Early Immune Activation in Acute Dengue Illness Is Related to Development of Plasma Leakage and Disease Severity

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T lymphocyte activation and increased cytokine levels have been described in retrospective studies of children presenting with dengue hemorrhagic fever (DHF). Serial plasma samples obtained in a prospective study of Thai children presenting with <72 h of fever were studied. Plasma levels of 80-kDa soluble tumor necrosis factor receptors (sTNFRs) were higher in children who developed DHF than in those with dengue fever (DF) or other nondengue febrile illnesses (OFIs) and were correlated with the degree of subsequent plasma leakage. Soluble CD8 and soluble interleukin-2 receptor levels were also elevated in children with DHF compared with those with DF. Interferon-γ and sTNFR 60-kDa levels were higher in children with dengue than in those with OFIs. TNF-α was detectable more often in DHF than in DF or OFIs (\(P < .05\)). These results support the hypothesis that immune activation contributes to the pathogenesis of DHF. Further studies evaluating the predictive value of sTNFR80 for DHF are warranted.

Dengue viruses are arthropodborne flaviviruses that cause significant morbidity and mortality in tropical and subtropical regions of the world. There are four serotypes (dengue 1–4) of dengue viruses. Classical dengue fever (DF) is a self-limited illness characterized by fever, headache, myalgia, arthralgia, and abdominal pain. Since the 1950s, a more severe form of the disease, dengue hemorrhagic fever (DHF), has been recognized. Patients who develop DHF present clinically in a similar fashion to DF patients, but around the time of deferves-

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2 receptor (sIL2R), in the sera of children with DHF compared with DF, and elevated levels of IL-2 and interferon (IFN)-γ in children with dengue infections compared with healthy controls [12]. Other investigators have found elevated levels of tumor necrosis factor (TNF)-α, IL-1β, IL-6, and sTNF receptor p75 in patients with severe dengue illness [13–18]. In those reports, samples were obtained late in the course of illness, patient numbers were usually small, serial samples were seldom studied, and there were no concurrently enrolled comparison groups with other febrile illnesses.

The purpose of this study was to investigate the levels of cytokines and soluble receptors in children with dengue prior to the overt manifestations of DHF in order to elucidate the immunologic pathways that ultimately lead to plasma leakage. The duration of fever in dengue is typically 3–5 days, and plasma leakage tends to occur at or around the time of defervescence. We therefore enrolled children with fever of <72 h duration so that our observations would precede the period of maximal plasma leakage [19]. As a result of this design, the study included a cohort of controls with other nondengue febrile illnesses (OFIs), enabling us to determine whether the observed phenomena were specific to dengue virus infection.

Methods

Study design. The Dengue Hemorrhagic Fever Project enrolled Thai children aged 6 months to 14 years with fever of <72 h duration and no obvious source of infection [19]. Children were hospitalized, observed without study-specific intervention, and had a venous blood sample drawn daily until the day after defervescence and an outpatient blood sample obtained between study days 8 and 11. On the day after defervescence, a right lateral decubitus chest radiograph was obtained. The pleural effusion index (PEI) was calculated as follows: 

\[
\text{PEI} = \left( \frac{\text{width of effusion}}{\text{width of hemithorax}} \right) \times 100
\]

A total of 189 children were enrolled between 25 April and 14 December 1994. Sixty patients had dengue (28 with DHF, 32 with DF) and 112 children had OFIs (presumed to be viral). A subset of these enrolled subjects was evaluated in the present study (see below).

Study definitions. Study day 1 was the day a child was enrolled in the study. Fever day 0 was the day of defervescence, when the temperature dropped below 38°C without a subsequent elevation. Days before fever day 0 are designated fever day −1, −2, etc. A clinical diagnosis of DHF and severity grading (grades 1–4) were assigned according to WHO criteria [20]. All children with the clinical diagnoses of DF or DHF had evidence of acute dengue virus infection by dengue IgM ELISA [21], hemagglutination inhibition (HAI) antibody responses [22], or dengue virus isolation in Toxorhynchites splendens mosquitoes [23–25]. Any subject with evidence of acute dengue infection who did not meet criteria for DHF was assigned a clinical diagnosis of DF. OFIs were defined as those patients who had no dengue virus–specific IgM or HAI antibody responses, no dengue virus isolated from plasma, and no obvious bacterial, rickettsial, or protozoal etiology for their illness and were presumed to most likely have self-limited viral illnesses.

