MAJOR ARTICLE

Relationship of Preexisting Dengue Virus (DV) Neutralizing Antibody Levels to Viremia and Severity of Disease in a Prospective Cohort Study of DV Infection in Thailand

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Background. Infection with any 1 of the 4 dengue viruses (DVs) can produce several illnesses, ranging from a mild febrile illness to classic dengue fever (DF) to dengue hemorrhagic fever (DHF), a potentially life-threatening disease. Most DHF cases occur after sequential heterotypic DV infections. The role of preexisting humoral immunity in modifying severity of dengue disease is not well understood.

Methods. We conducted a prospective cohort study of children in a region where dengue disease is hyperendemic and examined the role of preexisting neutralizing anti-DV antibodies (Abs) in modifying secondary dengue-3 virus (D3V), dengue-2 virus (D2V), and dengue-1 virus (D1V) infections.

Results. In secondary D3V infection, higher levels of preexisting neutralizing Ab directed against D3V (reference virus strain and patient's virus isolate) were associated with lower viremia levels and milder disease. Preexisting neutralizing Ab levels against D2V were not associated with severity of secondary D2V infection. The levels of preexisting neutralizing Ab against the infecting virus isolates were not associated with viremia levels in secondary D2V or D1V infections.

Conclusions. Cross-reactive memory humoral immune responses appear to be beneficial in symptomatic secondary D3V infection, but not in secondary D2V or D1V infection. These results may have important implications for the development of live attenuated tetravalent dengue vaccines.

Dengue is an emerging arboviral disease caused by infection with 1 of the dengue viruses (DVs), a group of 4 antigenically related mosquito-borne flaviviruses (D1V, D2V, D3V, and D4V) [1]. Infection with any 1 of the 4 DV serotypes can produce a spectrum of clinical illness, ranging from an asymptomatic or mild febrile illness to classic dengue fever (DF) to the most severe form of illness, dengue hemorrhagic fever (DHF). A primary infection with 1 serotype typically produces long-term protective immunity to reinfection with the homologous serotype. After a short period of cross-protection, individuals who have recovered from a primary DV infection are fully susceptible to infection and disease by heterologous serotypes [2, 3]. In fact, the relative risk of developing DHF is enhanced 5–500-fold in a secondary, sequential, heterotypic DV infection [4–6]. On the basis of these observations, observations of DHF in infants born to mothers with preexisting dengue immunity [7], and results of in vitro studies [8, 9], antibody (Ab)–dependent enhancement of infection (ADE) has been postulated to be a primary mechanism of immune enhancement of severity of disease. It is thought that, with ADE, preexisting DV serotype cross-reactive Abs facilitate virus entry into Fc-receptor–bearing cells, thereby in-

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creasing the virus burden and severity of disease. An exaggerated cellular immune response to DV infection, driven by DV serotype cross-reactive memory T lymphocytes, may also lead to increased severity of disease and to DHF [10]. These 2 mechanisms are not mutually exclusive and may interact with other viral and host factors to shape the clinical manifestations of dengue disease.

The development of an effective tetravalent dengue vaccine is a key step toward controlling the increasing morbidity and mortality of dengue disease throughout the tropical and subtropical regions of the world. The protective capability of dengue vaccine candidates has been inferred by their ability to induce neutralizing Ab responses, as has been reported with other viral vaccines [11, 12]. However, anti-DV neutralizing Ab titers that would be considered to be protective and the in vivo roles of Ab in ameliorating or augmenting severity of dengue disease are not well defined. Clinical evidence directly implicating humoral immunity in modifying the severity of dengue disease has been limited and restricted to D2V infections [7, 13].

We conducted a prospective cohort study of primary schoolchildren in Thailand, to examine some of the factors that contribute to severity of disease in subjects with acute DV infection and secondary, or anamnestic, Ab responses. The present report focuses on the contribution of preexisting anti-DV neutralizing Abs to the degree of severity of disease. Our study is novel and differs from the previously reported prospective studies of dengue disease [4, 14–16] in several key respects: first, our cohort for analysis included subjects with virologically defined acute D1V, D2V, and D3V infections and well-defined anamnestic Ab responses; second, we measured preillness neutralizing Ab levels to reference DV strains and to the clinical virus isolates; and third, we correlated preexisting neutralizing Ab capacity against the infecting DV serotypes with viremia levels during the subsequent DV infections (a proximate effect of Ab-virus interactions) and clinical severity of disease.

SUBJECTS AND METHODS

Subject enrollment and study design. Full details of the investigational protocol have been published elsewhere [17]. The present study was conducted during 1998–2000 among ~2000 primary schoolchildren in Kamphaeng Phet province, Thailand. Parents or guardians of all study subjects gave written, informed consent. A blood plasma sample was obtained every January in all subjects. Active case surveillance for acute febrile illnesses occurred during the dengue disease season every year, from 1 June to 15 November. Acute- and convalescent-phase (day 14) blood samples were obtained from students who missed school or who visited the school nurse and who had a history of fever (temperature $\geq$38.0°C) $\leq$7 days or a fever on evaluation. The investigational protocol was approved by the institutional review boards of the Thai Ministry of Public Health, the Office of the US Army Surgeon General, and the University of Massachusetts Medical School.

Identification of DV infections. Secondary infections refer to those in children with an anamnestic serologic response to their DV infection and may represent secondary or tertiary infections with DVs or other cross-reactive flaviviruses. Secondary, or anamnestic, DV infections were identified by use of previously established serologic criteria for IgM/IgG ELISAs and hemagglutination-inhibition assays to D1V–D4V and Japanese encephalitis virus (JEV) in paired acute- and convalescent-phase serum samples [18, 19]. A symptomatic secondary DV infection was defined as a febrile illness identified during the active surveillance period and by serologic identification of a secondary DV infection. The infecting DV serotype was identified from acute-phase serum samples by use of a serotype-specific reverse-transcriptase polymerase chain reaction (RT-PCR) [20], which was positive in 104 of 106 cases, or by virus isolation in *Toxorhynchites splendens* mosquitoes [21]. There was 100% concordance in serotype identification when the RT-PCR and mosquito isolation assays were both positive.

