Strong HLA Class I–Restricted T Cell Responses in Dengue Hemorrhagic Fever: A Double-Edged Sword?

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Dengue is an increasingly important cause of morbidity and mortality in the tropics, but vaccine development has been impeded by a poor understanding of disease pathogenesis and, in particular, of immunologic enhancement. In a large case-control study of Vietnamese patients with dengue hemorrhagic fever (DHF), variation at the HLA-A locus was significantly associated with susceptibility to DHF ($P = .02$), and specific HLA-A susceptibility and resistance alleles were identified. HLA-A–specific epitopes were predicted from binding motifs, and ELISPOT analyses of patients with DHF revealed high frequencies of circulating CD8 T lymphocytes that recognized both serotype-specific and cross-reactive dengue virus epitopes. Thus, strong CD8 T cell responses are induced by natural dengue virus infection, and HLA class I genetic variation is a risk factor for DHF. These genetic and immunologic data support both protective and pathogenic roles for dengue virus–specific CD8 T cell responses in severe disease. The potentially pathogenic role of serotype–cross-reactive CD8 T cells poses yet another obstacle to successful dengue vaccine development.

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Inform consent was obtained from all patients or their parents or guardians. The study was approved by the local ethics committee.

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The pathogenesis of DHF is poorly understood. The favored antibody-dependent enhancement theory of DHF pathogenesis [5] is based on epidemiologic studies demonstrating that prior infection with a different viral serotype predisposes to DHF, whereas circulating dengue virus–specific IgG antibodies represent a major risk factor for DHF [6, 7], and on the in vitro finding that dengue virus–specific IgG can enhance viral infection of cells [5]. Another possibility is that viral virulence factors might mediate DHF pathogenesis by causing tissue damage and secreting cytokines that aggravate vascular leakage, has been suggested by analyses of cross-reactivity patterns of CD8 T cell responses to dengue virus [11–13], which is consistent with the higher level of soluble CD8 in severe dengue [14]. Here, we provide support for this cellular immune mechanism of pathogenesis by genetic and immunologic analysis of Vietnamese patients with DHF.

Human genetic factors have been little studied in DHF, but the small proportion of antibody-positive persons who develop DHF [15], a possible racial difference in susceptibility [16], and 2 small studies suggesting HLA associations [17, 18] provide support for some genetic component to variable susceptibility. Dengue virus–specific antibody responses have been well studied in areas of endemicity [19]; however, dengue vaccine development has been impeded by the possibility that vaccination could actually increase disease susceptibility by producing enhancing antibodies. Little is known about cellular immune responses during dengue virus infection. Most of the available information on dengue virus–specific T cell responses derives from immunization studies with dengue candidate vaccines that induced specific CD4 and CD8 T cell responses [11, 20] in volunteers who were not from areas of endemicity. In principle, vaccines that induced mainly a protective cellular immune response might avoid the danger of antibody-dependent enhancement of DHF; however, such cellular immune responses also could contribute to the pathogenesis of DHF.

Here, we report a large case-control study in which we used molecular HLA typing of patients with DHF in Vietnam. An important role for variation in the HLA-A gene was identified,
and we proceeded to search for CD8 T cell responses that are determined by this gene. Strong immune reactivity to both conserved and strain-specific dengue virus epitopes was observed, which indicates that these HLA class I–restricted CD8 T cell responses may be relevant to both protection and immunopathology in this infectious disease.

Population, Materials, and Methods

Patients with DHF. The DHF genetic susceptibility study was carried out at Dong Nai Paediatric Centre, a provincial pediatric hospital 40 km north of Ho Chi Minh City, Vietnam. Patients with DHF grades III and IV were classified according to World Health Organization criteria [1]. A subset of patients also was diagnosed serologically, to confirm the clinical diagnosis. Patients with DHF (grades I–IV) were diagnosed clinically, and the diagnoses for a subset of ~50 patients were confirmed serologically by means of conventional capture ELISA techniques for IgM and IgG on paired serum samples. During both the genetic study undertaken in 1993–1994 and the cellular immunologic studies undertaken in 1997–1998, the predominant dengue virus serotypes were 2 and 3. Three hundred fifteen patients with DHF grade III, 37 patients with DHF grade IV, and 251 healthy control subjects were recruited. The latter were patients from the surgical wards of the same hospital without dengue and with noninfectious illnesses. All case patients and control subjects, who were ethnically matched as a group, originated from Dong Nai Province. Patients with DHF were recruited for the cellular immunology study at the Centre for Tropical Diseases, Ho Chi Minh City, Vietnam. Patients with DHF (grades I–IV) were diagnosed clinically, and a subset of patients also had a confirmatory serologic diagnosis.

