Dengue in the Early Febrile Phase: Viremia and Antibody Responses

David W. Vaughn, Sharone Green, Siripen Kalayanarooj, Bruce L. Innis, Suchitra Nimmannitya, Saroj Suntayakorn, Alan L. Rothman, Francis A. Ennis, and Ananda Nisalak

A multicenter effort was begun in 1994 to characterize the pathophysiology of dengue using a study design that minimized patient selection bias by offering enrollment to all children with undifferentiated fever for <72 h. In the first year, 189 children were enrolled (age range, 8 months to 14 years). Thirty-two percent of these children had dengue infections (60 volunteers). The percentage of children with a secondary dengue infection was 93%, with only 4 (7%) having a primary dengue infection. The virus isolation rate from the plasma of children with dengue was 98%. Viremia correlated highly with temperature. All four dengue virus serotypes were isolated at both study sites. This study demonstrates that all four serotypes of dengue virus can cause dengue hemorrhagic fever, that all dengue patients as defined by serology experience viremia during the febrile phase, and that as fever subsides, so does viremia.

Dengue is a major cause of morbidity in tropical and subtropical areas. It is estimated that >250,000 cases of severe dengue infections, called dengue hemorrhagic fever (DHF), and at least 100 million cases of uncomplicated dengue fever (DF) occur annually [1]. Dengue viruses consist of four serotypes and are transmitted by mosquitoes, particularly Aedes aegypti [2]. DF in adults is typically a flu-like illness characterized by fever, headache, eye pain, myalgia, arthralgia, and rash [3, 4]. Most dengue infections in children are mild or asymptomatic but may present as undifferentiated fever, classic DF, or DHF [5, 6]. DHF includes plasma leakage that may lead to shock, and defects in hemostasis including thrombocytopenia that may result in hemorrhage. Mortality rates for hospitalized DHF patients in dengue-endemic areas range from <1% to 40% [7]. There is no treatment other than careful fluid management [8]. No preventive measures are available beyond mosquito avoidance. Dengue is an emerging disease throughout tropical and subtropical regions as the territory of its principal vector, A. aegypti, expands to include Asia, Africa, Central America, South America, and the Pacific [9].

Preexisting dengue cross-reactive antibodies and T cells are central to the immune enhancement theory of dengue pathogenesis, which attempts to explain why sequential or secondary dengue infections are more likely to result in severe disease than first or primary infections. According to this hypothesis, cross-reactive nonneutralizing antibodies from a previous heterologous dengue infection facilitate virus entry into Fc receptor-bearing cells such as monocyte/macrophages. This increase in the number of antigen-presenting cells activates precursor cross-reactive memory T cells, which are present in high numbers as a result of previous heterologous infection. This leads to the release of chemical mediators that cause plasma leakage and results in increased disease severity [10, 11].

The purpose of this study was to characterize the early virologic and antibody responses of dengue infection in an effort to better define the pathophysiology of dengue disease. This clinical investigation examined several aspects of dengue pathogenesis: Are the duration and level of viremia related to outcome? Do certain dengue virus serotypes cause more severe disease than others? Do the kinetics of the antibody response early in infection correlate with the severity of disease? Detailed clinical and cellular immunologic aspects of this study are reported elsewhere [12] (Green S, et al., unpublished data).

Patients and Methods

Study design. Children with fever for <3 days without localizing signs of infection were enrolled and were observed in
hospital until 1 day following defervescence. Data were collected and treatment was provided according to standard guidelines. Blood samples were obtained daily up to a maximum of 5 consecutive collections, and a follow-up specimen was obtained ~8 days after enrollment. A right lateral decubitus chest radiograph was obtained on the day after defervescence. Children who had antibody responses diagnostic of an acute dengue virus infection had additional blood samples collected at 6 months and 1 year after enrollment.