Severity of dengue disease categories. Among symptomatic DV infections, 3 categories of severity of dengue disease were defined: first, dengue hemorrhagic fever (DHF)—hospitalized and evidence of DHF grades I–IV, according to World Health Organization (WHO) criteria [19]; second, dengue fever–hospitalized (hDF)—hospitalized and did not meet the WHO criteria for DHF; and third, dengue fever–mild, not hospitalized (nhDF)—a febrile illness that did not result in hospitalization.

Plaque reduction neutralizing Ab assay. Plaque reduction neutralizing Ab titers against reference strains of D1V–D4V and JEV were performed on the plasma samples obtained during the January immediately preceding the secondary DV infection by use of standard methods [22]. In brief, a monolayer of the LLC-MK2 cell line was infected with a constant amount of virus in the presence of 4-fold serial dilutions of heat-inactivated plasma from patients (1:10, 1:40, 1:160, 1:640, and 1:2560). The 50% plaque reduction neutralization titer ($PRNT_{50}$) was calculated by use of a log probit regression method (SPSS software; version 10.0; SPSS) and was reported as a reciprocal titer. $PRNT_{50}$ values <10 (undetectable) were recorded as 5 for the purposes of analysis. The reference virus strains were D1V 16007, D2V 16681, D3V 16562, D4V 1036, and JEV Nakayama. The sources and passage histories of the parental DV reference virus strains have been described elsewhere [23]. Specimens were tested in a blinded fashion, and all sample dilutions were performed in duplicate. All assays had high positive, low positive, and negative control serum samples included. The coefficients of variance (CV) of the $\log_{10}$ $PRNT_{50}$ for the high positive control serum samples against D1V–D3V and JEV were

Table 1. Demographic characteristics of a cohort of primary schoolchildren with secondary dengue virus (DV) infections in Kamphaeng Phet province, Thailand, by DV type.

Characteristic	D1V (n = 24)	D2V (n = 37)	D3V (n = 45)
Age, median (5th, 95th percentiles), years	10 (8, 13)	10 (8, 12)	10 (8, 13)
Sex, M:F	13:11	15:22	19:26
Severity of disease classification			
nhDF	21	29	30
hDF	1	3	7
DHF	2	5	8

NOTE. The Mann-Whitney U test was used for comparisons between nonnormally distributed continuous variables. The χ^2 analysis was used for comparisons among proportional data. All P values were not significant ($P > .10$). D1V, dengue-1 virus; D2V, dengue-2 virus; D3V, dengue-3 virus; DHF, dengue hemorrhagic fever (hospitalized); hDF, dengue fever–hospitalized; nhDF, dengue fever–mild, not hospitalized.

≤7% (there was no high positive control against D4V). The CV of the $\log_{10}$ $PRNT_{50}$ for the low positive control serum samples against D1V–D4V and JEV were <15%. The $PRNT_{50}$ values against D1V–D4V and JEV for the negative control serum samples were always <10.

In cases in which DV was isolated, we also measured the $PRNT_{50}$ against the patient's virus isolate by use of the methods described above. The clinical virus isolates were amplified in *T. splendens* mosquitoes and passaged twice in C6/36 mosquito cells before being used in the plaque reduction neutralization Ab assay.

Preexisting neutralizing Ab patterns. On the basis of previously published criteria [4], we defined 3 patterns of the preillness $PRNT_{50}$ results: undetectable ($PRNT_{50}$ <10 to all 4 DV serotypes), monotypic ($PRNT_{50}$ >10 to only 1 DV serotype or $PRNT_{50}$ >10 to >1 DV serotype with a $PRNT_{50}$ ≥80 to only 1 DV serotype), and multitypic ($PRNT_{50}$ >10 to >1 DV serotype without a $PRNT_{50}$ ≥80 to only 1 DV serotype). In subjects with a preexisting monotypic pattern of anti-DV neutralizing Abs, the DV serotype with the highest $PRNT_{50}$ was assumed to be the serotype of the previous DV infection. In those subjects with preexisting undetectable or multitypic patterns, we could not determine the serotype of any prior DV infections.

Quantification of DV viremia. The circulating level of DV RNA (viremia) was quantified in acute-phase serum samples by use of a serotype-specific fluorogenic RT-PCR assay [24]. Amplicons (143 nt) of the D1V–D3V 3′-noncoding regions (the RT-PCR products) [24] were cloned into pNOT vectors, and serial dilutions were used as quantification standards (provided by Dr. H. S. Houng, Department of Virus Diseases, Walter Reed Army Institute of Research, Silver Spring, MD). Interassay precision was monitored by positive and negative controls on every 96-well plate. All samples were assayed in triplicate in a blinded fashion and were quantified on the linear portion of standard curves. Viremia levels were expressed as DV genome equivalent cDNA copies (genome eq) per milliliter.

Statistical analysis. We used the Student's t test for comparisons between normally distributed continuous variables and the Mann-Whitney U test for comparisons between nonnormally distributed continuous variables. The χ^2 analysis was used for comparisons between proportional data. We used the Spearman's correlation test to examine associations between nonnormally distributed ordinal variables or continuous variables. $P < .05$ was considered to be significant, $.05 \leq P \leq .10$ was considered to be a nonsignificant trend, and $P > .10$ was considered to be not significant. The statistical software package SPSS (version 10.0; SPSS) was used for all statistical analyses.