Sample processing. Blood was drawn into EDTA tubes (Becton Dickinson, Franklin Lakes, NJ), immediately placed on ice, and transported to the blood processing laboratory. Samples were maintained at 4°C throughout processing. Samples were initially centrifuged at 300g for 10 min; platelet-rich plasma was transferred to polystyrene centrifuge tubes (Becton Dickinson) and centrifuged at 800g for 10 min. The platelet-poor plasma was divided into aliquots and frozen at −70°C until analysis.

Assays for IL-1β, TNF-α, IL-6, IL-4, IFN-γ, sIL2R, sCD8, sCD4, sTNFR60, and sTNFR80. Cytokines and receptors were measured by commercial ELISAs (IL-1β and TNF-α: Cistron Biotechnologies, Pine Brook, NJ; IL-4, IL-6, and IFN-γ: Endogen, Cambridge, MA; sCD4, sCD8, and sIL2R: T Cell Diagnostics, Cambridge, MA; sTNFR60 and sTNFR80 [for measurement of sTNFR-55 and -75 kDa, respectively]: Bender MedSystems, Vienna) according to manufacturers’ recommendations. The lower limits of detection were as follows: IL-1β, <2 pg/mL; TNF-α, <10 pg/mL; IL-4 and -6, <1 pg/mL; IFN-γ, <5 pg/mL; sCD4, 1.1 U/mL; sCD8, 50 U/mL; sIL2R, 24 U/mL; sTNFR60, <80 pg/mL; and sTNFR80, <150 pg/mL. Samples with levels above the maximum optical density were diluted and retested.

Sample selection. A subset of study subjects was selected from each of the three diagnostic categories: DHF, DF, and OFIs for immunoassay testing. Because the volume of plasma obtained was limited, specimens from the same patients could not be assayed for all immune response parameters. These subset populations were selected without knowledge of clinical data other than final diagnosis. For each immunoassay, the ranges of patients tested were as follows: DHF, 17–24; DF, 13–20; OFIs, 8–20. We tested 2–6 serial specimens for each subject in a given immunoassay. For each subject, we included samples from study day 1, fever days −1 and 0, and the next outpatient follow-up visit whenever available. Plasma samples from a 6-month follow-up visit from study subjects with acute dengue virus infection were tested in each immunoassay as healthy controls (n = 14–19). All samples were tested under code.

Statistical analysis. Mean plasma levels of the measured parameters were initially compared by two-factor analysis of variance (ANOVA) using final diagnosis (DHF, DF, or OFIs) and fever day. When significant differences were noted between groups based on final diagnosis, we tested for significant differences on each fever day with general linear models, an extension of ANOVA, using conservative imputation procedures. Comparisons were made between all subjects with acute dengue virus infection (combined DF and DHF) and those with OFIs and also between subjects with DHF and DF. We used both Pearson’s and Spearman’s correlations. Raw values were log transformed to stabilize the variances. Differences between diagnostic groups in the proportion of samples with detectable levels of TNF-α were tested using Fisher’s exact test. Due to the small number of primary dengue cases (4/60), an analysis comparing subjects with primary and secondary dengue infection was not done. Analyses were done on the Harvard School of Public Health computer using an SAS package [26, 27]. P < .05 was considered significant.

Results

Clinical data at study entry. Clinical findings for this study have been reported [19]. The subset population included 26
children with DHF, 24 with DF, and 27 with OFIs. Among the children with DHF, 5 (19%) were grade 1, 12 (46%) were grade 2, 9 (35%) were grade 3, and none were grade 4. Four children had primary dengue virus infection, and all 4 were classified as DF. There were no significant differences between subjects selected for study and those not selected for study in any diagnostic group for sex, age, duration of fever before enrollment, symptoms, platelet count, hematocrit level, absolute monocyte
Mean plasma sTNFR80 levels were significantly higher in children with DHF than DF from 2 days before defervescence ($P < .01$) until the day of defervescence ($P < .01$). Mean plasma levels of sTNFR80 and sTNFR60 were significantly elevated in children with dengue compared with those with OFIs on the same days ($P < .001$ and $< .05$, respectively) (figures 1, 2). Levels of sTNFR80 measured 2 days before defervescence in children with dengue virus infections correlated with the pleural effusion indices measured 1 day after defervescence (Pearson’s $r = 0.788$, $P = .0001$; Spearman’s $r = 0.620$, $P = .0003$) (figure 3).

