and soluble receptors in the plasma or serum are an indirect measure of immune activation and do not reveal which immune cells are activated. We previously reported evidence of dengue-specific cross-reactive CD4 and CD8 memory cytotoxic T lymphocyte responses in persons immunized with experimental live-attenuated dengue vaccines and, more recently, in children following natural dengue virus infections [8–11]. While these studies suggested a role for immune activation, the presence of activated cytotoxic T cells has not been studied during acute infection.

The goal of this study was to directly examine the in vivo activation of the various components of the cellular immune system during acute dengue infection. CD69 is an early activation marker [12] and is expressed on activated hematopoietic cells, including T cells [13], NK cells [14], and monocytes [15]. CD69 expression on in vitro–activated peripheral blood mononuclear cells (PBMC) correlates with the ability to induce proliferative T cell responses [16, 17].

Thai children with fever of ≤72 h and no obvious source of infection were enrolled in an ongoing study of dengue pathogenesis in Bangkok [18, 19]. Fresh whole blood samples obtained on study day 2 and 1 day after defervescence were stained with immunofluorescent monoclonal antibodies (MAbs) to PBMC subset markers and the activation markers CD69 and CD25 and were analyzed by flow cytometry (FACS). We analyzed the relationship between the flow cytometry findings and disease severity.
Methods

Study design. The design of the prospective cohort clinical study has been described elsewhere [18]. In brief, Thai children presenting to the outpatient department or located on the ward at the Queen Sirikit National Institute for Child Health with fever of ≤72 h duration, flushed face, and no other localizing signs of infection were enrolled and admitted to the ward for observation with no study-specific intervention. Venous blood samples were obtained daily until 1 day after defervescence (temperature <38°C) and on study day 9. All subjects with serologic or virologic evidence of dengue (see below) had additional blood samples obtained 6 months after enrollment. These latter samples were utilized as healthy controls.

Study definitions. Study day 1 is the day of enrollment and when the first blood sample was obtained. Fever day 0 is the day of defervescence (temperature <38°C). One day after defervescence is fever day +1. Children were assigned a final diagnosis of dengue only if confirmed by at least two of the following criteria: dengue serology (ELISA and/or hemagglutination inhibition [HAI]), virus isolation, or dengue reverse transcription–polymerase chain reaction (RT-PCR; for methods, see below). DHF was classified by WHO criteria into 4 grades, with all cases meeting minimal criteria of plasma leakage (hemocentration of 20%, pleural effusion on right lateral decubitus chest radiograph, or ascites), and thrombocytopenia [20]. Any subject who did not meet criteria for DHF with serologic or virologic evidence of acute infection was classified as having DF.

Dengue serology and virus detection. Evidence of acute dengue virus infection was determined by dengue ELISA [21], HAI antibody responses [22], dengue virus isolation in Toxorhynchites splendens mosquitoes [19], and dengue RT-PCR [23].

Sample preparation and selection. All blood specimens were drawn by venipuncture into Vacutainer EDTA tubes (Becton Dickinson, San Jose, CA) each morning and transported to the specimen processing laboratory on ice. On study day 1, whole blood was centrifuged at 800 g for 10 min at 4°C. Plasma for RT-PCR was aliquotted into an Eppendorf tube containing lysis solution (see [23]) and transported to the PCR laboratory on ice for further studies. RT-PCR results were available by the morning of study day 2. For PCR-positive subjects and a small number of randomly selected PCR-negative subjects, on study day 2, 2 mL of whole blood was removed from EDTA tubes for flow cytometry studies. A second sample was obtained for staining on the day following defervescence (fever day +1). A subset of these study cases (n = 20) was selected at random and had a control sample stained from a healthy 6-month-follow-up visit.

MABs. The following fluorescent MABs were used: peridinin chlorophyll protein–conjugated Leu 4 (CD3; pan T); phycoerythrin-conjugated Leu-3a (CD4; T lymphocytes), Leu-2a (CD8 T and NK lymphocytes), Leu-11c (CD16; NK lymphocytes), Leu-19 (CD56; NK lymphocytes, T lymphocyte subset), Leu-12 (CD19; B lymphocyte), CD45 (monocyte/macrophages), fluorescein isothiocyanate-labeled Leu 23 (CD69; NK, B, and activated T cells), and anti–IL-2 receptor (CD25; B cell subset and activated T cells). All MABs were purchased from Becton Dickinson. IgG1 isotype control antibody conjugates were included in all assays to determine background fluorescence.