RESULTS

Study population characteristics. During the 3 years of the study, 154 children with symptomatic DV infections were identified. More than 90% of children in the study with symptomatic DV infections had secondary serologic responses. We selected for analysis the children with symptomatic secondary DV infections in which the infecting serotype was identified (total, $n = 106$; secondary D3V infections, $n = 45$; secondary D2V infections, $n = 37$; secondary D1V infections, $n = 24$). One child with a secondary D4V infection was excluded from further analysis. There were no differences with regard to age, sex, or severity of disease among the children with symptomatic secondary D1V, D2V, or D3V infections (table 1). The majority of children (76%) had a mild illness.

Preexisting neutralizing Ab patterns against D1V–D4V and development of DHF. We examined the pattern of $PRNT_{50}$ to D1V–D4V in blood samples obtained 6–9 months before the development of acute, symptomatic, secondary dengue disease ($n = 104$; 2 samples were excluded because of incomplete

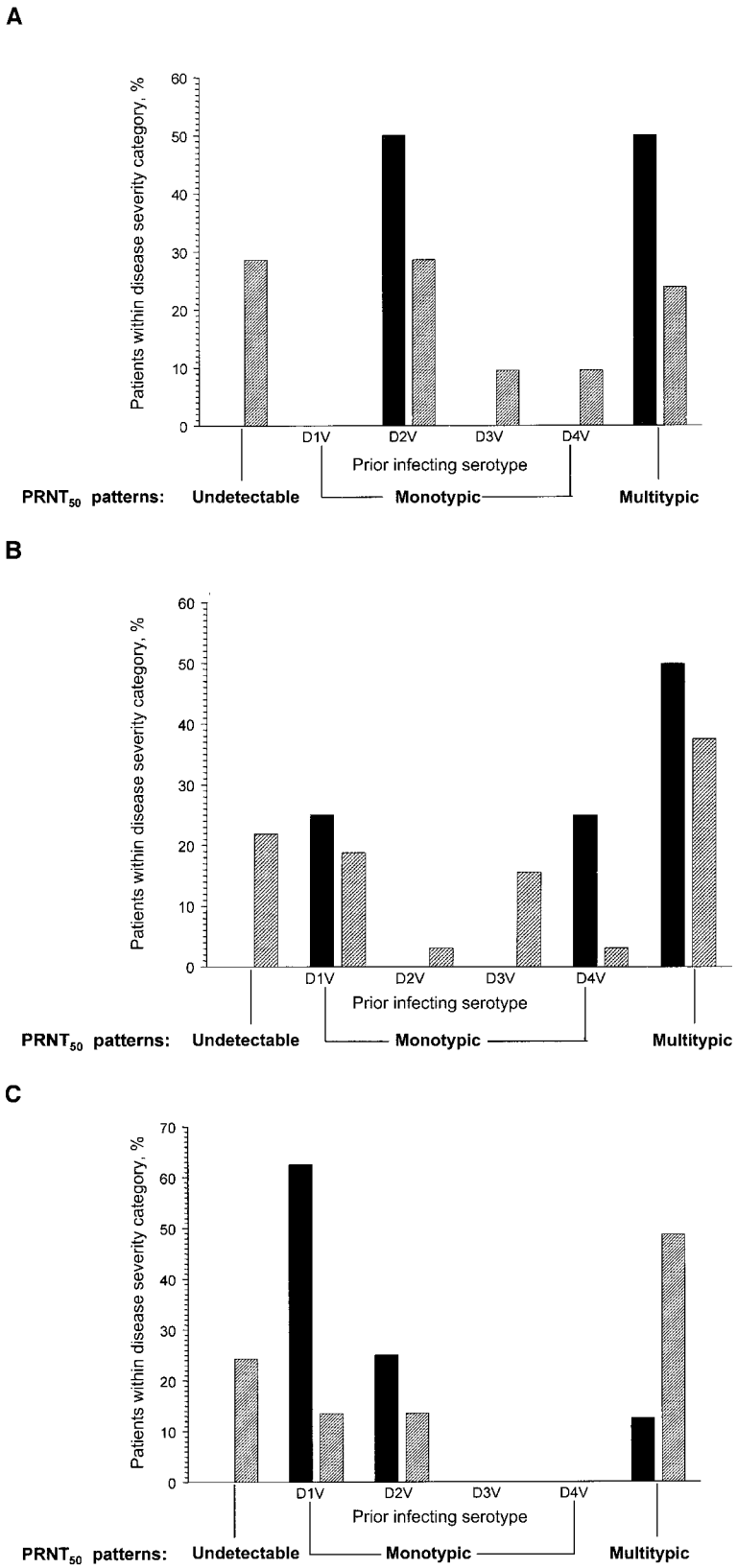

Figure 1. Preexisting neutralizing antibody patterns against dengue virus serotypes 1–4, stratified by severity of disease category: dengue hemorrhagic fever (DHF, *solid bars*) vs. dengue fever (DF, *striped bars*). DF consists of hospitalized (hDF) or mild, not hospitalized (nhDF) cases. $PRNT_{50}$ (50% plaque reduction neutralizing reciprocal titer) patterns are labeled as undetectable, monotypic, or multitypic (see Subjects and Methods for definitions). D1V, dengue-1 virus; D2V, dengue-2 virus; D3V, dengue-3 virus; D4V, dengue-4 virus. *A,* secondary D1V infections (DHF, $n = 2$; DF, $n = 21$); *B,* secondary D2V infections (DHF, $n = 4$; DF, $n = 32$); *C,* secondary D3V infections (DHF, $n = 8$; DF, $n = 37$).

data) (figure 1). In D1V, D2V, and D3V infections, DHF occurred only in subjects with a preexisting detectable $PRNT_{50}$ pattern. Among subjects with a preexisting detectable $PRNT_{50}$ pattern, the proportion with DHF was 17%; among subjects with an undetectable pattern, the proportion with DHF was 0% (for all serotypes combined; $P = .04$). In D3V infection, a preexisting monotypic $PRNT_{50}$ pattern, which reflects prior infection with D1V or D2V, was associated with DHF, compared with a preexisting multitypic $PRNT_{50}$ pattern; 41% of subjects with preexisting monotypic $PRNT_{50}$ patterns developed DHF, compared with 5% of subjects with multitypic patterns ($P = .02$). In secondary D2V and D1V infections, there was no significantly increased risk of DHF in those with preexisting monotypic $PRNT_{50}$ patterns directed against heterotypic DVs.

