Dengue Viremia Titer, Antibody Response Pattern, and Virus Serotype Correlate with Disease Severity

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Viremia titers in serial plasma samples from 168 children with acute dengue virus infection who were enrolled in a prospective study at 2 hospitals in Thailand were examined to determine the role of virus load in the pathogenesis of dengue hemorrhagic fever (DHF). The infecting virus serotype was identified for 165 patients (DEN-1, 46 patients; DEN-2, 47 patients; DEN-3, 47 patients, DEN-4, 25 patients). Patients with DEN-2 infections experienced more severe disease than those infected with other serotypes. Eighty-one percent of patients experienced a secondary dengue virus infection that was associated with more severe disease. Viremia titers were determined for 41 DEN-1 and 46 DEN-2 patients. Higher peak titers were associated with increased disease severity for the 31 patients with a peak titer identified (mean titer of $10^{7.6}$ for those with dengue fever vs. $10^{8.5}$ for patients with DHF, $P = .01$). Increased dengue disease severity correlated with high viremia titer, secondary dengue virus infection, and DEN-2 virus type.

Dengue fever (DF), classically characterized by fever, headache, eye pain, myalgia, arthralgia, and rash, has been recognized for more than 200 years [1]. It was not until the 1950s that a more severe manifestation of dengue virus infection, dengue hemorrhagic fever (DHF), characterized by defects in hemostasis and plasma leakage, became widely recognized [2, 3]. Dengue is an emerging disease throughout tropical and subtropical regions as the principal vector, *Aedes aegypti,* expands its habitat across Asia, Africa, Central America, South America, and the Pacific [4]. It is estimated that at least 100 million dengue infections and >250,000 cases of DHF occur annually [5].

Although most dengue infections result in a self-limited febrile illness [6], dengue is a public health problem because DHF can be fatal unless its attendant plasma leakage is treated early. Current practice is to hospitalize patients with early signs of disease for observation. Lenient hospitalization policies improve outcome but require increased health resources. If the early determinants of dengue disease severity were understood in detail, more effective and less costly case management might be devised.

Dengue viruses are flaviviruses, of which there are 4 different serotypes [7]. It has been observed in several studies that sequential or secondary dengue virus infections are more likely to produce severe disease [6, 8–11]. This epidemiologic observation can be explained by the theory of immune enhancement. Cross-reactive, nonneutralizing antibodies from a previous heterologous dengue virus infection bind to the new infecting serotype and facilitate virus entry via Fc-receptor-bearing cells [5, 12, 13]. This mechanism can serve to increase the number of antigen-presenting cells infected during secondary dengue, which can lead to the activation of preexisting cross-reactive dengue virus-specific T lymphocytes from the primary flavivirus infection [14, 15]. This self-amplifying cascade can then lead to the release of cytokines and chemical mediators that may cause plasma leakage [16, 17]. Other factors have been postulated as important in the pathogenesis of DHF: (1) specific virulent virus genotypes that replicate to high levels resulting in an increased

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Written informed consent was obtained from parents or guardians of patients who met enrollment criteria. The study was approved by the Ethical Review Subcommittee of the Ministry of Public Health, Thailand, the institutional review board of the University of Massachusetts Medical Center, and the human subjects research review board of the Office of the US Army Surgeon General, Washington, DC.

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immune response and increased disease [18–20]; (2) a genetic predisposition to severe disease among certain populations [21, 22]; and (3) other risk factors, such as age [23], sex [9, 23], and nutrition [24, 25]. Although high virus loads may be necessary to develop DHF, there is no direct evidence that this occurs.

The results of the clinical investigation presented in this article extend our group's efforts to better define the pathophysiology of dengue disease through a prospective study in Thailand [15, 20, 26–32]. We examined the relationship between peak viremia titer and disease severity, measured as clinical grade and by direct quantitation of plasma leakage (pleural effusion). To explore whether pathogenicity was limited to a subset of dengue viruses, we determined virus serotype and explored its relationship to disease severity. We present evidence that establishes a direct correlation between peak viremia titer and disease severity.

Patients and Methods

Patient enrollment and data collection. Investigational methods can be found in previous publications [27, 28]. In brief, children seen at the Queen Sirikit Institute of Child Health (formally the Bangkok Children's Hospital) and the Kamphaeng Phet Provincial Hospital from 24 April 1994 to 16 December 1996 were eligible for enrollment in the study. Enrollment criteria were age 6 months through 14 years, weight >6 kg, temperature ⩾38.5°C, history of fever for <72 h, and flushed face. Exclusion criteria were fever for >72 h, focal source of infection (e.g., otitis media, pneumonia, meningitis), coryza, chronic illness, including anemia, or unstable vital signs. Children were observed in-hospital until 1 day after defervescence. A right-lateral decubitus chest radiogram was performed on the day after defervescence to measure plasma leakage manifest as a pleural effusion. The volume of pleural fluid was expressed as an index (PEI = [depth of pleural effusion/diameter of right hemithorax] × 100). Blood samples were obtained daily up to a maximum of 5 consecutive collections, and a follow-up specimen was obtained 8–10 days after enrollment.