Definitions. Study day 1 is the day on which a child was enrolled in the study and the first blood sample was obtained. Fever day 0 is the day of defervescence, that is, the day that the temperature fell below 38°C without further significant temperature elevation. Days prior to fever day 0 are designated fever day −1 (1 day before defervescence), fever day −2, etc. The day after defervescence is fever day 1. Assessment of disease severity followed the World Health Organization grading system [13]. Children with a confirmed diagnosis of dengue based on viremia or antibody responses (or both) without evidence of plasma leakage were considered to have DF; those with a fall in platelets and plasma leakage manifested by either a 20% increase in hematocrit, a pleural effusion, or ascites without shock were diagnosed as having DHF grade 1 (DHF-1, no spontaneous hemorrhage) or DHF-2 (spontaneous hemorrhage). Dengue patients experiencing peripheral vascular collapse with a pulse pressure of <20 mm Hg or clinical signs of shock (or both) were considered to have DHF-3; those with undetectable blood pressure were diagnosed as having DHF-4. Other febrile illnesses (OFI) were defined as cases with no antibody evidence of dengue infection, no isolation of dengue virus, and no obvious bacterial, rickettsial, or protozoal etiology. OFI was a diagnosis of exclusion; most illnesses were self-limited and presumed to be viral infections other than dengue.

Selection of patients. Pediatric patients seen at the Bangkok Children’s Hospital (a 538-bed tertiary-care facility) and the Kamphaeng Phet Provincial Hospital (a 335-bed secondary-level facility 270 km north of Bangkok) from 24 April to 14 December 1994 were enrolled. Dengue is transmitted year-round in Thailand but with a distinct seasonal peak each August through October. Children enrolled were in stable condition and were early in the course of a nonspecific febrile illness [12]. Briefly, febrile children were evaluated consecutively to identify those who met screening criteria (age 6 months through 14 years, weight ≥6 kg, temperature ≥38.5°C, history of fever for ≤72 h, and flushed face). Patients meeting screening criteria were examined by a study physician to exclude those with fever >72 h, focal source of infection (e.g., otitis media, pneumonia, meningitis), coryza, chronic illness including anemia, or unstable vital signs. At each hospital, a maximum of 6 children were enrolled per week.

Data and specimen collection. Study participants were examined daily by a study physician, and a case report form was completed. Daily blood specimens were collected into EDTA anticoagulant and maintained on wet ice until received in the processing laboratory. There, while at 4°C throughout, samples were centrifuged at 300 g for 10 min, the resulting platelet-rich plasma was recentrifuged at 800 g, and the platelet-poor plasma supernatant was frozen at −70°C until assays were performed.

Virus isolation. Virus isolation in Toxorhynchites splendens mosquitoes [14, 15] was attempted with each plasma sample from all patients during the first 3 study days and then with the remaining specimens from patients whose plasma samples contained virus during the first 3 days. In brief, viremia was assessed by injecting 20 live mosquitoes with 0.34 μL of undiluted patient sera. After 14 days, ~15 of the surviving mosquitoes were tested for flavivirus antigen by IFA of the head [16]. The number of positive tests over the number of mosquitoes tested was recorded. Virus-positive mosquitoes were used to infect C6/36 cell cultures for identification of virus type using a panel of monoclonal antibodies against dengue and Japanese encephalitis virus (JEV) in an EIA [17].

Antibody responses. IgM and IgG to dengue and JEV were measured in all specimens by antibody capture EIA [18]. For single specimens, 40 U of IgM to dengue (with dengue IgM greater than JEV IgM) was considered evidence of a dengue infection (30 U if from paired sera with <15 U of antibody in the acute specimen). A dengue IgM-to-IgG ratio ≥1.8:1 defined a primary dengue infection. A ratio <1.8:1 defined a secondary dengue infection. With serial specimens, a 2-fold increase in IgG to dengue with an absolute value of ≥100 U indicated a secondary infection in the absence of IgM to dengue of ≥40 U.

Hemaggulutination-inhibition (HAI) antibody against dengue virus types 1–4 and JEV were measured in all specimens [19]. A 4-fold increase was considered positive for acute flavivirus infection. The infection was diagnosed as primary if titers ≥1 week after onset of illness were ≤1:1280 or as secondary if antibody titers were >1:1280 [13].

A serologic diagnosis was assigned to each patient on the basis of EIA and HAI results. Patients in whom the serologic diagnosis was suggestive of a recent (rather than acute) dengue infection (falling IgM level, fixed HAI titer of ≥2560, or both) or for whom no convalescent specimen was available and no virus was isolated were diagnosed as “unknown” and were excluded from the analysis.

Statistical analysis. Data were entered and manipulated using FoxPro for Windows software (Microsoft, Redmond, WA) and analyzed using SPSS for Windows version 6.1.3 (SPSS, Chicago, IL).