Flow cytometry analysis. Whole blood (150 μL) was incubated on ice with 10 μL of each antibody for 15 min in the dark. Two milliliters of FACS lysis solution (Becton Dickinson) was added and incubated at room temperature for 10 min. The cells were then washed with PBS and fixed with 0.5 mL of 0.1% glutaraldehyde solution in PBS. Whole blood samples were analyzed by three-color analysis using a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA). For two-color analyses, lymphocyte or monocyte gates were used based on forward and side scatter. For three-color staining, CD3 gates were used with secondary lymphocyte gating based on forward and side scatter results. Data were acquired and analyzed by CellQuest software (version 1; Becton Dickinson).

Statistical analysis. Means for lymphocyte parameters were compared between cases of DHF and DF on study day 2 and fever day +1. If a study day 2 sample coincided with fever day +1, it was defined as a fever day +1 sample. Therefore, all study day 2 samples were from children who were febrile on the day of sample acquisition. We compared both the percentage and absolute counts for each cellular immunophenotype and for activated populations by linear regression analysis adjusting for fever day, serologic response (primary vs. secondary), and final diagnosis. Statistical analyses were performed with SPSS for Windows software (version 8.0.1.; SPSS, Chicago). P < .05 was considered statistically significant.

Results

Patient population. Between 16 September and 22 December 1997, 51 volunteers with dengue were enrolled: 22 were subsequently diagnosed with DHF and 29 with DF. DHF cases were divided into the following DHF grades according to WHO criteria: grade 1, 4 subjects; grade 2, 13 subjects; and grade 3, 5 subjects. There were no statistically significant differences between children with DHF and DF with respect to age, sex, duration of fever prior to study entry, or duration of illness (table 1). We determined the viral serotype in 50 of 51 children and demonstrated that dengue virus types 1, 2, and 3 were present in this population. No differences were seen in the frequency of dengue serotype in children with DF and DHF (P not significant by χ² analysis). Subjects with DF were more likely than those with DHF to be experiencing a primary dengue virus infection (P < .01, Fisher’s exact test), consistent with the results of others [3, 4, 24]. Therefore, we adjusted for serologic response (primary vs. secondary) in subsequent statistical analyses.

Lymphocyte counts in whole blood samples. On study day 2, mean white blood cell counts were 2605 and 3214 cells/mm³ in children with DHF and DF, respectively, lower than in controls (7390 cells/mm³; P < .001; table 2). The mean number of absolute lymphocytes was lower in children with DHF (811 cells/mm³) than in those with DF (11,134 cells/mm³; P < .05), and both were lower than in controls (2883 cells/mm³; P < .001). Children with DHF and DF had lower mean absolute monocytes (181 and 168 cells/mm³, respectively) than controls.
Table 1. Demographic data for population studied for dengue hemorrhagic fever (DHF) and dengue fever (DF).

<table>
<thead>
<tr>
<th></th>
<th>DHF (n = 22)</th>
<th>DF (n = 29)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years, mean (95% CI)</td>
<td>9.1 (7.7–10.4)</td>
<td>7.6 (6.6–8.6)</td>
</tr>
<tr>
<td>Sex, % male (95% CI)</td>
<td>55 (32–77)</td>
<td>38 (19–57)</td>
</tr>
<tr>
<td>Days ill at study entry, mean (95% CI)</td>
<td>2.1 (1.8–2.4)</td>
<td>2.1 (1.8–2.4)</td>
</tr>
<tr>
<td>Duration of fever for entire illness, mean (95% CI)</td>
<td>4.8 (4.2–5.3)</td>
<td>4.2 (3.7–4.7)</td>
</tr>
</tbody>
</table>

Dengue serotype (primary/secondary)a
- Dengue-1: 5 (1/4) vs. 9 (6/3)
- Dengue-2: 8 (0/8) vs. 5 (1/3)
- Dengue-3: 9 (3/6) vs. 14 (11/3)
- Dengue-4: 0 vs. 0
- Not determinedb: 0 vs. 1 (0/1)

NOTE. CI, confidence interval.
a Serologic diagnosis of primary and secondary flavivirus infection were determined as in Materials and Methods. In 1 case (dengue type 2 by both mosquito inoculation and dengue reverse transcription-polymerase chain reaction [RT-PCR]), serologic response was indeterminate as outpatient follow-up day 9 specimen was not available for study.
b Attempts at virus isolation and detection of virus by RT-PCR (see Methods) were not successful.