Definitions. Illness day 1 was the 1st day of reported illness. Study day 1 was the day on which a child was enrolled and the first blood sample was obtained. Fever day 0 was the day of defervescence. Days prior to fever day 0 were designated fever day −1 (1 day prior to defervescence), fever day −2, and so forth. The day after defervescence was fever day +1. The World Health Organization grading system [28, 33] was the clinical measure of disease severity. Children with 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 over recovery value, a pleural effusion, or ascites without shock were given a diagnosis of DHF grade 1 (no spontaneous hemorrhage) or grade 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 grade 3; those with undetectable blood pressure were given a diagnosis of DHF grade 4.

Antibody responses. IgM and IgG to dengue virus and Japanese encephalitis virus (JEV) were measured in all specimens by antibody capture enzyme immunoassay (EIA) [27, 34]. Hemagglutination inhibition antibody against dengue virus types (DEN) 1–4 and JEV were measured in all specimens [33, 35]. Determination of primary versus secondary dengue virus infection was determined as described elsewhere [27].

Virus serotypes. Virus isolation in *Toxorhynchites splendens* mosquitoes [27, 36, 37] was attempted with each plasma sample from all patients during the first 3 study days and with the remaining blood specimens from patients whose plasma sample contained virus during the first 3 days. Isolates were identified in C6/36 cell cultures by using a panel of monoclonal antibodies against dengue and JEV in an EIA [38]. Reverse transcriptase–polymerase chain reaction (RT-PCR) was used to attempt to identify the infecting serotype in specimens for which a virus was not isolated [39].

Quantitation of peak viremia. Viremia titers were determined in samples containing DEN-1 and DEN-2 as follows: $\log_{10}$ dilutions of plasma were made up to 10^{-6}, and each dilution was injected intrathoracically into 20 mosquitoes. Fifteen survivors were assessed for virus replication at 14 days. The quantity of dengue virus was calculated as the median mosquito infectious doses per milliliter (MID_{50}/mL) by probit analysis. A virus peak was defined for cases where the virus titer increased from the first specimen or if the titer decreased by a half log or less from study day 1 to study day 2.

Viremia duration. To estimate the total duration of viremia, researchers assumed detectable viremia started on the day prior to the onset of illness [40] and ended on the last day in which it was detected. For example, if a child was admitted on the third day of illness and virus was detected until the fifth day of illness (study days 1–3), then the viremia duration was 5 days.

Rate of virus clearance. To estimate the rate of virus clearance, researchers calculated the slope of the descending portion of the viremia curve for DEN-1– and DEN-2–infected patients between fever day −2 and fever day 0. This represented the linear portion of the descending viremia curve by using a $\log_{10}$ scale for viremia titer. The slope was calculated as the change in virus log-titer divided by the number of days over which the change was observed (2 days).

Statistical analysis. Data were entered and managed by use of FoxPro for Windows software (Microsoft, Redmond, WA), and analysis was performed by use of SPSS for Windows version 8.5 (SPSS, Chicago). For analysis, disease severity was measured by PEI (continuous) and by 3 severity groups (ordinal) based on clinical assessment (DF, DHF grade 1/2, DHF grade 3/4). Student's *t* test (unpaired) was used to compare mean values. If variance was unequal (Levin), the *t* test used independent variance. Fisher's exact test was used to compare proportions. The Spearman rank correlation test was used to assess the degree of correlation between severity and other measures. Multivariate logistic regression was used to assess the association of viremia titer (peak or maximum as a continuous variable), antibody response pattern (primary vs. secondary), and dengue virus serotype (DEN-1 vs. DEN-2) on the occurrence of disease severity (DF vs. DHF). All reported *P* values are 2-sided.

Table 1. Age and sex of children enrolled from April 1994 to December 1996, by diagnosis.

Diagnosis	Number	Mean age, y	% male
Nonviral	13	8.1	54
OFI	254	6.4	60
Unknown	21	5.2	52
DF	88	7.8	50
DHF Gr 1	23	7.5	78
DHF Gr 2	44	8.8	73
DHF Gr 3	13	8.1	46
All children	456	7	59

NOTE. Nonviral, probable bacterial or parasitic etiology for fever; OFI, other febrile illness, presumed viral (nondengue) illness; Unknown, dengue virus infection during previous few months but probably not cause of current illness ($n = 6$) or no dengue virus isolated and no convalescent plasma for a serological diagnosis; DF, dengue fever; DHF Gr, dengue hemorrhagic fever grade.

Results

Children enrolled and disease severity. A total of 456 children aged 18 months through 14 years (table 1) were enrolled. Mean age (7 years) and sex distribution (59% male) did not vary over the 3 years of the study. One hundred sixty-eight patients (37%) had dengue. The final diagnosis was DF for 88 patients and DHF for the remaining 80, including 67 grade 1/2 and 13 cases of grade 3 (dengue shock syndrome). A PEI from fever day +1 was available for 158 (94%) of the 168 patients with dengue. The mean PEI for all patients with dengue was 7.6 (range, 0–78; 52% with pleural effusion) versus 0.5 for the 238 patients with a diagnosis of presumed viral (nondengue) infection (30% with pleural effusion). A pleural effusion was detected in 2% of 81 patients with DF, 90% of 64 patients with DHF grade 1 and 2, and 92% of 13 patients with DHF grade 3. For dengue patients, the PEI correlated with clinical grade with $r = .9$, $P < .001$.