JEV is endemic in the Kamphaeng Phet region, and preexisting JEV immune responses may diminish the severity of subsequent dengue disease [25]. The association between a preexisting detectable DV $PRNT_{50}$ pattern and DHF remained a trend, although not statistically significant, in the absence of preexisting JEV neutralizing Ab ($n = 50$; among subjects with a preexisting detectable DV $PRNT_{50}$ pattern, the proportion with DHF was 22%, and, among subjects with an undetectable pattern, the proportion with DHF was 0%; $P = .05$) and in the presence of JEV neutralizing Ab ($n = 54$; among subjects with a preexisting detectable DV $PRNT_{50}$ pattern, the proportion with DHF was 12%, and, among subjects with an undetectable pattern, the proportion with DHF was 0%; $P = .09$).

Only 1 of the 43 subjects with monotypic $PRNT_{50}$ patterns had preexisting Ab directed against the same DV serotype as the infecting one (figure 1*B*). In 1 subject with D2V infection and with mild nhDF, preexisting neutralizing Abs against D1V, D3V, D4V, and JEV were undetectable, but low levels of neutralizing Ab against the reference D2V strain ($PRNT_{50}$, 13) and against the patient's D2V isolate ($PRNT_{50}$, 11) were detected.

Preexisting neutralizing Ab against the infecting DV serotype: comparison of reference virus strains and the patient's virus isolate. In cases of secondary D3V ($n = 40$), D2V ($n = 35$), and D1V ($n = 22$) infection from which DV was isolated, preexisting neutralizing Ab titers against the infecting DV serotype were determined against a reference DV strain and the patient's virus isolate (a virus isolate of sufficient titer was not available for all patients). Preexisting neutralizing Ab titers directed against the reference viruses correlated with the neutralizing Ab titers directed against the patient's virus isolates (Spearman's $r = 0.8$, $r = 0.8$, and $r = 0.9$, for log-log comparisons in D3V, D2V, and D1V, respectively; all $P < .001$). $PRNT_{50}$ values to the reference D3V strain were higher than $PRNT_{50}$ values to the clinical D3V isolates (mean ± SE $\log_{10}$ $PRNT_{50}$ reference strain minus the $\log_{10}$ $PRNT_{50}$ clinical isolate, 0.45 ± 0.08), $PRNT_{50}$ values to the reference D2V strain were nearly equivalent to the titers against the clinical D2V isolates (mean ± SE $\log_{10}$ $PRNT_{50}$ reference strain minus the $\log_{10}$ $PRNT_{50}$ clinical isolate, −0.17 ± 0.07), and $PRNT_{50}$ values to the D1V strain were generally lower than the titers against the clinical D1V isolates (mean ± SE $\log_{10}$ $PRNT_{50}$ reference strain minus the $\log_{10}$ $PRNT_{50}$ clinical isolate, −0.30 ± 0.08).

Preexisting neutralizing Ab titers against the infecting DV serotype and severity of subsequent dengue disease. Preexisting neutralizing Ab directed against the reference strain of the infecting DV serotype was detected in 51%, 60%, and 29% of the D3V, D2V, and D1V infections, respectively. Preexisting neutralizing Ab directed against the patient's subsequent virus isolate was detected in 36%, 67%, and 46% of the D3V, D2V, and D1V infections, respectively. In secondary D3V infection, the preillness $PRNT_{50}$ geometric mean titers (GMTs) to the reference D3V strain and clinical D3V isolates were lowest in those who developed DHF (figure 2*C*). A preexisting $PRNT_{50}$ >100 against the reference D3V strain was associated with milder severity of disease ($PRNT_{50}$ >100 in 46% of DF cases [hDF or nhDF] vs. 0% of DHF cases; $P = .02$). However, in secondary D2V and D1V infections, there were no significant associations between the levels of preexisting neutralizing Ab to the infecting DV serotype (reference virus strain or clinical virus isolate) and severity of subsequent disease (figure 2*B* and 2*A*, respectively).

Preexisting neutralizing Ab titers against the infecting DV serotype and viremia levels. Plaque reduction neutralizing Ab titers in patient blood samples presumably reflect the capacity of circulating Abs to control viral replication in vivo. We therefore compared preexisting neutralizing Ab titers directed against the patient's virus isolate and viremia levels. On the basis of results from our earlier studies [26–28], we took the viremia level in an acute illness serum sample to be an estimate of peak viremia when the serum sample was obtained within 2 days of fever onset (D3V, $n = 27$; D2V, $n = 32$; and D1V, $n = 14$). In secondary D3V infection, subjects with more-severe disease had lower preexisting $PRNT_{50}$ GMTs against D3V and higher viremia levels (table 2). A higher level of preexisting neutralizing Ab against the patient's D3V isolate was associated with lower viremia levels (Spearman's $r = -0.63$; $P = .001$) (figure 3*C*). All subjects with detectable preexisting neutralizing Ab against their own subsequent D3V isolate had viremia levels ≤ 7 $\log_{10}$ D3V RNA genome eq/mL.