As compared with controls.

However, the mean absolute counts of CD4 T cells, CD8 T cells, CD16/56-expressing lymphocytes (NK cells), and γδ T cells were significantly lower in children with DHF than in those with DF, and both groups had significantly lower counts than controls (table 3). The percentage of B lymphocytes in children with DHF was significantly higher than that in controls (P < .001).

One day after defervescence, the absolute counts of all lymphocyte subsets increased in children with DHF and in those with DF (table 3). The percentage of B lymphocytes was significantly higher in children with DHF compared with those with DF (P < .05), and both groups had significantly higher percentages of B cells than controls.

Quantitation of activated lymphocyte subsets. On study day 2, the percentage of cells expressing CD69 among T cells, CD8 T cells, and CD16/56-expressing lymphocytes (NK cells) was significantly higher in children later found to have DHF than in those with DF (table 4). The percentage of CD69 expression on these cell types remained elevated on fever day +1 (P < .001 vs. controls), but the levels of expression no longer differed between children with DHF and those with DF. Representative data for CD69 expression on NK and CD8 T cells are shown in figure 1. There were no significant increases in CD69 expression on B lymphocytes or monocytes in children with DHF or DF compared with controls (data not shown). There were also no significant differences in percent expression of CD25 on CD4 or CD8 T cells or NK cells between children with DHF, DF, and controls.

Table 2. Mean values of total white blood cells (WBC) and lymphocyte counts expressed in percentage and absolute cell count.

<table>
<thead>
<tr>
<th></th>
<th>Study day 2</th>
<th>Fever day +1</th>
<th>Study day 2</th>
<th>Fever day +1</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total WBCs, absolute countb</td>
<td>2605 ± 878c</td>
<td>4489 ± 2101f</td>
<td>3214 ± 1702e</td>
<td>3459 ± 12178</td>
<td>7281 ± 1839</td>
</tr>
<tr>
<td>Typical lymphocytes</td>
<td>30.6 ± 12.2</td>
<td>44.1 ± 11.8</td>
<td>40.6 ± 14.6</td>
<td>43.9 ± 13.4</td>
<td>39.9 ± 11.9</td>
</tr>
<tr>
<td>% total WBCd</td>
<td>811 ± 539c</td>
<td>1987 ± 795f</td>
<td>1134 ± 591c</td>
<td>1444 ± 560d</td>
<td>2883 ± 1162</td>
</tr>
<tr>
<td>Atypical lymphocytes</td>
<td>5.3 ± 4.0</td>
<td>20.7 ± 8.48</td>
<td>5.2 ± 4.7d</td>
<td>19.4 ± 10.8e</td>
<td>2.95 ± 2.4</td>
</tr>
<tr>
<td>% total WBCd</td>
<td>127 ± 106c</td>
<td>996 ± 754f</td>
<td>160 ± 229</td>
<td>732 ± 627d</td>
<td>217 ± 174</td>
</tr>
<tr>
<td>Total monocytes</td>
<td>6.1 ± 4.6</td>
<td>5.2 ± 2.9</td>
<td>5.3 ± 3.4</td>
<td>5.5 ± 2.3</td>
<td>4.7 ± 2.2</td>
</tr>
<tr>
<td>% total WBCd</td>
<td>181 ± 162c</td>
<td>226 ± 196d</td>
<td>168 ± 145c</td>
<td>193 ± 119d</td>
<td>334 ± 197</td>
</tr>
</tbody>
</table>

NOTE. CI, confidence interval.
a Study day 2 vs. study day 1.
b Control specimens were obtained from study volunteers at healthy 6-month follow up visit.
c Results expressed as mean cells/mm³ ± SD.
d Results expressed as mean count/mm³ ± SD.
e P < .001 vs. DHF or DF and controls.
f Results expressed as mean percent total WBC ± SD.
g P < .05, DHF vs. DF.
h P < .05 between DHF or DF and controls.