Virological diagnosis. A dengue virus was isolated from 162 of 168 dengue patients (96%). Dengue RT-PCR was positive for 3 additional patients from whom a virus could not be isolated, for an overall virus identification rate of 98%. Virus types identified were DEN-1 ($n = 46$), DEN-2 ($n = 47$), DEN-3 ($n = 47$), and DEN-4 ($n = 25$). All 3 patients for whom no infecting virus was identified were enrolled late (1 day prior to or on the day of defervescence) and had a secondary dengue virus infection.

Antibody response patterns. A secondary dengue virus infection was diagnosed in 133 patients (79%), and 32 patients (19%) had a primary dengue virus infection. Three other patients (2%) had a virus isolated in an acute specimen, but no follow-up blood specimens were available to classify the antibody response pattern. DEN-2 and DEN-4 infections were almost exclusively associated with a secondary or anamnestic flavivirus antibody response (table 2).

Peak viremia. Viremic plasma from 42 of the 46 DEN-1 infected patients and the 47 DEN-2 infected patients were titrated in live *T. splendens* mosquitoes. A peak titer was clearly identified (increasing titer before decreasing) for 16 patients. For another 15 patients, the titer was stable for the first 2 collections with a 0.5 log decrease in titer or less from the 1st to the 2nd study day. Among the 31 patients with a peak titer as defined (mean peak titer, $10^{8.2}$ MID_{50}/mL), the median and modal day for the peak to occur was illness day 3 (fever day −2).

Duration of viremia. The study protocol limited blood collections to the day following defervescence or a maximum of 5 acute blood collections, whichever came first. Dengue viremia was detected in the last acute blood sample for 28 (17%) of 165 patients. Duration of viremia ranged from 1 to 7 days (mean, 4.5 days; median, 5 days). Viremia during primary infection was prolonged compared with secondary infections. The mean duration of viremia for all patients experiencing a primary dengue virus infection ($n = 32$) was 5.1 days versus 4.4 days for those experiencing a secondary dengue virus infection ($n = 133$, $P = .002$). This relationship held for DEN-1 patients (5.5 days for 16 patients with primary infections vs. 4.5 days for 30 patients with secondary dengue virus infections, $P = .002$) but not for DEN-3 patients (4.6 days for 15 primary patients vs. 4.4 days for 32 secondary patients, $P = .5$).

Rate of virus clearance. From fever day −2 until fever day 0, viremia titers decreased an average of 2.5 logs per day (DEN-1 and DEN-2 patients, $n = 51$). The slope of this descending portion of the viremia curve was steeper for patients with secondary infections ($n = 41$, mean loss of 2.7 logs of virus/day) versus those who experienced primary dengue virus infections ($n = 10$, mean loss of 1.4 logs of virus/day, $P = .006$)

Disease severity by DEN. Patients with DEN-2 were more likely to have DHF (66%) than those infected by other virus serotypes (table 3). When only patients with secondary antibody responses were considered, patients with DEN-2 were more likely to have DHF than those with DEN-4. There was a trend toward more severe disease (DF vs. DHF) in DEN-2 patients, compared with those with DEN-1 and DEN-3 infections. On average, patients with DEN-2 infections had a larger PEI than those infected by other virus serotypes. When only patients with secondary antibody responses were considered, those with DEN-2 infections had a larger PEI than patients

Table 2. Antibody response patterns by type of infecting dengue virus (DEN).

Virus type	Primary	Secondary	Uncharacterized	Totals
DEN-1	16	30	0	46
DEN-2	1[a]	44	2	47
DEN-3	15	32	0	47
DEN-4	0	24	1	25
Unknown	0	3	0	3
Totals	32	133	3	168

[a] While this patient's conditions met enzyme immunoassay and hemagglutination inhibition assay criteria for a primary dengue virus infection, neutralizing antibody assay revealed preexisting antibody to Japanese encephalitis virus (i.e., this was also a secondary flavivirus infection).

with DEN-1 and DEN-4 infections with a trend toward a larger PEI compared with patients with DEN-3 infections (see table 3 and figure 1).

Disease severity by antibody response pattern. Patients with a secondary antibody response were twice as likely to have DHF, compared with those with a primary antibody response (53% vs. 23%, respectively, $P < .001$, table 4). Patients with a secondary dengue virus infection had a larger mean PEI than patients with primary infections. For the subset of patients with DEN-1 and DEN-3 infections, those with secondary infections were more likely to experience DHF and had a larger mean PEI than those with primary infections (table 4). We were unable to perform this analysis for DEN-2 and DEN-4 patients, because all, or nearly all, patients had a secondary antibody response. Dengue shock syndrome (DHF grade 3, $n = 13$) was associated with a secondary dengue virus infection in all cases.

Disease severity by peak virus titer. For patients with a peak titer identified ($n = 31$), disease severity correlated with peak titer with a mean titer of $10^{7.6}$ for patients with DF compared with $10^{8.4}$ for those with DHF grades 1 or 2 and $10^{9.0}$ for those with DHF grade 3 ($r = 0.5$, $P = .006$). When looked at by virus type, this association held up for the DEN-2 patients ($10^{7.0}$ for 6 DF patients, compared with $10^{8.3}$ for 11 DHF grade 1/2 patients and $10^{9.0}$ for 3 DHF grade 3 patients; $r = .6$, $P = .002$). For DEN-1 patients, the mean peak virus titer for 6 DF patients was $10^{8.2}$ compared with $10^{8.7}$ for 5 DHF patients ($P = .3$; there were no DEN-1 DHF grade 3 patients with a peak titer identified). As peak viremia titer increased so did the pleural effusion ($r = 0.4$, $P = .03$; see figure 2). There was little or no pleural effusion until higher peak levels of viremia were experienced.