Results

Children enrolled. The total enrollment at Bangkok Children’s Hospital and Kamphaeng Phet Provincial Hospital between 24 April and 14 December 1994 was 189 volunteers. The distribution by clinical diagnosis and corresponding demographic information are shown in table 1. Sixty of the 189 children had acute dengue infection.

Virus isolation. A dengue virus was isolated from the plasma of 59 of the 60 dengue patients (table 2). The remaining patient (KPP94-029) had clear serologic evidence for an acute secondary dengue infection; he defervesced within 3 h of treatment. There, while at 4°C until centrifugation for the first blood sample, which had elevated antibody levels (dengue IgM = 95 U, HAI = 1:1280). Of the 129 children who did not have serologic test results diagnostic of acute dengue infection, none had a dengue virus isolated.

Virus identification. The serotypes of the 59 dengue viruses isolated are shown in table 3. All 4 serotypes of dengue virus...
that were positive decreased as fever day 0 approached (figure 2A), and the rest had secondary infections (e.g., figure 2B). All 4 of the patients with primary dengue infections had DF; but 49% of patients with secondary dengue infections had DHF.

There was excellent agreement between EIA and HAI for diagnosing acute dengue infection. Of the 55 secondary cases, the rise in IgM to dengue did not meet test criteria for an acute dengue infection in 3 (change from 15 to 28 U; 0 to 17 U; and 5 to 22 U). However, in each of these 3 patients, the rise in IgG to dengue was dramatic (change from 41 to 143 U; 33 to 5 to 22 U). In each of these 3 patients, the rise in IgG to dengue was dramatic (change from 41 to 143 U; 33 to 5 to 22 U). Thus, all secondary dengue infections had positive serologic test results by both EIA and HAI. The time sequence for serial specimens to be considered positive for acute secondary dengue infection is shown in figure 3. All patients with secondary dengue infections acquired diagnostic levels of anti-

### Table 1. Diagnosis and demographic information for enrolled patients.

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No. (range)</th>
<th>Mean age, years</th>
<th>Sex (% male)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dengue fever</td>
<td>32 (2-14)</td>
<td>8.1</td>
<td>47</td>
</tr>
<tr>
<td>Dengue hemorrhagic fever</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade 1</td>
<td>5 (1-9)</td>
<td>6.0</td>
<td>60</td>
</tr>
<tr>
<td>Grade 2</td>
<td>14 (2-11)</td>
<td>8.7</td>
<td>71</td>
</tr>
<tr>
<td>Grade 3</td>
<td>9 (4-11)</td>
<td>8.2</td>
<td>44</td>
</tr>
<tr>
<td>Bacterial*</td>
<td>3 (1-14)</td>
<td>7.0</td>
<td>33</td>
</tr>
<tr>
<td>Other febrile illness†</td>
<td>112 (0.7-14)</td>
<td>6.2</td>
<td>55</td>
</tr>
<tr>
<td>Unknown†</td>
<td>14 (0.8-11)</td>
<td>5.7</td>
<td>43</td>
</tr>
<tr>
<td>Totals</td>
<td>189 (0.7-14)</td>
<td>6.8</td>
<td>53</td>
</tr>
</tbody>
</table>

* Haemophilus influenzae meningitis, Salmonella gastroenteritis, urinary tract infection.
† Nondengue self-limited febrile illness; presumed viral.
‡ Inadequate specimens to rule out dengue (n = 10) or recent dengue infection rather than acute (n = 4).

were isolated from patients at each hospital. There was no temporal pattern of virus type isolated at either hospital. At Bangkok Children’s Hospital, dengue virus types 1, 2, and 4 were isolated in at least 5 of the 8 study months and type 3 was isolated from August through November. In Kamphaeng Phet, types 2 and 4 were isolated from April through July. Dengue virus type 3 was isolated in May and June, and the single type 1 virus was isolated in September. There were no dengue isolates in Kamphaeng Phet after September in this study.

All four dengue virus types caused both DF and DHF. No correlation between virus type and disease severity could be demonstrated (table 3). While types 2 and 3 were almost twice as likely to cause DHF rather than DF in this sample, the difference was not significantly different (Pearson’s χ², P = .3). In comparing the amount of plasma leakage, judged by the level of the pleural effusion in a right lateral decubitus chest radiograph 1 day after defervescence, there was no difference among dengue virus serotypes (analysis of variance, P = .3).