In secondary D2V infection, subjects with more-severe disease had higher viremia levels but did not have lower preexisting $PRNT_{50}$ GMTs against D2V (table 2). Unlike patients with secondary D3V infections, there were no significant associations between preexisting neutralizing Ab levels to the patient's D2V isolate and subsequent viremia levels (Spearman's $r = 0.03$; $P = .9$) (figure 3*B*). We could not analyze the association between viremia, $PRNT_{50}$ GMTs, and severity of disease for secondary D1V infection, because there were no hDF

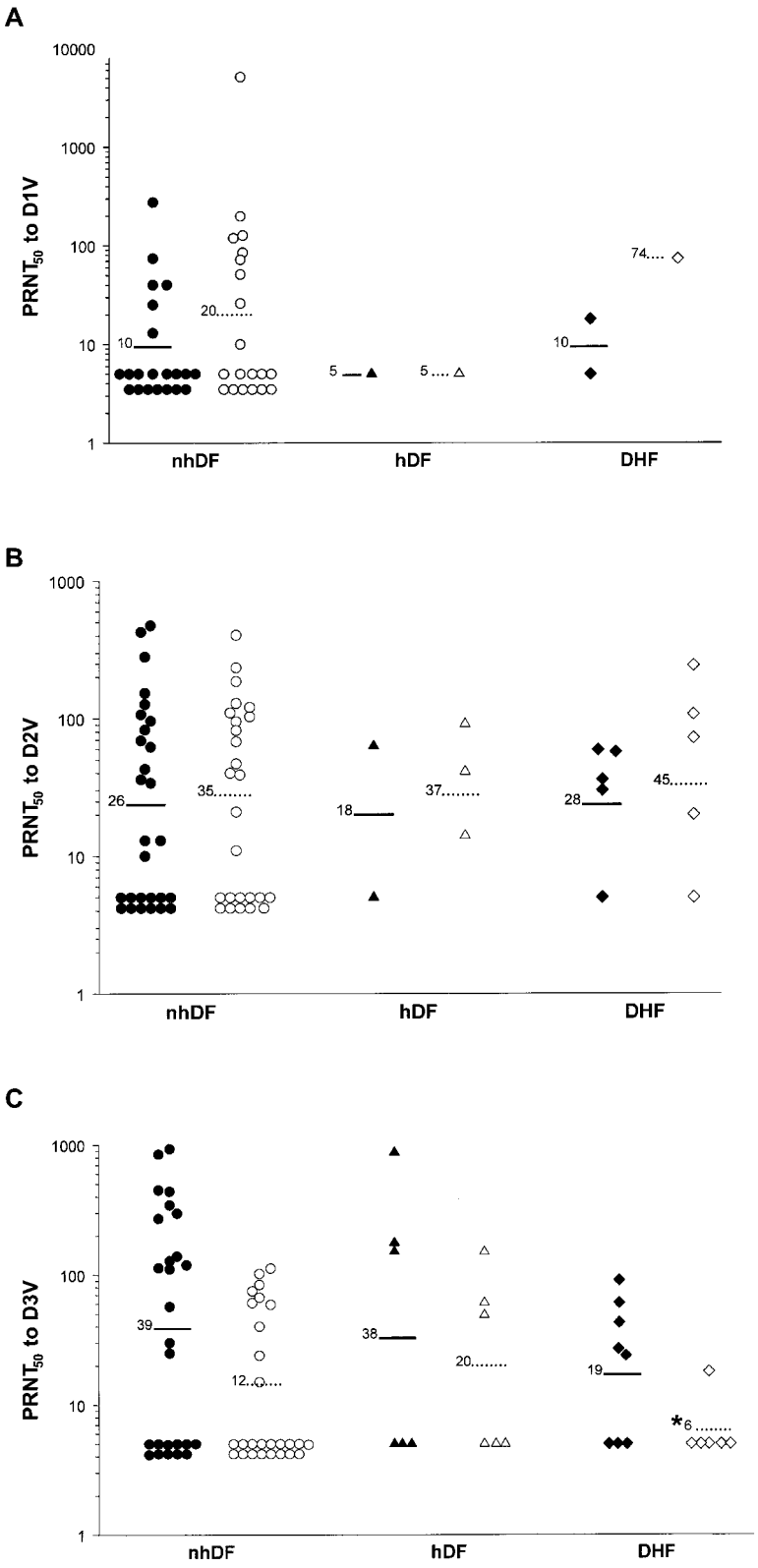

Figure 2. Relationship between preexisting neutralizing antibody titers against the infecting dengue virus (DV) serotype and subsequent dengue severity of disease in secondary dengue-1 virus (D1V) *(A),* dengue-2 virus (D2V) *(B),* and dengue-3 virus (D3V) *(C)* infections. $PRNT_{50}$ (50% plaque reduction neutralization reciprocal titer) values <10 (undetectable) are recorded as 5. *Solid symbols,* $PRNT_{50}$ against the reference DV strain; *open symbols,* $PRNT_{50}$ against the patient's DV isolate; *bars,* geometric mean titers of the $PRNT_{50}$. DHF, dengue hemorrhagic fever (hospitalized); hDF, dengue fever–hospitalized; nhDF, dengue fever–mild, not hospitalized. $*P = .05$, DHF vs. nhDF (log-log comparison).

Table 2. Preexisting neutralizing antibody levels and estimates of peak viremia levels stratified by severity of disease in secondary dengue-1 virus (D1V), dengue-2 virus (D2V), and dengue-3 virus (D3V) infections.

Virus type, severity of disease classification	Viremia level, mean ± SE, $\log_{10}$ virus genome eq/mL	Preexisting $PRNT_{50}$ GMT to reference virus	Preexisting $PRNT_{50}$ GMT to clinical virus isolate
D1V			
nhDF	7.85 ± 0.20 (n = 14)	10 (n = 14)	19 (n = 14)
hDF	NA	NA	NA
DHF	NA	NA	NA
D2V			
nhDF	7.50 ± 0.19 (n = 25)	31 (n = 25)	42 (n = 24)
hDF	8.51 ± 0.79[a] (n = 3)	18 (n = 2)	37 (n = 3)
DHF	8.41 ± 0.31[a,b] (n = 4)	44 (n = 4)	78 (n = 4)
D3V			
nhDF	6.84 ± 0.30 (n = 19)	29 (n = 17)	13 (n = 19)
hDF	7.96 ± 0.43[c] (n = 5)	18 (n = 4)	12 (n = 4)
DHF	8.36 ± 0.66[c,d] (n = 3)	8 (n = 3)	5[e] (n = 3)

NOTE. The Mann-Whitney U test was used for all comparisons. DHF, dengue hemorrhagic fever (hospitalized); eq, genome equivalents; hDF, dengue fever–hospitalized; nhDF, dengue fever–mild, not hospitalized; NA, not available; $PRNT_{50}$ GMT, 50% plaque reduction neutralizing reciprocal titer, geometric mean titer.