**IFN-γ levels.** Mean plasma IFN-γ levels were significantly higher in children with dengue than in those with OFIs as early as fever day –2 ($P < .05$), and this difference became more apparent on fever days –1 and 0 ($P < .05$ and $< .001$, respectively). Mean IFN-γ levels were not significantly different in patients with DHF than in those with DF on any given fever day. However, we noted that IFN-γ levels increased and decreased abruptly in patients with DHF and that by fever day +1 (after defervescence), the mean level in children with DHF (20.9 pg/mL) was similar to that of children with DF (19.0 pg/mL) or OFIs (16.2 pg/mL). The day of peak IFN-γ production for all patients occurred before or on the day of defervescence (figure 4).

**TNF-α, IL-1β, IL-4, and IL-6 levels.** There were no differences between groups in mean plasma levels of TNF-α, IL-1β, IL-4, or IL-6 (data not shown). However, the proportion of samples with detectable TNF-α was greater in children with DHF than in those with DF or OFIs (table 1). By Fisher’s exact test, the difference in the proportion of samples with
Table 1. Proportion of samples with detectable levels of tumor necrosis factor (TNF)-α.

<table>
<thead>
<tr>
<th>Fever day</th>
<th>Diagnosis</th>
<th>0/2</th>
<th>0/10</th>
<th>3/12</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Other febrile illnesses</td>
<td>1/18</td>
<td>1/19</td>
<td>2/16</td>
</tr>
<tr>
<td>1</td>
<td>Dengue fever (DF)</td>
<td>1/17</td>
<td>2/11</td>
<td>4/25</td>
</tr>
<tr>
<td>2</td>
<td>Dengue hemorrhagic fever (DHF)</td>
<td>1/13</td>
<td>1/6</td>
<td>5/19</td>
</tr>
</tbody>
</table>

NOTE: No. of specimens with detectable TNF-α/total specimens tested (%) comparison of DHF vs. DF by Fisher’s exact test (P < .05 for fever day 0 and <.10 for fever days −2 and −1 combined.

detectable TNF-α was statistically significant on the day of defervescence (P < .05), and there was a trend toward statistical significance before defervescence (P < .10). Logistic regression analysis showed that the proportion of samples with detectable TNF-α was significantly higher (P < .05) in subjects with DHF over this whole time period.

sIL2R, sCD8, and sCD4 levels. Levels of sIL2R and sCD8 were significantly higher in children with dengue than in children with OFIs on the day of defervescence (P < .05) (figures 5, 6). There was a trend (P < .10) toward higher levels of sIL2R and sCD8 in children with DHF compared with those with DF from as early as fever day −1, and this achieved statistical significance on the day following defervescence (P < .001 and < .05, respectively). sCD4 levels were not significantly different between children with OFIs, DF, or DHF (data not shown).

Cytokine-to-albumin ratios. In the 1998 study of Bethell et al. [18], cytokine concentrations were adjusted to albumin levels in order to account for the translocation of small proteins during plasma leakage. Our data were analyzed as a ratio of cytokine-to-albumin levels (data not shown). No new associations were noted using these procedures, but the relationship between sIL2R and sCD8 and disease severity (DF vs. DHF) were strengthened. The sIL2R:albumin ratio was significantly higher in children with DHF than in those with DF on fever days −3, −1, and 0 (P < .05). Similarly, the sCD8:albumin ratio was significantly higher in children with DHF than in those with DF 1 day before and on the day of defervescence (P < .01). There was no change in the relationship between sTNFR80 and disease severity utilizing this ratio.

Discussion

The prospective design of the present study permitted us to measure cytokines and T cell activation markers early in the course of dengue illness and to evaluate the kinetics of these responses in individual patients during the course of their illness. Samples from a cohort of children with nondengue febrile illnesses were available for comparison. While circulating levels of cytokines may or may not reflect secretion at the tissue level, our study design enabled us to evaluate the relationship of these markers to acute dengue virus infection and to the development of the plasma leakage seen in DHF.