### Kinetics of viremia.

The blood samples of children who had serologic evidence of an acute dengue infection were dengue virus-positive in nearly all inoculated mosquitoes early in the illness. As an estimate of the virus titer, the mean percentage of inoculated *T. splendens* mosquitoes that were positive for virus was calculated. The percentage of inoculated mosquitoes that were positive decreased as fever day 0 approached (figure 1). Viremia correlated highly with temperature (Spearman’s correlation coefficient, P < .001). All blood specimens collected ≥2 days prior to defervescence yielded a virus, whereas all specimens collected ≥2 days following defervescence did not. There was an abrupt and rapid disappearance of virus by live mosquito culture at the time of defervescence.

### Duration of viremia.

The duration of viremia was calculated by assuming that a patient was continuously viremic from the reported onset of fever through the day of the last positive culture. For 7 patients, the last day of viremia, when no inoculated mosquitoes were IFA-positive, was not identified. In 44% of the remaining patients, the percentage of mosquitoes positive was <100% on the day prior to the day that no virus was isolated. In 56% of the patients, the percentage of mosquitoes positive fell from 100% to 0% in 24 h. Therefore, it was assumed for the 7 patients for whom the percentage of mosquitoes positive was 100% on the last day measured that the viremia would persist for another half day. The mean number of days of viremia for primary cases was 5.4 days (95% confidence interval [CI], 4.0–6.7) ending on fever day 1.6 (range, 0–2.5), and the mean number of days of viremia for secondary cases was 4.5 days (95% CI, 4.3–4.8) ending on fever day 0.6 (range, 0–2.5). Mean duration of fever for patients with primary infections was 3.8 days (95% CI, 1.4–6.1), and for patients with secondary infections the mean was 4.1 days (95% CI, 3.8–4.4). The duration of viremia did not correlate with dengue virus serotype or disease severity (grade).

### Antibody responses.

Fifty-nine of the 60 dengue patients were diagnosed with dengue infection by antibody responses (table 2). One remaining patient (KPP94-011) had a dengue virus type 4 isolated in the single blood specimen collected 2 days before defervescence; no convalescent blood sample was available for antibody analysis. Only 4 of the 59 children with diagnostic antibody responses had primary infections (e.g., figure 2A), and the rest had secondary infections (e.g., figure 2B). All 4 of the patients with primary dengue infections had DF; but 49% of patients with secondary dengue infections had DHF.

### Table 2. Dengue virus isolation rate by serologic diagnosis.

<table>
<thead>
<tr>
<th>Serology</th>
<th>No. of virus isolates</th>
<th>Isolation rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary infection</td>
<td>4/4</td>
<td>100</td>
</tr>
<tr>
<td>Secondary infection</td>
<td>54/55</td>
<td>98</td>
</tr>
<tr>
<td>Nondiagnostic</td>
<td>1/1</td>
<td>100</td>
</tr>
<tr>
<td>Total</td>
<td>59</td>
<td>98</td>
</tr>
</tbody>
</table>
Table 3. Serotypes of dengue viruses isolated from patients at the two study hospitals, by grade of illness.

<table>
<thead>
<tr>
<th>Dengue virus serotype</th>
<th>Bangkok Children's</th>
<th>Kamphaeng Phet</th>
<th>Both hospitals</th>
<th>Total viruses</th>
<th>% DHF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DHF</td>
<td>DF</td>
<td>DHF</td>
<td>DF</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>13</td>
<td>8</td>
<td>1</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>25</td>
<td>3</td>
<td>5</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>3</td>
<td>35</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>45</td>
<td>5</td>
<td>2</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>Totals</td>
<td>18</td>
<td>18</td>
<td>9</td>
<td>14</td>
<td>59</td>
</tr>
</tbody>
</table>

NOTE. DHF, dengue hemorrhagic fever; DF, dengue fever.

body by EIA by 1 day following defervescence in serial testing. If assay results were considered individually without the benefit of previous results (single specimen analysis), the EIA was diagnostic in just 29% (15/51) of patients on fever day 0 and 67% (28/42) on fever day 1 (in a single specimen, IgM antibody against dengue must be ≥40 U). Diagnostic serology by HAI (79% positive on fever day 1) lagged behind that by EIA. This is because a diagnosis of secondary dengue infection based on HAI antibody responses requires a rising titer to >1:1280. None of 4 cases of primary dengue infections acquired diagnostic levels of dengue antibody by fever day 1. IgM antibody, as measured by EIA, developed later in primary infections, and for a diagnosis by HAI, a specimen must be obtained at least 1 week into the illness with a maximum titer of ≤1:1280.