The maximum virus titers were observed on study day 1, except for the 16 patients where virus titer increased after study day 1, as described earlier. Maximum titer also correlated with increasing clinical grade for all DEN-1 and DEN-2 patients (DF, $10^{6.3}$; DHF grade 1 or 2, $10^{7.2}$; DHF grade 3, $10^{8.5}$; $r = .4$, $P = .001$). This pattern held for DEN-2 patients ($r = .5$, $P = .001$; figure 3). The trend was the same for all DEN-1 patients ($n = 42$, $r = 0.3$, $P = .09$; figure 3), although it was more convincing if only DEN-1 patients with secondary infections were considered (13 DF patients, $10^{5.5}$; 10 DHF grade 1 or 2 patients, $10^{7.3}$; 3 DHF grade 3, $10^{8.6}$; $r = .5$, $P = .01$). For patients with primary DEN-1 infections, the mean maximum titer was $10^{8.0}$ for 13 DF patients versus $10^{8.5}$ for 3 DHF grade 1/2 patients, $P = .3$). Figure 4 shows mean viremia curves over several days for DEN-2 patients by clinical diagnosis.

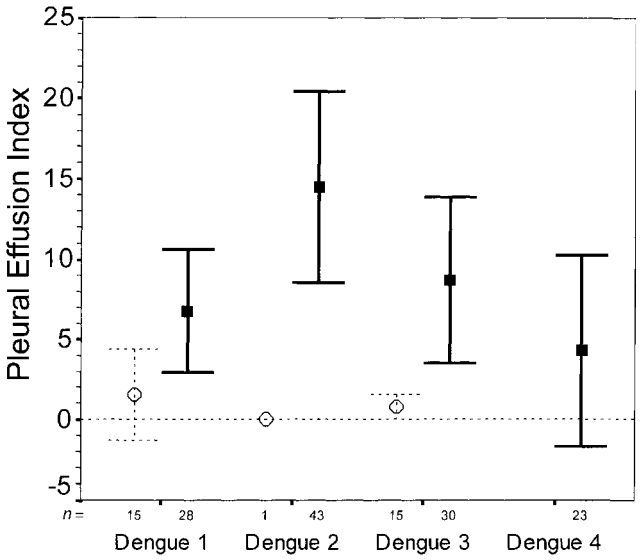

Figure 1. Mean plural effusion index and 95% confidence intervals by dengue virus type and antibody response pattern (○ and *dashed lines,* primary; ■ and *heavy lines,* secondary).

Disease severity by duration of viremia and rate of virus clearance. There was no correlation between duration of viremia and disease severity by clinical grade ($r = 0.07$, $P = .4$) or PEI ($r = 0.03$, $P = .7$). There was an association between disease severity and the slope of the descending portion of the viremia curve (see figure 4 for patients with DEN-2 infections). The mean rate of virus clearance (fever day −2 to day 0) was greater in patients with DHF than in those with DF (2.9 logs of virus/day vs. 1.8 logs/day, $P = .002$).

Table 3. Disease severity and pleural effusion index (PEI) in children infected with dengue virus type (DEN) 2 compared with those infected with other dengue virus serotypes.

	All dengue patients						Patients with secondary dengue virus infections					
Virus type	*n*	% with DHF	P^a	*n*	Mean PEI	P^b	*n*	% with DHF	P^a	*n*	Mean PEI	P^b
DEN-1	46	39	.01	43	4.9	.005	30	50	.1	28	6.8	.03
DEN-2	47	66	—	44	14.2	—	44	68	—	43	14.5	—
DEN-3	47	40	.02	45	6	.02	32	47	.1	30	8.7	.1
DEN-4	25	40	.05	23	4.3	.02	24	38	.02	23	4.3	.02
Totals	165			155			130			124		

NOTE. DHF, dengue hemorrhagic fever.

[a] Fisher's exact test vs. patients with dengue virus type 2 (DEN-2) infections.

[b] Student's *t* test vs. patients with DEN-2 infections.

Table 4. Disease severity by clinical grade and pleural effusion index (PEI) vs. antibody response pattern for all patients and for patients with dengue virus type (DEN) 1 and DEN-3 infections.

Patient category	*n*	% with DHF	P^a	*n*	Mean PEI	P^b
All primary dengue	32	23	.001	31	1.2	.004
All secondary dengue	133	53		127	9.2	
Primary, DEN-1 and DEN-3	31	23	.008	30	1.2	<.001
Secondary, DEN-1 and DEN-3	60	50		58	7.7	

NOTE. DHF, dengue hemorrhagic fever.

[a] Fisher's exact test: all primary dengue patients vs. all secondary dengue patients, and primary vs. secondary antibody response pattern for patients with DEN-1 and DEN-3 infections only.

[b] Student's *t* test: all primary dengue patients vs. all secondary dengue patients and primary vs. secondary antibody response pattern for patients with DEN-1 and DEN-3 infections only.