Molecular genetic analyses. DNA was extracted from 3–5 mL of blood from each subject by means of Nucleon II DNA extraction kits (Scotlab). All subjects were typed for the 5 most common HLA-A alleles and 10 most frequent HLA-B alleles in this population by means of a polymerase chain reaction (PCR) phototyping method making use of sequence-specific primers [21]. All subjects were typed for the 11 most common HLA-DRB1 alleles and for single nucleotide polymorphisms at positions −238 [22] and −308 [23] of the tumor necrosis factor (TNF)–α promoter by a PCR method with sequence-specific oligonucleotide probes, as described elsewhere [24, 25]. In brief, PCR products containing the region of interest are immobilized, and the immobilized product is hybridized with 3′-digoxigenin–labeled (Boehringer Mannheim) sequence-specific oligonucleotide probes. Oligonucleotides for HLA class II alleles [24] and TNF-α polymorphisms [25] have been described elsewhere. Results were analyzed by logistic regression analysis to assess the overall effect of a locus and χ² tests for a particular HLA type by means of SPSS (SPSS) and Statcalc (EpiInfo) software packages, respectively.

Interferon (IFN)-γ ELISPOT assay. Peripheral blood mononuclear cells (PBMC) were separated from 4–8 mL of blood by use of Vacutainer CPT tubes (Becton Dickinson), were resuspended in R-10 (RPMI 1640, 10% heat-inactivated human AB serum, 100 U/mL penicillin, 100 mg/mL streptomycin, and 4 mM L-glutamine), and were assayed in Vietnam by means of an 18-h ELISPOT assay for IFN-γ secretion, as described elsewhere [26]. In brief, 96-well polyvinylidene difluoride–backed plates (MAIP, Millipore) were coated with monoclonal antibody to IFN-γ (1-DIK, 50 µL of 15 µg/mL in PBS; Mabtech), overnight at 4°C. The wells were washed 3 times with PBS, before blocking for 1 h with R-10. Peptides (final concentration, 2 µg/mL) and 3–5 × 10⁵ freshly isolated PBMC were added to each well, and wells were incubated in a humidified, 5% CO₂, 37°C chamber for 18–24 h. In each assay, mitogen leukoagglutinin (phytohemagglutinin, 2 µg/mL; Sigma) was used as the positive control, and an irrelevant peptide, pb9 (2 µg/mL, an epitope from a rodent malaria parasite), was used as a negative control.

Cells and media were removed by washing the wells 3 times with PBS, before incubating for 2 h with biotinylated IFN-γ detection antibody, 7-B6-1–biotin (1 µg/mL; Mabtech). Subsequently, PBS washing was repeated before adding alkaline phosphatase–conjugated streptavidin (1 µg/mL; Mabtech) for another 2 h. After washing, 50 µL of alkaline phosphatase conjugate substrate (BioRad) was added to visualize spots formed by IFN-γ–secreting cells. Spots were counted by eye with use of a dissecting microscope.

Dengue virus serotype 2 peptide epitopes. Nonamer peptides were selected from the dengue virus serotype 2 New Guinea C (NGC) polypeptide sequence (Genbank accession no. M29095), based on published peptide-binding motifs for HLA-A*11, A*24, and A*33 (reviewed in [27]). This motif-based selection procedure is an efficient, but not comprehensive, means of identifying new CD8 T cell epitopes and will identify only a subset of all peptides to which persons with 3–6 HLA class I molecules respond. Peptides were synthesized by use of a semiautomated peptide synthesizer (Zinsser Analytical) or were purchased from Research Genetics. A total of 47 peptides was studied: 16 were synthesized on the basis of the peptide-binding motif for HLA-A*11, 11 on the motif for HLA-A*24, and 20 on the motif for HLA-A*33.