The relative reactivity of IgM for the dengue virus complex was evaluated by determination of levels of IgM to equivalent amounts of tetravalent dengue antigen and JEV antigen. JEV is the only other flavivirus known to infect people in Thailand. EIA identified each of the 60 flavivirus infections as dengue rather than JEV. With 1 exception, the ratio of dengue IgM to JEV IgM in the follow-up specimen (n = 49) was >1, ranging from 1.3 to 22 (mean, 4.8), supporting a serologic diagnosis of dengue rather than Japanese encephalitis (JE). This ratio for the remaining patient was 0.93 on fever day 4, although it had been 1.3 on the day of defervescence (fever day 0) and even higher earlier in the illness (4.3, 2.6, and 2.9 on fever days −3, −2, and −1, respectively). The ratio of dengue IgM to JEV IgM was most useful to distinguish dengue from JE from fever day 0 onward (figure 4). Levels of IgG against

Figure 1. Mean values by fever day for 55 patients with secondary dengue infections for maximum daily temperature, % of mosquitoes positive by indirect IFA for dengue antigen, dengue IgM and IgG antibody levels, and reciprocal titer of hemagglutination-inhibition antibody (HAI) (average of all 4 dengue types, log scale).
Figure 3. % of 55 patients with acute secondary dengue infections acquiring diagnostic levels of dengue antibody by fever day and assay. Plasma was collected daily through fever day 1 and again on study day 8 per study protocol (here grouped as fever day 6). All 4 children with primary dengue infections acquired dengue antibody levels consistent with acute dengue infection after fever day 1 and are not included in this figure. Fever day 0 = day that temperature dropped to \( <38^\circ\text{C} \). EIA measured IgM and IgG antibody against dengue viruses. HAI = hemagglutination-inhibition antibody assay against 4 serotypes of dengue virus.

The timing of the antibody increases early in secondary dengue infections is shown in figure 1. The HAI antibody titer increased before the antibodies measured by EIA. The IgG

dengue and JEV were similar \( (r = .94) \); therefore, they failed to distinguish between infection with dengue viruses and JEV.

The HAI antibody responses to homologous and heterologous dengue virus antigens were similar. The HAI antibody responses to JEV antigen commonly lagged behind the response to dengue antigens (figure 2B). Among the four primary infections, the homologous antibody response was the first to appear in 2 patients and first along with antibody to two other serotypes in 1 patient (figure 2A). In the fourth patient, HAI antibody was not seen on fever day 6 (9 days after onset of

Figure 4. Mean ratio of IgM to dengue (EIA units) to IgM to Japanese encephalitis virus (JEV) with 95% confidence intervals by fever day (fever day 0 = day of defervescence). Reference (dashed) line is at 1.0, where levels of IgM to dengue and to JEV are about equal.
antibodies as measured by EIA increased before IgM and rose to higher peak levels.

**Virus-antibody interactions.** As antibodies increased, the virus titer, as estimated by the percentage of mosquitoes positive for dengue virus, decreased (figure 1). The first day in which virus isolation was not possible was identified in 46 patients with secondary dengue infections. For the remainder, a dengue virus was isolated on the last day that blood was obtained during the acute illness. On the last day a virus was isolated, the mean level of IgM antibody against dengue virus was 19 U (range, 0–213) and the geometric mean HAI antibody titer (average of all four dengue types) was 1:140 (range, <1:10–1:2560). On the first day a virus was not isolated, the mean level of IgM against dengue virus was 46 U (range, 2–160), and the geometric mean HAI titer was 1:860 (range, 1:24–1:7240).