[a] P = .02, DHF or hDF vs. nhDF.
[b] P = .06, DHF vs. nhDF.
[c] P = .03, DHF or hDF vs. nhDF.
[d] P = .07, DHF vs. nhDF.
[e] P = .006, DHF vs. nhDF (log-log comparison).

or DHF D1V cases with viremia determinations. However, in the nhDF D1V cases, there was no significant association between preexisting neutralizing Ab levels to the patient's D1V isolate and subsequent viremia levels (Spearman's $r = 0.23$; $P = .4$) (figure 3*A*).

DISCUSSION

The results of the present study are consistent with those of earlier prospective studies [4, 14–16] in finding that preexisting immunity to dengue disease detected by anti-DV neutralizing Abs was associated with subsequent development of DHF. This pattern held true for secondary D2V infections and D3V infections, but, given the smaller numbers, some caution must be exercised when interpreting this pattern in secondary D1V infection. As in other prospective studies [14, 15], the preillness neutralizing Ab titers were measured at a fixed time point during the nadir of seasonal DV transmission, 6–9 months before the onset of acute symptomatic dengue disease. We believe that these preillness blood samples reasonably reflect the serologic status of the study subjects at the time of their symptomatic DV infection. In our study location, the period between the preillness blood sampling in January and the initiation of active surveillance at the beginning of the rainy season in June is a time of very low to nonexistent dengue [17]. In the absence of intercurrent DV infection, we assume that preillness neutralizing Ab titers measured in healthy subjects are not significantly altered over the course of a 6–9-month period and that any decay in Ab titers would occur at a uniform rate across the study cohort.

In Kamphaeng Phet, Thailand, multiple flaviviruses cocirculate. The subjects in the present report with preexisting undetectable $PRNT_{50}$ to all 4 DV serotypes had anamnestic flavivirus serologic responses during their acute symptomatic DV infection. We could not determine whether the anamnestic responses were due to earlier DV infections that produced $PRNT_{50}$ values below the assay's limit of detection, earlier JEV infections (59% of subjects [13/22] had detectable $PRNT_{50}$ to JEV), or exposure to other flaviviruses in the region, such as Tembusu or Langat [29, 30]. A potential effect of preexisting JEV immunity on the development of DHF did not appear to be a confounding factor but could not be completely excluded.

In secondary D3V infection, we observed that a preexisting monotypic $PRNT_{50}$ pattern, reflecting prior infection with the heterotypic D1V or D2V, was associated with development of DHF. Perhaps because of smaller sample sizes, we could not draw similar conclusions in the secondary D2V and D1V infection cases. Among the previous prospective cohort studies of dengue disease [4, 14–16], the definitions of undetectable, monotypic, or multitypic neutralizing Ab patterns have varied. We based our definitions on previously published criteria [4], except that our minimum threshold of detection was a $PRNT_{50}$