Results

Association of HLA class I variation with DHF. Polymorphism was studied for class I, II, and III region genes of the major histocompatibility complex. These were the 2 major class I genes, HLA-A and -B, which restrict the immune response of CD8 T lymphocytes, the major HLA class II gene, HLA-DRB1, and 2 promoter polymorphisms in the class III region gene, TNF-α. To rule out the loss of statistical power associated with typing and correcting for numerous rare alleles at the HLA loci, we determined the higher-frequency alleles in this Vietnamese population in a pilot study and compared only these alleles for all case patients and control subjects. We initially assessed the overall effect of variation at the loci on the likelihood of developing DHF by logistic regression analysis (table 1).

Polymorphism at the HLA class I loci was significantly associated with DHF disease susceptibility (P = .03), but polymorphism in the HLA-DRB1 or TNF genes was not. Further analysis of the HLA class I association found that the association is confined to the HLA-A and not the HLA-B gene (P = .02) and
that 2 particular alleles were relevant (table 2). Children with HLA-A*33 (P = .01; odds ratio, 0.56; 95% confidence interval, 0.34–0.93) were less likely to develop DHF, and children with HLA-A*24 allele were at increased risk of developing DHF (P = .02; odds ratio, 1.54; 95% confidence interval, 1.05–2.25).

**Dengue virus–specific CD8 T cells in patients with DHF.**

The association of HLA-A variants with susceptibility to DHF and the lack of association of flanking genes suggested that CD8 T lymphocytes that are restricted by this locus may be relevant to disease susceptibility. However, CD8 T cells have been characterized from only a few naturally infected persons [13], and no T cells restricted by HLA-A*24 or -A*33 have been identified. We thus aimed to search for such T cells with peptide recognition and subsequent IFN-γ release are dependent on CD8 T cells (data not shown). Response rates (number of positive responses/number of peptides tested) were higher to peptides from the NS3 (6/21) and NS5 proteins (3/8) than to other antigens (0/18).

Of the 47 peptides studied, 3 peptides from the NS3 protein and 2 from NS5 protein were recognized by CD8 T lymphocytes of patients with DHF (table 3). Comparison of the sequence of the dengue virus serotype 2 NGC peptide epitopes with sequences from different geographic strains of dengue virus serotype 2, other dengue virus serotypes, and other flaviviruses showed that peptide 11-13 from the NS3 protein is conserved between all dengue virus serotypes but is not shared with other flaviviruses and that the NS3 peptide 24-5 and the NS5 peptide 11-20 are conserved among different geographic strains of dengue virus serotype 2 but not between among serotypes. Peptide 33E is conserved among strains of dengue virus serotype 2 and in dengue virus serotype 4 but not in dengue virus serotype 1 and 3 sequences. Peptide 11-18 is shared among some geographic strains of dengue virus serotype 2 and also dengue virus serotype 1. Thus, there are varying degrees of conservation of these CD8 T lymphocyte epitopes between dengue virus strains and serotypes, but none are found in related flaviviruses, such as Japanese B encephalitis and yellow fever viruses.

**Discussion**

Studies of DHF pathogenesis have focused mainly on the phenomenon of antibody-dependent enhancement and, to a lesser extent, viral virulence. We show here that polymorphism in the HLA class I region, particularly of the HLA-A gene, is a significant determinant of genetic susceptibility to DHF. This is the first study to document an overall effect of HLA class I locus variation on susceptibility to an infectious disease. These Vietnamese data on HLA class I associations with DHF support the relevance of class I variation suggested by 2 previous small studies [17, 18], even though the allelic associations in each study differ, possibly as a result of viral and other host gene polymorphism. Collectively, these genetic data underscore the potential importance of HLA class I–restricted responses in DHF.