Discussion

We believe that this study is the first to demonstrate direct evidence of early immune activation of lymphocytes in children with DHF, compared with those with DF. We found a significa-
Figure 1. CD69 activation in child with dengue hemorrhagic fever (DHF) and child with dengue fever (DF). A, Two-color analysis of CD69 expression in CD16+/CD56+ lymphocytes (NK cells) in lysed fresh whole blood specimens from child with DHF and child with DF on study day 2, fever day +1, and at convalescent visit (healthy 6-month follow-up). B, Three-color analysis of CD69 expression in CD3+/CD8+ lymphocytes in lysed fresh whole blood specimens from same children with DHF and DF. T cells were gated by CD3 staining. Data in brackets, % of CD16+/CD56+ or CD3+/CD8+ lymphocytes staining for CD69. PE, phycoerythrin; FITC, fluorescein isothiocyanate.
CD69 Expression in Acute Dengue

Our previous studies demonstrated early immune activation in DHF, characterized by elevated levels of soluble CD8, soluble IL-2 receptors, interferon (IFN)−γ, TNF−α, and soluble TNF receptors in plasma from children with dengue [6, 7]. The present findings extend these results and give insight to the potential cellular sources of these proteins. T and NK cells express CD8 and IL-2 receptors [25–27] and can produce IFN−γ upon activation [28]. Activated T cells can produce TNF−α [29] and shed soluble TNF receptors [30]. γδ T cells can produce early bursts of IFN−γ and can prime macrophages to produce TNF−α [31].

We have confirmed reports by others [32–35] that the absolute lymphocyte count, CD4 and CD8 T cell subsets, and NK cell counts are significantly decreased during acute DHF.

Table 3. Lymphocyte phenotype marker analysis in acute whole blood specimens from children hospitalized with dengue hemorrhagic fever (DHF) and dengue fever (DF).

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Study day 2</th>
<th>Fever day +1</th>
<th>Study day 2</th>
<th>Fever day +1</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4 T</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% total lymphocytes</td>
<td>30.1 ± 6.1</td>
<td>29.9 ± 5.7</td>
<td>29.0 ± 6.2</td>
<td>28.0 ± 5.8e</td>
<td>32.1 ± 5.2</td>
</tr>
<tr>
<td>Absolute count</td>
<td>281 ± 184f</td>
<td>834 ± 357</td>
<td>379 ± 195d</td>
<td>561 ± 258f</td>
<td>1004 ± 428</td>
</tr>
<tr>
<td>CD8 T</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% total lymphocytes</td>
<td>24.1 ± 5.6b</td>
<td>29.8 ± 5.3</td>
<td>26.5 ± 6.8e</td>
<td>33.9 ± 9.6</td>
<td>30.9 ± 5.4</td>
</tr>
<tr>
<td>Absolute count</td>
<td>208 ± 144g</td>
<td>829 ± 386</td>
<td>329 ± 134e</td>
<td>732 ± 387</td>
<td>962 ± 442</td>
</tr>
<tr>
<td>CD16/CD56 NK</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% total lymphocytes</td>
<td>18.6 ± 9.0</td>
<td>15.1 ± 5.5</td>
<td>23.1 ± 8.2</td>
<td>20.5 ± 7.0</td>
<td>20.2 ± 6.8</td>
</tr>
<tr>
<td>Absolute count</td>
<td>164 ± 97g</td>
<td>430 ± 258</td>
<td>306 ± 207f</td>
<td>391 ± 128f</td>
<td>609 ± 275</td>
</tr>
<tr>
<td>CD4 T</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% total lymphocytes</td>
<td>6.7 ± 2.5</td>
<td>5.3 ± 2.3</td>
<td>6.8 ± 4.0</td>
<td>7.3 ± 3.7</td>
<td>9.4 ± 6.5</td>
</tr>
<tr>
<td>Absolute count</td>
<td>61 ± 38f</td>
<td>149 ± 90f</td>
<td>87 ± 57f</td>
<td>124 ± 51f</td>
<td>275 ± 154</td>
</tr>
</tbody>
</table>

NOTE. Fever day +1 is day after defervescence.

Table 4. Percent expression of activation markers on lymphocyte subsets in acute whole blood specimens from children hospitalized with dengue hemorrhagic fever (DHF) and dengue fever (DF).