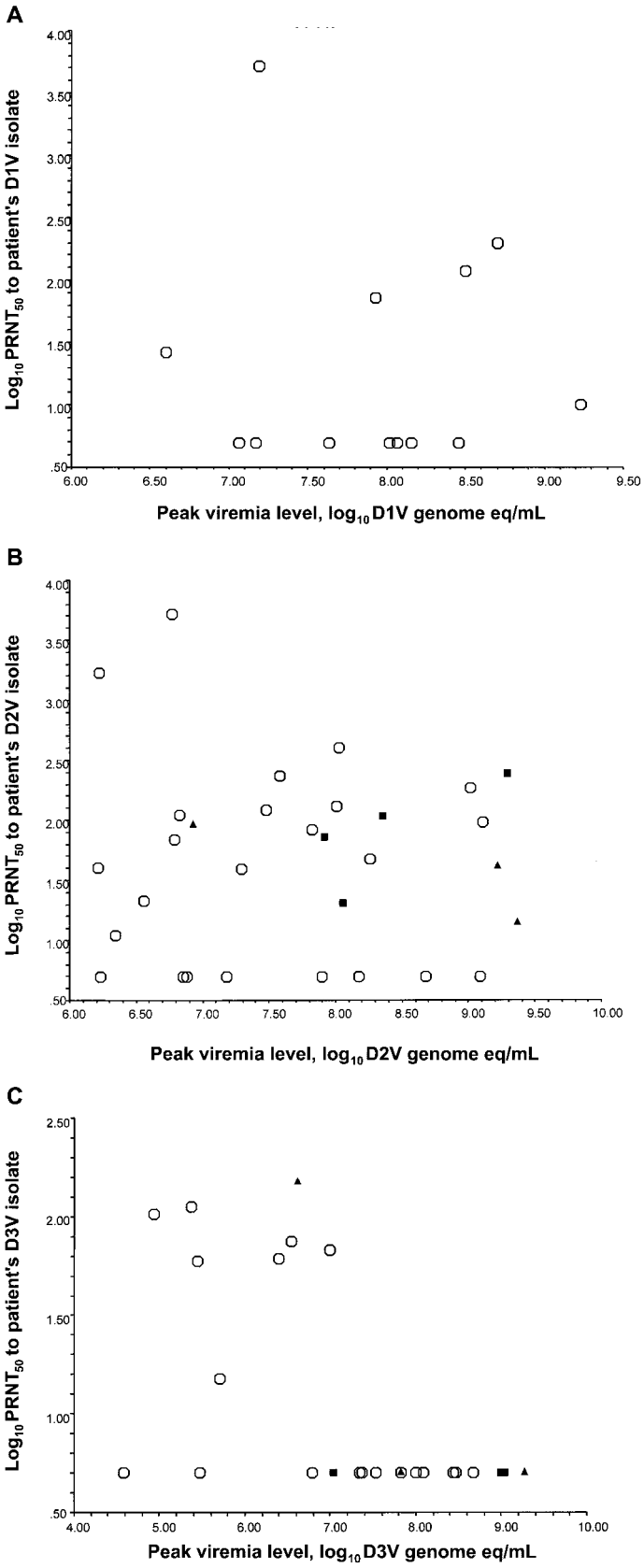

Figure 3. Relationship of preexisting neutralizing antibody titers against the patient's virus isolate and subsequent peak viremia levels in secondary dengue-1 virus (D1V) *(A),* dengue-2 virus (D2V) *(B),* and dengue-3 virus (D3V) *(C)* infections. ○, Dengue fever–mild, not hospitalized (nhDF); ▲, dengue fever–hospitalized (hDF); ■, dengue hemorrhagic fever (hospitalized) (DHF) cases. eq, genome equivalents; $PRNT_{50}$, 50% plaque reduction neutralization reciprocal titer.

of 10 instead of 20. Our $PRNT_{50}$ threshold that was considered to be positive ($\geq$10) was taken from the original article describing the neutralization assay [22] and from another article [23] and was used to identify preexisting Ab reactive to the infecting serotype with high sensitivity. When we used criteria with higher specificity (i.e., undetectable [$PRNT_{50}$ <80 to all 4 DV serotypes], monotypic [$PRNT_{50}$ $\geq$80 to only 1 DV serotype], and multitypic [$PRNT_{50}$ $\geq$80 to >1 DV serotype]), a preexisting monotypic $PRNT_{50}$ pattern remained associated with the development of DHF in all serotypes combined and in D3V alone (data not shown). By using the lower $PRNT_{50}$ threshold, as defined in Subjects and Methods, we identified 1 case in which D2V reinfection was suspected, implying that low-level homotypic immunity does not necessarily produce sterile immunity [2]. However, we cannot entirely exclude the possibility that the low level of preexisting monotypic D2V neutralizing Abs in this case were cross-reactive from earlier occult non-D2V, JEV, or other flavivirus infections.

By use of either reference virus strains or clinical virus isolates, we detected neutralizing Abs against the infecting DV serotype in a significant proportion of the preillness plasma samples obtained from patients with secondary D1V, D2V, and D3V infection. One earlier prospective study, which looked at presumed secondary D2V infections, made similar observations by use of only reference virus strains [13]. The absence of detectable preillness neutralizing Abs directed against the infecting DV serotype in the other prospective studies [4, 14–16] likely reflects the different detection thresholds for the neutralizing Ab assays. For many different viruses, animal models, and challenge routes, passive Ab transfer experiments have consistently shown a good correlation between in vivo protection and Ab neutralizing activity measured in vitro [31, 32]. This correlation does not necessarily imply that the mechanism of neutralization in vitro is the only, or even predominant, mechanism of protective activity in vivo. In secondary D3V infection, we found that the preexisting neutralizing Abs against D3V were associated with protective activity in vivo. Our results suggest that, after a primary D1V, D2V, or possibly D4V infection, cross-reactive Abs are directed against neutralizing epitopes on the D3V E or preM structural proteins [33, 34] but do not necessarily produce sterile immunity. At D3V $PRNT_{50}$ levels of 100–1000, neutralizing Abs are of sufficient titer and affinity to diminish replication of D3V and, consequently, severity of disease. At low or undetectable D3V $PRNT_{50}$ levels, ADE may be occurring and cannot be excluded. Alternatively, the conditions for developing DHF in a secondary D3V infection may be optimal when the preexisting neutralizing capacity for D3V is low or absent, and partial agonist, cross-reactive, anamnestic cell-mediated immune responses to D3V dominate [10, 35, 36].

The role of preexisting Abs directed against the infecting DV serotype appears to be different in secondary D2V infection than in secondary D3V infection. We found that the levels of preexisting neutralizing Ab against D2V were not associated with severity of disease or with subsequent D2V viremia levels. These data suggest that, after primary D1V, D3V, or D4V infections, cross-reactive Abs produced against the E or preM structural proteins are typically not of sufficient titer or affinity to dampen D2V replication. A less likely possibility is that the cross-reactive Abs are directed against different epitopes on D2Vs that are nonneutralizing in vivo. In either scenario, preexisting, cross-reactive, neutralizing Abs to D2V in symptomatic secondary D2V infection were not associated with protective activity in vivo. Kliks et al. [13] also found no difference in the preexisting D2V $PRNT_{50}$ in LLC-MK2 cells between symptomatic subjects (8/9 were classified as having DHF) and asymptomatic subjects in a prospective study that examined presumed secondary D2V infections [4]. By use of an assay combining human monocytes, low dilutions of preillness patient serum samples, and D2V, they described a significant positive association between D2V "enhancement outcome" in the culture supernatants and DHF/symptomatic disease. They also reported a significant negative association between D2V "neutralization outcome" and DHF/symptomatic disease [13]. In our experience, interassay variability has been an obstacle in performing similar studies with human monocytes to address the potential role of ADE in this cohort. The absence of a correlation between preexisting D1V $PRNT_{50}$ and viremia levels suggests that the nature of preexisting Abs reactive to the infecting DV serotype in secondary D1V infection is similar to what was observed in D2V infection, rather than in D3V infection. In the absence of neutralizing Ab capacity, cellular immune responses to DV may play a greater role in shaping severity of disease in symptomatic secondary D2V and D1V infection.