A role for cytotoxic T lymphocytes in DHF pathogenesis has been discussed elsewhere [11], but there has been remarkably little characterization of cellular immune responses during natural dengue virus infections, especially of CD8 T cell responses. Here, we document and characterize circulating dengue virus–specific CD8 T cells during the acute and convalescent phases of DHF. The circulating frequencies of dengue virus peptide-specific CD8 T cells (1 : 3900–1 : 34,500) are comparable to those seen in other acute viral diseases, such as influenza virus infection (1 : 6000–1 : 111,000) [26], but lower than in chronic viral infections, such as that due to Epstein-Barr virus (1 : 100–

**Table 1.** Polymorphism at class I, II, and III loci in the major histocompatibility complex and susceptibility to dengue hemorrhagic fever.

<table>
<thead>
<tr>
<th>Class, locus</th>
<th>No. of alleles studied</th>
<th>df</th>
<th>Residual χ²</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class I</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLA-A and B</td>
<td>15</td>
<td>15</td>
<td>26.767</td>
<td>.0307</td>
</tr>
<tr>
<td>HLA-A</td>
<td>5</td>
<td>5</td>
<td>13.335</td>
<td>.0204</td>
</tr>
<tr>
<td>HLA-B</td>
<td>10</td>
<td>10</td>
<td>14.780</td>
<td>.1403</td>
</tr>
<tr>
<td>Class II, HLA-DR</td>
<td>11</td>
<td>11</td>
<td>10.083</td>
<td>.5229</td>
</tr>
<tr>
<td>Class III, TNF-α</td>
<td>4</td>
<td>4</td>
<td>4.628</td>
<td>.3277</td>
</tr>
</tbody>
</table>

NOTE. Boldface indicates statistical significance. TNF-α, tumor necrosis factor-α.

**Table 2.** HLA-A variation and susceptibility to dengue hemorrhagic fever.

<table>
<thead>
<tr>
<th>HLA-A allele</th>
<th>Case patients (n = 309)</th>
<th>Control subjects (n = 251)</th>
<th>Odds ratio (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>131 (42.4)</td>
<td>124 (49.4)</td>
<td>0.98</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>151 (48.9)</td>
<td>115 (45.8)</td>
<td>0.472</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>108 (35.0)</td>
<td>65 (25.9)</td>
<td>1.54 (1.05–2.25)</td>
<td>.021</td>
</tr>
<tr>
<td>29</td>
<td>45 (14.6)</td>
<td>42 (16.7)</td>
<td>0.481</td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>34 (11.0)</td>
<td>46 (18.3)</td>
<td>0.56 (0.34–0.93)</td>
<td>.014</td>
</tr>
</tbody>
</table>

NOTE. Data are no. (%) of case patients or control subjects with allele. Boldface indicates statistical significance. CI, confidence interval.
We have identified 5 new epitopes of dengue virus serotype 2 that are recognized by CD8 T lymphocytes of patients with DHF. In keeping with studies in mice [29] and of human vaccinees [30], the NS3 protein appears to be the most immunogenic antigen for cellular responses to dengue and other flaviviruses. Epitopes were also found in the NS5 protein, which is the most conserved flavivirus protein and possibly is important for viral replication [31].

This combined genetic and immunologic study suggests an important role for CD8 T cell responses in dengue virus infections and development of DHF. The data suggest that T cells may contribute both to protection against and development of DHF. A protective role for CD8 T cells is plausible, because these cells may limit viral infection by cytolytic destruction of infected cells and secretion of antiviral cytokines such as IFN-γ. The mechanism by which T cells contribute to DHF pathogenesis is not clear. However, serotype cross-reactivity is likely to be important and has been demonstrated for CD8 T cell responses to dengue virus in both mice [12] and humans [13, 32], with cross-reactivity demonstrated to both completely and incompletely conserved epitopes. Inflammatory cytokines secreted by CD8 T cells could directly affect vascular permeability [11]; epitopes from dengue virus might mimic host proteins, resulting in excessive tissue damage; or more complex interactions between virus strains and serotypes could result in altered peptide-ligand antagonism, impairing protective virus-specific T cell responses [33]. Further studies of CD8 T cell responses to natural infection will be required to assess these possibilities.