Virus titer by serologic response. Viremia titers of patients infected with DEN-1 and DEN-2 were higher among patients with secondary dengue virus infection early in the course of illness but were higher among patients with primary dengue at the time of defervescence. At fever day −4 the mean virus titer was $10^{6.2}$ MID_{50}/mL for patients with primary infections (n = 7) versus $10^{8.5}$ for those with secondary dengue virus infections (n = 7, P = .05). On fever day 0, the mean virus titer was $10^{4.4}$ for patients with primary infections (n = 13) versus $10^{1.9}$ for those with secondary dengue virus infections (n = 70, P = .003).

Logistic regression. Based on logistic regression, higher peak viremia titer (n = 29 patients with complete data) was more likely to result in DHF (odds ratio [OR], 4.4 for each log increase in peak viremia titer, P = .03). Secondary antibody response predicted DHF (OR, 6.3, P = .1). The odds ratio for DEN-2 infection to result in DHF versus DEN-1 infection was 2.5, P = .4. Repeating the analysis by using maximum viremia titer rather than peak titer (n = 89) gave similar results. Higher maximum viremia titer predicted DHF (OR, 1.6, P = .004). Secondary dengue virus infection predicted DHF (OR, 9.7, P = .004). The OR for more severe disease with a DEN-2 infection was 1.6, P = .4.

Discussion

Earlier studies of dengue hypothesized a relationship between disease severity and the virus load as reflected in viremia titer [5, 41]. In this study, quantification of daily viremia in patients with secondary DEN-1 and DEN-2 infections demonstrated a correlation between dengue viremia early in the course of illness and disease outcome. Peak virus titers were 100- to 1000-fold higher for patients who developed dengue shock syndrome, compared with those with the more benign DF.

Although there was an association between disease severity and peak viremia titer in our full data set and for secondary DEN-1 and DEN-2 patients, no association was seen for primary DEN-1 patients. It may be that our sample size was inadequate to reveal this relationship (13 DF patients and 3 DHF patients). Alternatively, there may be other critical determinants of DEN-1 primary disease severity.

There was no association between disease severity and the estimated duration of viremia. One interpretation of virus load is that it is related both to the height of the viremia and the duration (area under the curve). In fact, the estimated duration of dengue viremia was prolonged in patients experiencing primary dengue virus infections, compared with those experiencing secondary infections (5.1 days vs. 4.4 days, respectively). It should be noted that precise measurement of the duration of viremia was not possible. We were not able to identify the onset of viremia; children were enrolled up to 72 h after the onset of their fever. Siler et al. demonstrated that viremia (as measured by the ability to transmit virus from patient to mosquito to a healthy volunteer) started 6–18 h prior to the onset of illness [40]. With regard to the termination of viremia, our study protocol limited blood collections to the day after defervescence or a maximum of 5 acute blood collections, whichever came first. This resulted in 28 patients with viremia documented on the final acute blood collection. This included 15 (47%) of 32 patients who experienced a primary dengue virus infection versus 10 (8%) of 133 of those who experienced a secondary dengue virus infection (P< .001). This suggested that the slope of the dengue viremia was steeper for secondary cases and that many children with primary dengue may have had detectable viremia

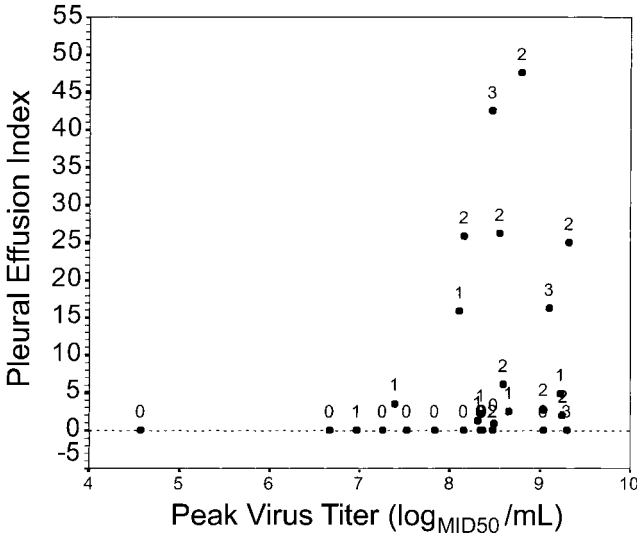

Figure 2. Peak virus titer (log mosquito infectious doses per milliliter [MID_{50}/ml]) by pleural effusion index ([depth of pleural effusion/diameter of right hemithorax as read from right lateral decubitus chest radiogram obtained on the day after defervescence] × 100) for patients who experienced dengue virus type (DEN) 1 (n = 11) and DEN-2 infections (n = 20). 0, dengue fever (n = 12); 1, dengue hemorrhagic fever grade 1 (n = 8); 2, dengue hemorrhagic fever grade 2 (n = 8); 3, dengue hemorrhagic fever grade 3 (n = 3).