**Long-term antibody responses.** The long-term IgG, IgM, and HAI titers for the 55 secondary dengue patients are shown in figure 5, with data at fever days −3, 6 (during the second week of illness), 180, and 365. Dengue IgM antibodies fell to low levels by 6 months (mean, 9 U; range, 0–40). All children with secondary dengue infections had intermediate HAI antibody levels at 6 months (mean titer, 1:174; n = 48) and at 12 months (mean titer, 1:82; n = 48). The 4 children with primary dengue infections had lower levels of HAI antibody at 6 months (mean titer, 1:17) and 12 months (mean titer, 1:18).

**Discussion**

In this study of children with acute fever, all children who had a dengue virus isolated from their blood had antibody responses consistent with an acute dengue virus infection, and all children with an acute dengue antibody response had detectable viremia (with the exception of 1 child who was evaluated <3 h prior to defervescence). Our data demonstrate the occurrence of viremia early in the febrile period of dengue and its rapid elimination as fever abates and antibody levels rise.

Ashburn and Craig [20] working in Manila in 1906 first demonstrated that patients with dengue infections were viremic early in their illness. Subsequently, virus isolation was confirmed to be more likely early in the illness [21]. Kuberski et al. [22] isolated dengue virus type 1 in 73% of patients on the first day of illness, with decreasing rates until no isolations were made after 6 days of illness. Higher isolation rates, from 51% to 100%, have also been reported for patients experiencing first or primary dengue infections [23, 24]. After the first day of illness, Kuberski et al. [22] was more likely to isolate virus and demonstrate higher virus titers in children experiencing primary dengue infections. While Gubler et al. [24] also noted that virus isolation was more common in primary dengue infections, they found that among dengue type 3 infections, the titer of type 3 virus was 5-fold higher in secondary infections.

Our data confirm and extend previous data on the relative ease of virus isolation early in the illness. Our study has several features that probably explain the high rate of viremia we found compared with most published studies of DF and DHF [23–27]. We followed children with fever and obtained serial blood samples from the same patient during the course of illness. Furthermore, children were enrolled in the study shortly after the onset of illness, during the febrile phase when virus isolation is more likely. On the other hand, Gubler et al. [28] isolated dengue type 2 virus from only 6 of 18 patients on the island of Tongatapu in 1974 despite specimen collection early in the illness. In that study in the Kingdom of Tonga and another study by the same group in central Java in 1978, specimens were collected (live *Aedes aegypti* mosquitoes fed on patients) during the first days of illness over several days, yet the isolation rate was only 33% [28, 29]. The illnesses in both the 1974 and 1978 outbreaks "were remarkable for their mildness and relative short duration"; the lower isolation rate may represent either a difference in virus isolation technique or the presence of a less virulent strain of dengue type 2 virus, resulting in lower virus titer and mild disease. Partial genome sequencing of the 1974 Tonga dengue type 2 virus has shown it to be significantly different from Thai isolates including isolates from our study [30, 31] (Rico-Hesse R, personal communication).

The typical duration of viremia in our patients was 4–5 days, as has been seen previously [32]. While not statistically significant, our data are consistent with the findings of Kuberski et al. [22] and Gubler et al. [32] in showing that viremia persists longer in children experiencing primary infections rather than secondary dengue infections. Our results suggest that this may be due to continued viremia into the afebrile phase, because viremia lasted 1 day longer than the duration of fever in primary dengue patients. If viremia extends further into the afebrile
period in primary dengue infections, it may be due to more gradual antibody or cellular immune responses.

Some observers have found that certain serotypes of dengue virus (serotypes 2 and 3) are more likely to cause severe disease [23, 29, 33]. However, Gubler et al. [24] could not demonstrate a relationship between dengue serotype and disease severity in Indonesia. In these and other earlier studies, a possible confounder arises from the generally low isolation rates of 15%–65% [23–27]. If, for example, dengue type 2 virus is easier to recover than the other serotypes of dengue virus, then more severe disease would be falsely biased toward dengue type 2.

In the present study, all four dengue virus serotypes were isolated during a single season in an urban children’s hospital and a rural provincial general hospital. The circulation of three or more serotypes of dengue throughout Thailand has been observed consistently for 2 decades. This promotes the occurrence of sequential dengue infections and appears to sustain endemic DHF. The isolation of virus from nearly 100% of patients removes one potential bias in evaluating differences in disease outcomes by infecting dengue serotype. While patients infected with serotypes 2 and 3 were nearly twice as likely to progress from DF (undifferentiated febrile illness) to DHF as patients infected with serotypes 1 and 4, these differences were not statistically significant. Continued enrollment in this study may allow adequate numbers of patients to provide a more definitive answer.