These observations have particular relevance to dengue vaccine design. The specter of antibody-dependent enhancement after vaccination has generated interest in designing dengue vaccines that might induce only protective T cell responses, comprising, for example, a string of CD8 T cell epitopes. However, the finding of HLA class I susceptibility associations with severe dengue suggests that, in addition to antibody-dependent enhancement, vaccine-induced cross-reactive T cell responses could also result in a predisposition to DHF. This concern may apply to new subunit vaccines currently in clinical trials [34], and T cell cross-reactivity with the currently deployed flavivirus vaccines also may be relevant [35]. On the other hand, our data also support an important protective role for CD8 T cell response that may be exploitable for vaccine development. The critical question is whether protective responses can be induced without predisposing a minority to more severe disease.

Acknowledgments

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References


Table 3. CD8 T lymphocyte responses to dengue virus in patients with dengue hemorrhagic fever (DHF).

<table>
<thead>
<tr>
<th>Peptide label (epitope sequence), patient</th>
<th>HLA-A type(s)</th>
<th>HLA-B type(s)</th>
<th>Age, years</th>
<th>Sex</th>
<th>DHF grade</th>
<th>Time from DHF onset to study point, weeks</th>
<th>Dengue virus protein</th>
<th>Peptide-specific response, sfc/10⁶ PBMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>11-13 (TVWFVPSIK)</td>
<td>DID 109</td>
<td>1, 2</td>
<td>57, 46</td>
<td>16</td>
<td>F</td>
<td>III</td>
<td>10</td>
<td>NS3</td>
</tr>
<tr>
<td>24-5 (NYADRRWCF)</td>
<td>DID 22</td>
<td>24</td>
<td>35</td>
<td>10</td>
<td>F</td>
<td>III</td>
<td>16</td>
<td>NS3</td>
</tr>
<tr>
<td></td>
<td>DID 63</td>
<td>2, 24</td>
<td>46, 35</td>
<td>7</td>
<td>F</td>
<td>III</td>
<td>11</td>
<td>NS3</td>
</tr>
<tr>
<td></td>
<td>DID 122</td>
<td>2, 11</td>
<td>60</td>
<td>30</td>
<td>M</td>
<td>II</td>
<td>Acute</td>
<td>NS3</td>
</tr>
<tr>
<td></td>
<td>DID 93</td>
<td>24</td>
<td>35</td>
<td>5</td>
<td>F</td>
<td>III</td>
<td>4</td>
<td>NS5</td>
</tr>
<tr>
<td>11-20 (YILRDVSKK)</td>
<td>DID 93</td>
<td>24</td>
<td>35</td>
<td>5</td>
<td>F</td>
<td>III</td>
<td>4</td>
<td>NS5</td>
</tr>
<tr>
<td></td>
<td>DID 89</td>
<td>ND</td>
<td>ND</td>
<td>10</td>
<td>F</td>
<td>III</td>
<td>8</td>
<td>NS5</td>
</tr>
<tr>
<td>11-18 (DVFFTPPEK)</td>
<td>DID 206</td>
<td>2, 11</td>
<td>ND</td>
<td>Child</td>
<td>ND</td>
<td>ND</td>
<td>Acute</td>
<td>NS5</td>
</tr>
<tr>
<td></td>
<td>DID 95</td>
<td>1, 33</td>
<td>57, 58</td>
<td>14</td>
<td>M</td>
<td>III</td>
<td>7</td>
<td>NS3</td>
</tr>
</tbody>
</table>

NOTE. Patients are grouped according to the peptide epitope recognized by their T cells. The first 2 digits of the peptide label correspond to the HLA-A type to which the peptide was predicted to bind. Peptide-specific T cells were enumerated as interferon-γ-secreting spot-forming cells (sfc) per million peripheral blood mononuclear cells (PBMC). ND, not determined.

* Patient DID 95’s cells were frozen, thawed, and cultured with peptide for 14 days before assay; all others were done on freshly isolated cells.