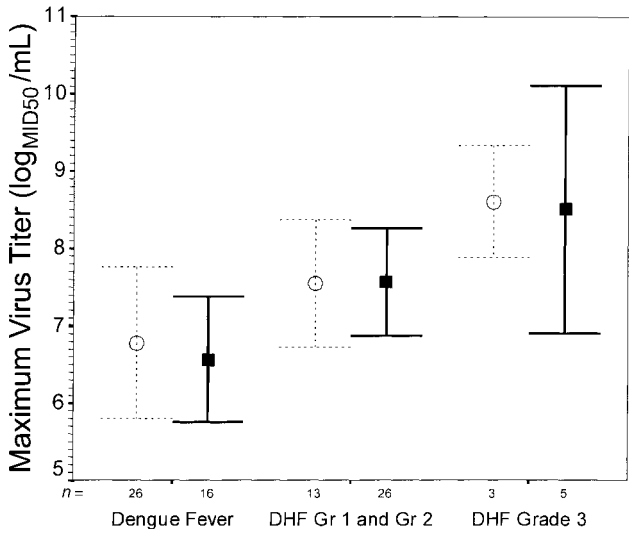

Figure 3. Mean maximum virus titer (log mosquito infectious doses/ml, log MID_{50}/mL) by dengue virus type and clinical grade. Dengue virus type (DEN) 1 shown with ○ and *dashed lines* for 95% confidence levels. DEN-2 shown with ■ and *heavy lines* for confidence intervals. DHF, dengue hemorrhagic fever.

well beyond our last acute blood collection, further underestimating the duration of viremia for patients with primary infections. In fact, rapid virus clearance for patients with secondary dengue virus infection was identified directly as a steeper slope for the descending portion of the viremia curve. The difference in viremia duration and rate of virus clearance between primary and secondary antibody response patterns was presumably due to the relatively slower response of a nonprimed immune system to clear virus for those who experienced a primary dengue virus infection.

Several earlier reports identified secondary dengue virus infection as a risk factor for severe dengue disease [6, 8–11]. We observed the same in this study, both overall and after stratification by virus serotype, by using either clinical grade as the traditional ordinal severity measure or PEI as a continuous quantitative severity measure. A secondary antibody response provides evidence for the circulation of enhancing antibodies able to amplify dengue virus by facilitating virus entry into susceptible cells. Increased peak viremia titer has been documented in rhesus monkeys with a secondary DEN-2 infection and confirmed here for the first time in patients [42]. Immune clearance of virus presumably is mediated by preexisting neutralizing antibody and cross-reactive lymphocytes and is accelerated in persons with secondary infections.

Earlier studies in Thailand found that DEN-2 caused more severe disease among hospitalized patients [6, 11]. Those studies may have been biased, as they failed to identify virus serotype in most patients and lacked quantitation of viremias. We identified virus serotype in essentially all patients and stratified our analysis by type of antibody response. We found that secondary DEN-2 caused more severe disease than DEN-1 and DEN-4, but not DEN-3. The differential severity associated with virus serotype suggests that virus phenotype influences outcome. We speculate that greater virus replication for DEN-2 in primed hosts confers the enhanced pathogenicity, compared with that seen in DEN-1 secondary or primary infections. While suggestive, our data set failed to demonstrate that viremia titer was higher in secondary DEN-2 than in secondary DEN-1 patients (mean maximum titer, $10^{7.3}$ MID_{50}/mL for DEN-2 [$n = 44$] vs. $10^{6.5}$ MID_{50}/mL for DEN-1 [$n = 26$], $P = .1$). It must be recalled that these data are based on children coming to the hospital for care. Our data do not address the much larger population of children infected with dengue viruses who do not seek medical attention.

Another difference among dengue virus serotypes emerged from this study. We identified primary infections only among patients with DEN-1 and DEN-3; almost all patients with DEN-2 and DEN-4 had secondary infections. While a single DEN-2 patient met EIA and hemagglutination inhibition assay (HAI) criteria for a primary dengue virus infection, HAI antibody was widely cross-reactive, and neutralizing antibody assay revealed preexisting antibody to JEV (i.e., this was also a secondary flavivirus infection). Apparently DEN-2 and DEN-4 viruses circulating in Bangkok and Kamphaeng Phet lacked sufficient pathogenicity to bring children to the hospital clinic, except in hosts with heterologous immunity. This same observation has been made in a data set spanning 25 years of sur-

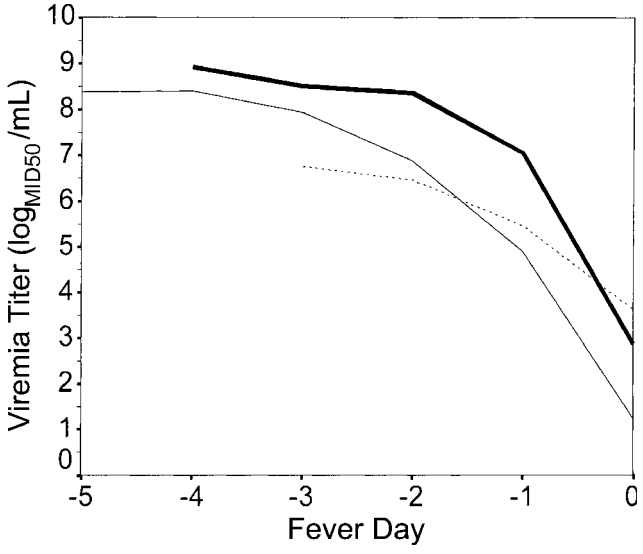

Figure 4. Mean dengue virus titer (log mosquito infectious doses/ml [log MID_{50}/mL]) by fever day for dengue 2 virus patients experiencing dengue fever (*thin dashed line, n* = 16), dengue hemorrhagic fever grades 1 and 2 (*solid thin line, n* = 26), and dengue hemorrhagic fever grade 3 (*heavy solid line, n* = 5).

veillance at the Bangkok Children's Hospital, where preliminary analysis shows that in children aged >12 months, 97% of patients with DEN-2 or DEN-4 infections have a secondary antibody response pattern, compared with 72% of patients with DEN-1 or DEN-3 ($P < .001$; A. Nisalak, unpublished data).