The major findings of this study were the demonstration of higher levels of sTNFR80, IFN-γ, sCD8, and sIL2R and the more frequent detection of TNF-α in children who developed DHF during the course of the study (table 2). Activated T cells release TNF-α [28], IFN-γ [29], sTNF receptors [30], sIL2R [31], and sCD8 [32, 33]. Our present findings of higher levels of sIL2R and sCD8 in children who develop DHF are consistent with those of our prior study in Thai children later in the course of dengue infections [12] and support a role for CD8+ T cell activation in the pathogenesis of DHF. In addition, we have found higher levels of sTNFR80 and IFN-γ and were more frequently able to detect TNF-α in children with DHF, which indicates that immune activation precedes defervescence and the onset of plasma leakage and that the increased immune activation is associated with the severity of dengue illness. Although others have reported elevated levels of sTNF receptors [17, 18] and TNF-α in DHF [13, 14, 16], our study is the first to compare serial levels in children with DF and DHF and to include a febrile control group without dengue infection.

IFN-γ levels were elevated early in children with dengue compared with children with OFIs. The curve of IFN-γ production in some children with dengue was very abrupt. This increase did not occur in all patients, but was seen more often in children with DHF than in those with DF. The most likely explanation for these results is the transient nature of cytokine production and the short plasma half-life of these compounds. Our daily blood sampling could have missed a short-lived burst of IFN-γ activity. Although peak IFN-γ levels occurred on different days, the peak occurred before or on the day of defervescence. Presumably, dengue virus–specific cross-reactive memory T cells are activated by dengue-infected APCs to produce IFN-γ early in infection [34]. The abrupt decline in IFN-γ levels after defervescence coincides with the disappearance of viremia [25] and may be due to lysis of dengue-infected APCs. Production of IL-4 and IL-10 can also down-regulate IFN-γ production, but we found no increase in IL-4 in these patients.

The elevated levels of IFN-γ, TNF-α, and sTNFRs in children with DHF are of great interest. IFN-γ and TNF-α have a synergistic effect on endothelial cell cultures in vitro by increasing monolayer permeability, which might play a role in capillary leakage [35, 36]. In addition, they activate endothelial cells as demonstrated by up-regulation of both major histocompatibility complex class I and expression of soluble intracellular adhesion molecule-1 on the cell surface, thereby enabling lymphocyte binding and migration into the tissues [37]. IFN-γ up-regulates expression of TNF receptors on myeloid and epithelial cells, which may render these cells more sensitive to the effects of TNF-α [38–40]. In vivo evidence of the interaction between these cytokines also exists. Mouse models of cerebral malaria and bacterial sepsis have demonstrated that the TNF-α-mediated morbidity or mortality is regulated by IFN-γ [41–44].

A recent study in Vietnam [18] reported that sTNFR80 >55 pg/mL had a sensitivity of 93% and a specificity of 34% for
Figure 5. Plasma soluble interleukin (IL)-2 receptors in dengue hemorrhagic fever (DHF), dengue fever (DF), and other febrile illnesses (OFIs). Fever day 0 represents day of defervescence. Horizontal lines indicate mean values. Outpt = outpatient follow-up sample at study days 8–13. OFIs vs. dengue (DHF + DF): * P < .05; DHF vs. DF: ¶ P < .10; ‡‡‡ P < .01.

 predicting the development of shock in children with suspected dengue. We were unable to assess prediction of shock as there were too few shock cases; however, we analyzed the potential value of sTNFR80 to differentiate DF and DHF. On the day of defervescence only, when we utilized a cutoff value of 1.6 ng/mL, we found a sensitivity of 94%, specificity of 25%, positive predictive value (PPV) of 53% and negative predictive value (NPV) of 83%. However, when we examined sTNFR80

Figure 6. Plasma soluble CD8 in dengue hemorrhagic fever (DHF), dengue fever (DF), and other febrile illnesses (OFIs). Fever day 0 represents day of defervescence. Horizontal lines indicate mean values. Outpt = outpatient follow-up sample at study days 8–13. OFIs vs. dengue (DHF + DF): * P < .05, *** P < .001; DHF vs. DF: ¶ P < .10, ‡ P < .05.
levels 2 days before defervescence, the same cutoff had sensitivity decreased to 67%, specificity increased to 80%, and an NPV of 69%. Regardless of the cutoff value used, the sensitivity for predicting DHF decreased earlier in the course of illness.

If febrile children in an endemic area can be rapidly diagnosed with a dengue infection (e.g., by polymerase chain reaction) [45–47], an elevated immune marker such as sTNFR80 might help identify those at highest risk for developing DHF. If children at high risk for developing DHF can be identified early in the course of disease, clinical studies of therapeutic interventions to avert plasma leakage would be feasible. Further studies of immune activation markers should be done to determine their predictive value for DHF.

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