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Activation marker</th>
<th>Study day 2</th>
<th>Fever day +1</th>
<th>Study day 2</th>
<th>Fever day +1</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4 T</td>
<td>CD69</td>
<td>4.9 ± 1.9g</td>
<td>2.6 ± 1.1b</td>
<td>3.8 ± 2.3</td>
<td>3.6 ± 2.1b</td>
<td>1.1 ± 5.1</td>
</tr>
<tr>
<td></td>
<td>CD25</td>
<td>16.5 ± 0.7</td>
<td>13.4 ± 0.8</td>
<td>16.7 ± 0.9</td>
<td>15.8 ± 0.6</td>
<td>12.3 ± 0.03</td>
</tr>
<tr>
<td>CD8 T</td>
<td>CD69</td>
<td>22.5 ± 0.8h</td>
<td>12.2 ± 6.3b</td>
<td>17.0 ± 7.0b</td>
<td>13.9 ± 10.0b</td>
<td>3.3 ± 1.9</td>
</tr>
<tr>
<td></td>
<td>CD25</td>
<td>0.1 ± 0.07</td>
<td>2.0 ± 0.8</td>
<td>0.07 ± 0.09</td>
<td>1.2 ± 0.4</td>
<td>1.1 ± 0.6</td>
</tr>
<tr>
<td>CD16/CD56 NK</td>
<td>CD69</td>
<td>59.8 ± 15b</td>
<td>46.3 ± 16.0b</td>
<td>45.2 ± 17b</td>
<td>36.0 ± 21.9b</td>
<td>6.7 ± 3.4</td>
</tr>
<tr>
<td></td>
<td>CD25</td>
<td>2.1 ± 0.6</td>
<td>2.8 ± 0.08</td>
<td>1.4 ± 0.02</td>
<td>2.7 ± 0.5</td>
<td>1.7 ± 0.7</td>
</tr>
<tr>
<td>γδ T</td>
<td>CD69</td>
<td>40.2 ± 10.4b</td>
<td>31.1 ± 11.3b</td>
<td>33.0 ± 13.8b</td>
<td>30.3 ± 14.4b</td>
<td>4.2 ± 2.3</td>
</tr>
</tbody>
</table>

NOTE. Results expressed as mean percent total lymphocyte count ± SD. Fever day +1 is day after defervescence.

a Control specimens were obtained from study volunteers at healthy 6-month follow up visit.

b P < .001 between DHF and DF.

c P < .05 between DHF or DF.
Children in our study were enrolled with fever of \( \leq 72 \) h; therefore, we could extend these findings by demonstrating that these counts were lower early in illness in children with DHF than in those with DF.

The decrease in circulating lymphocytes during acute DHF may relate to the high level of cellular activation. In a murine influenza model, activated CD8 T cells were found to migrate to the liver and undergo apoptosis in the liver [36]. It is possible that in dengue, antigen-specific T cells and other activated cells migrate to tissues such as lymph nodes, spleen, and liver to clear dengue virus–infected cells and that these cells then undergo apoptosis at these distant sites. The mildly elevated levels of the enzymes alanine aminotransferase and aspartate aminotransferase that are typically associated with acute dengue [18] may reflect this migration and apoptosis.

On the day following defervescence, atypical lymphocytosis was present in children with DHF and DF. This finding has been reported by others [34, 37, 38] and was proposed by Boonpucknavig et al. [33] to represent B cells. Our study confirms that there is an increase in the proportion of B cells on the day after defervescence. In addition, when we gated on the “blast”–appearing cells, we found a mixed population of B and non-B lymphocytes (data not shown). Similarly, when we gated on the CD69-expressing cells, we found that the majority of cells were small by forward and side scatter analysis (data not shown). We hypothesize that the small early activated CD69-expressing CD4 T, CD8 T, NK, and γδ T lymphocytes progress into these blast forms in addition to the B cell blasts previously reported.

We previously showed that human PBMC are cytotoxic for dengue-infected Raji cells [39]. Homchampa et al. [35] found evidence of NK cell cytotoxicity in fresh cells from children with acute dengue and that the level of cytotoxicity was related to disease severity. Our present study demonstrates that the NK cell activation occurs early in disease and that the level of activation is higher in children with DHF than in those with DF.

This is the first report that absolute counts of γδ T cells are lower in children with DHF than in those with DF and that counts in children with both DHF and DF are lower than in healthy controls. In addition, we detected significant expression of CD69 on γδ T cells. The role of this cell population in human disease is not clear. However, one study reported that human γδ T cells exhibit herpes simplex virus–specific cytotoxicity that is not restricted by classical HLA class I or II molecules [40]. We hypothesize that dengue virus–infected cells can also induce γδ T cell–mediated cytotoxicity.

In conclusion, this study provides direct evidence that cellular immune activation occurs in the course of acute dengue. Our findings support our previous reports of elevations in cytokines and soluble receptors [6, 7] and suggest that the degree of activation early in clinical illness is related to ultimate disease severity. Further research to identify the possible etiology of these differences in cell activation between the DHF and DF populations, such as HLA haplotype or specific viral epitopes, is needed.

Acknowledgments

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References