How do our data fit with the various hypotheses on dengue pathogenesis? Our data indicate that viremia titer early in the course of infection is a key factor; higher viremia titer is associated with more severe disease. This finding is consistent with a role for viral virulence or replicative ability, as well as enhancing antibody in determining disease severity. In our regression analysis, secondary dengue virus infection and peak virus titer both correlated with disease severity. We also found that disease severity was associated with more rapid virus clearance, suggesting a greater importance for a primed immune system. In addition, other host factors apparently also play a role, leading to divergent disease outcomes given equivalent peak viremia titers. Girls, well-nourished children, and school-age children may have more robust immune systems that exaggerate the immune response to dengue virus, leading to more severe disease in some cases. Specific virus serotypes and genotypes may replicate more readily in specific population groups to cause DHF even in primary infections (rare) or enhance more readily in the presence of preexisting antibody.

In conclusion, we have shown that high viremia titers 3 days after the onset of fever correlates with severe disease 2 days later at the time of defervescence. Concurrent associations between disease severity and viremia titer and serological evidence of secondary dengue infections suggest that cross-reactive immune responses may be an important factor related to dengue pathogenesis among children in dengue hyperendemic regions.

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References

1. Rush B. An account of the bilious remitting fever, as it appeared in Philadelphia, in the summer and autumn of the year 1780. Medical Inquiries and Observations. 1st ed. Philadelphia: Prichard and Hall, **1789**: 89–100.
2. Hammon WM, Rudnick A, Sather GE, et al. Studies on Philippine hemorrhagic fever: relationship to dengue viruses. Proceedings of the Ninth Pacific Science Congress of the Pacific Science Association **1962**; 17:67–72.
3. Hammon WM, Rudnick A, Sather GE. Viruses associated with epidemic hemorrhagic fevers of the Philippines and Thailand. Science **1960**; 131: 1102–3.
4. Innis BL. Dengue and dengue hemorrhagic fever. In: Porterfield JS, ed. Kass Handbook of Infectious Diseases: Exotic Virus Infections. 1st ed. London: Chapman and Hall Medical, **1995**:103–46.
5. Halstead SB. Pathogenesis of dengue: challenges to molecular biology. Science **1988**; 239:476–81.
6. Burke DS, Nisalak A, Johnson DE, Scott RM. A prospective study of dengue infections in Bangkok. Am J Trop Med Hyg **1988**; 38:172–80.
7. Henchal EA, Putnak JR. The dengue viruses. Clin Microbiol Rev **1990**; 3: 376–96.
8. Halstead SB, Nimmannitya S, Yamarat C, Russell PK. Hemorrhagic fever in Thailand; recent knowledge regarding etiology. Jpn J Med Sci Biol **1967**; 20:96–103.
9. Halstead SB, Nimmannitya S, Cohen SN. Observations related to pathogenesis of dengue hemorrhagic fever. IV. Relation of disease severity to antibody response and virus recovered. Yale J Biol Med **1970**; 42:311–28.
10. Russell PK, Yuill TM, Nisalak A, Udomsakdi S, Gould D, Winter PE. An insular outbreak of dengue hemorrhagic fever. II. Virologic and serologic studies. Am J Trop Med Hyg **1968**; 17:600–8.
11. Sangkawibha N, Rojanasuphot S, Ahandrik S, et al. , Risk factors in dengue shock syndrome: a prospective epidemiologic study in Rayong, Thailand. I: the 1980 outbreak. Am J Epidemiol **1984**; 120:653–69.
12. Kliks SC, Nisalak A, Brandt WE, Wahl L, Burke DS. Antibody-dependent enhancement of dengue virus growth in human monocytes as a risk factor for dengue hemorrhagic fever. Am J Trop Med Hyg **1989**; 40:444–51.
13. Halstead SB, O'Rourke EJ. Dengue viruses and mononuclear phagocytes. I: infection enhancement by non-neutralizing antibody. J Exp Med **1977**; 146:201–17.
14. Kurane I, Meager A, Ennis FA. Dengue virus-specific human T cell clones: serotype crossreactive proliferation, interferon gamma production, and cytotoxic activity. J Exp Med **1989**; 170:763–75.
15. Green S, Vaughn DW, Kalayanarooj S, et al. Early immune activation in acute dengue illness is related to development of plasma leakage and disease severity. J Infect Dis **1999**; 179:755–62.
16. Kurane I, Innis BL, Nimmannitya S, et al. Activation of T lymphocytes in dengue virus infections: high levels of soluble interleukin 2 receptor, soluble CD4, soluble CD8, interleukin 2, and interferon-gamma in sera of children with dengue. J Clin Invest **1991**; 88:1473–80.
17. Kurane I, Ennis FA. Cytokines in dengue virus infections: role of cytokines in the pathogenesis of dengue hemorrhagic fever. Semin Virol **1994**; 5: 443–8.
18. Rosen L. The pathogenesis of dengue haemorrhagic fever: a critical appraisal of current hypotheses. S Afr Med J **1986**; (Suppl):40–2.
19. Rico-Hesse R, Harrison LM, Salas RA, et al. Origins of dengue type 2 viruses associated with increased pathogenicity in the Americas. Virology **1997**; 230:244–51.
20. Leitmeyer KC, Vaughn DW, Watts DM, et al. Dengue virus structural differences that correlate with pathogenesis. J Virol **1999**; 73:4738–47.
21. Chiewsilp P, Scott RM, Bhamarapravati N. Histocompatibility antigens and dengue hemorrhagic fever. Am J Trop Med Hyg **1981**; 30:1100–5.
22. Bravo Gonzalez JR, Guzman Tirado MG, Kouri Flores G. [Retrospective sero-epidemiological survey of dengue virus in the town of Cerro. Methodology] Encuesta seroepidemiologica retrospectiva a virus dengue en el municipio Cerro. Metodologia. Rev Cubana Med Trop **1985**; 37:259–68.