Studies of antibody responses in dengue began in 1930 with the development of a complement fixation test [34]. The use of HAI antibody assays against all four dengue virus serotypes in the early 1960s demonstrated that HAI antibody responses were often cross-reacting among the four. The results of HAI assays were used to distinguish between primary and secondary dengue infections [35–37]. More recent studies of antibody kinetics show that, in primary infections, IgM to dengue viruses increases to high levels in nearly all patients within 2 days of defervescence and peaks within 2 weeks. In nonprimary (secondary, tertiary, etc.) infections, the IgM response is variable, sometimes absent or lagging behind a dramatic increase in IgG antibodies [18, 38].

In the present study, both EIA and HAI antibody results were efficient in the diagnosis of acute dengue virus infections and were able to identify the infection as primary or secondary. In Southeast Asia, EIA had the further advantage of being able to distinguish dengue from JEV infections. Four patients (2%) not included in the analysis had serologic evidence of a recent rather than an acute dengue infection, that is, a fixed HAI titer of $\geq 2560$ [13] or EIA antibody levels that were high and level or falling within 72 h of the onset of illness (e.g., figure 2C). Given that 90% of dengue infections in Thai children are mild or asymptomatic [6], these children probably experienced an asymptomatic dengue infection or mild dengue illness prior to the febrile illness that brought them to the hospital. These patients illustrate the difficulties in interpreting serologic results from single specimens or failing to perform virus isolation (or both). However, in a nonresearch setting, patients often present later in the course of their illness, and a single high-titered specimen can be assigned a statistical probability of being associated with an acute secondary dengue infection. In addition, the consistent timing of fever, viremia, and antibody responses seen in the present study as well as the other clinical indicators of dengue illness [12] document that the children experienced acute illnesses due to dengue virus infection rather than illness due to another etiology with incidental dengue virus infection.

Concerning laboratory diagnosis of acute dengue virus infection, the message of this study is that if the patient with dengue fever has fever, then attempts at virus isolation (or dengue antigen detection or genome identification) should be successful while serologic assays may still be negative; conversely, if fever has abated, then virus isolation is less likely to be successful and serology is more likely to be diagnostic.

Previous studies of dengue HAI antibody duration following natural infection have been performed as cross-sectional antibody surveys in areas where clinical dengue has been absent for some time. These studies suggest that HAI antibody against dengue virus can be long-lived: Thellier et al. [39] and Rosen [40] found HAI antibody against dengue virus 30 and 45 years, respectively, after the dengue outbreaks of 1927 and 1928 in Greece; Rosen [41] also found HAI antibody 12 years after an epidemic in Panama; and Hammon et al. [42] found HAI antibody against dengue type 2 24 years after a dengue epidemic in 1934 in Florida. The previous studies assumed the HAI antibodies were due to dengue virus infection because the epidemic was clearly dengue on the basis of clinical criteria, and persons born since the epidemic did not have antibody. HAI antibody has also been shown to be long-lived in persons who were experimentally infected with dengue viruses by allowing infected mosquitoes to feed on them. Halstead [43] demonstrated HAI antibody 42–48 years following experimental dengue infections in volunteers infected in the Philippines between 1924 [44] and 1930 [34]. The present study followed HAI antibody levels in a prospective fashion to 1 year after infection and showed a slow decline in antibody titer between 6 and 12 months. We plan to follow children in this study yearly for at least 3 years after infection to further define long-term HAI antibody responses to dengue viruses.

All 4 children experiencing primary dengue infections were classified as having DF compared with secondary patients, of whom 50% demonstrated clear evidence of plasma leakage. These results are consistent with prior epidemiologic studies of severe dengue illness in secondary cases. In this prospective study, we demonstrated viremia in 98% of patients early in the course of disease. Further studies will be needed to validate major tenets of the immune enhancement theory, that is, the effects of preexisting cross-reactive antibody and T cell responses at the time of the secondary infection and the impor-
of the virus burden and immunologic responses in secondary infections that lead to DHF.

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