23. Guzman MG, Kouri G, Morier L, Soler M, Fernandez A. A study of fatal hemorrhagic dengue cases in Cuba, 1981. Bull Pan Am Health Organ **1984**; 18:213–20.
24. Anto S, Sebodo T, Sutaryo, Suminta, Ismangoen. Nutritional status of dengue haemorrhagic fever in children. Paediatrics Indonesia **1983**; 23:15–24.
25. Thisyakorn U, Nimmannitya S. Nutritional status of children with dengue hemorrhagic fever. Clin Infect Dis **1993**; 16:295–7.
26. Rico-Hesse R, Harrison LM, Nisalak A, et al. Molecular evolution of dengue type 2 virus in Thailand. Am J Trop Med Hyg **1998**; 58:96–101.
27. Vaughn DW, Green S, Kalayanarooj S, et al. Dengue in the early febrile phase: viremia and antibody responses. J Infect Dis **1997**; 176:322–30.
28. Kalayanarooj S, Vaughn DW, Nimmannitya S, et al. . Early clinical and laboratory indicators of acute dengue illness. J Infect Dis **1997**; 176: 313–21.
29. Mathew A, Kurane I, Green S, et al. Predominance of HLA-restricted cytotoxic T-lymphocyte responses to serotype–cross-reactive epitopes on nonstructural proteins following natural secondary dengue virus infection. J Virol **1998**; 72:3999–4004.
30. Mathew A, Kurane I, Green S, et al. Impaired T cell proliferation in acute dengue infection. J Immunol **1999**; 162:5607–15.
31. Green S, Vaughn DW, Kalayanarooj S, et al. Elevated plasma interleukin-10 levels in acute dengue correlate with disease severity. J Med Virol **1999**; 59:329–34.
32. Green S, Pichyangkul S, Vaughn DW, et al. Early CD69 expression on peripheral blood lymphocytes from children with dengue hemorrhagic fever. J Infect Dis **1999**; in press.
33. Anonymous. Dengue haemorrhagic fever: diagnosis, treatment, prevention and control. 2d ed. Geneva: World Health Organization, **1997**:12–47.
34. Innis BL, Nisalak A, Nimmannitya S, et al. An enzyme-linked immunosorbent assay to characterize dengue infections where dengue and Japanese encephalitis co-circulate. Am J Trop Med Hyg **1989**; 40:418–27.
35. Clarke DH, Casals J. Techniques for hemagglutination and hemagglutination inhibition with arthropod-borne viruses. Am J Trop Med Hyg **1958**; 7: 561–73.
36. Barth OM. Replication of dengue viruses in mosquito cell cultures—a model from ultrastructural observations. Mem Inst Oswaldo Cruz **1992**; 87: 565–74.
37. Rosen L, Shroyer DA. Comparative susceptibility of five species of Toxorhynchites mosquitoes to parenteral infection with dengue and other flaviviruses. Am J Trop Med Hyg **1985**; 34:805–9.
38. Kuno G, Gubler DJ, Santiago de Weil NS. Antigen capture ELISA for the identification of dengue viruses. J Virol Methods **1985**; 12:93–103.
39. Lanciotti RS, Calisher CH, Gubler DJ, Chang GJ, Vorndam AV. Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. J Clin Microbiol **1992**; 30:545–51.
40. Siler JF, Hall MW, Hitchens AP. Dengue: Its history, epidemiology, mechanism of transmission, etiology, clinical manifestations, immunity, and prevention. The Philippine Journal of Science **1926**; 29:1–304.
41. Gubler DJ. Dengue and dengue hemorrhagic fever: its history and resurgence as a global public health problem. In: Gubler DJ, Kuno G, eds. Dengue and dengue hemorrhagic fever. New York: CAB International, **1997**:1–22.
42. Halstead SB, Shotwell H, Casals J. Studies on the pathogenesis of dengue infection in monkeys. II: clinical laboratory responses to heterologous infection. J Infect Dis **1973**; 128:15–22.