The results of the present study have important implications for the development of live attenuated tetravalent dengue vaccines. A recent abstract presented the $PRNT_{50}$ to D1V–D4V in 71 healthy Thai children (6–13 years old), 8–12 months after they had received a 2-dose primary immunization series with 2 tetravalent dengue vaccine preparations [37]. The neutralizing Ab assays were performed with LLC-MK2 cells by use of the same reference DV strains as ours. The $PRNT_{50}$ GMT to D3V was >100 with both vaccine preparations, a level associated with lower viremia and milder D3V disease in the present study. Nevertheless, a significant number of the subjects had $PRNT_{50}$ to D3V $\leq$200, a level at which, in the present study, hDF and DHF developed with D3V infection. The $PRNT_{50}$ GMTs to D2V were 27–41 and nearly the same as the preexisting $PRNT_{50}$ GMTs to D2V seen in our patients with symptomatic secondary D2V infection (including hDF and DHF). These levels of neutralizing Ab to D2V did not appear to ameliorate viremia levels

or severity of disease. The $PRNT_{50}$ GMT to D2V increased early after a subsequent booster vaccination, and higher neutralizing Ab levels may provide a protective effect in vivo after D2V infection.

The present study has highlighted several important aspects of disease pathogenesis in dengue disease. The virus burden achieved after infection appears to be a key determinant of severity of disease, at least in D2V and D3V infections. However, it is not the sole determinant in an apparently multifactorial pathogenic process. Cases of DF in which viremia levels were high were observed. The role of preexisting neutralizing Abs reactive to the infecting DV serotype in modifying secondary DV infections varies depending on the infection sequence. In secondary D3V infections, preexisting D3V-reactive neutralizing Abs appear to ameliorate viral replication and severity of disease. By contrast, in secondary D2V infections, preexisting D2V-reactive neutralizing Abs do not ameliorate viral replication or severity of disease. Additional data are needed for secondary D1V and D4V infections. Whether ameliorating or augmenting virus burden and severity of disease, humoral immune responses represent only 1 arm of the immune-mediated processes that shape the clinical manifestations of dengue.

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References

1. Henchal EA, Putnak JR. The dengue viruses. Clin Microbiol Rev **1990**; 3:376–96.
2. Sabin A. Research on dengue during World War II. Am J Trop Med Hyg **1952**; 1:30–50.
3. Halstead SB. Pathogenesis of dengue: challenges to molecular biology. Science **1988**; 239:476–81.
4. Burke DS, Nisalak A, Johnson D, Scott RM. A prospective study of dengue infections in Bangkok. Am J Trop Med Hyg **1988**; 38:172–80.
5. Guzman MG, Kouri GP, Bravo J, Soler M, Vazquez S, Morier L. Dengue hemorrhagic fever in Cuba. Am J Trop Med Hyg **1990**; 42:179–84.
6. 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.
7. Kliks SC, Nimmannitya S, Nisalak A, Burke DS. Evidence that maternal dengue antibodies are important in the development of dengue hemorrhagic fever in infants. Am J Trop Med Hyg **1988**; 38:411–9.
8. Halstead SB, Porterfield JS, O'Rourke EJ. Enhancement of dengue virus infection in monocytes by flavivirus antisera. Am J Trop Med Hyg **1980**; 29:638–42.
9. Halstead SB. Antibody, macrophages, dengue virus infection, shock, and hemorrhage: a pathogenetic cascade. Rev Infect Dis **1989**; 11:S830–9.
10. Rothman AL, Ennis FA. Immunopathogenesis of dengue hemorrhagic fever. Virology **1999**; 257:1–6.
11. Sutter RW, Pallansch MA, Sawyer LA, Cochi SL, Hadler SC. Defining surrogate serologic tests with respect to predicting protective vaccine efficacy: poliovirus vaccination. Ann NY Acad Sci **1995**; 754:289–99.
12. Chen RT, Markowitz LE, Albrecht P, et al. Measles antibody: reevaluation of protective titers. J Infect Dis **1990**; 162:1036–42.
13. 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.
14. Sangkawibha N, Rojanasuphot S, Ahandrik S, et al. Risk factors in dengue shock syndrome: a prospective epidemiological study in Rayong, Thailand. I. The 1980 outbreak. Am J Epidemiol **1984**; 120:653–69.
15. Thein S, Aung MM, Shwe TN, et al. Risk factors in dengue shock syndrome. Am J Trop Med Hyg **1997**; 56:566–72.
16. Graham RR, Juffrie M, Tan R, et al. A prospective seroepidemiologic study on dengue in children four to nine years of age in Yogyakarta, Indonesia I. Studies in 1995–1996. Am J Trop Med Hyg **1999**; 61:412–9.
17. Endy TP, Chunsuttiwat S, Nisalak A, et al. Epidemiology of inapparent and symptomatic acute dengue virus infection: a prospective study of primary school children in Kamphaeng Phet, Thailand. Am J Epidemiol **2002**; 156:40–51.
18. Innis BL. Antibody responses to dengue virus infection. In: Gubler DJ, Kuno G, eds. Dengue and dengue hemorrhagic fever. New York: CAB International, **1997**:221–43.
19. World Health Organization. Dengue haemorrhagic fever: diagnosis, treatment, prevention and control. 2nd ed. Geneva: WHO, **1997**. Available at: http://www.who.int/emc/diseases/ebola/Denguepublication.
20. 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.
21. 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.
22. Russell PK, Nisalak A, Sukhavachana P, Vivona S. A plaque reduction test for dengue virus neutralization antibodies. J Immunol **1967**; 99: 285–90.
23. Sabchareon A, Lang J, Chanthavanich P, et al. Safety and immunogenicity of tetravalent live-attenuated dengue vaccines in Thai adult volunteers: role of serotype concentration, ratio, and multiple doses. Am J Trop Med Hyg **2002**; 66:264–72.
24. Houng HSH, Chen RCM, Vaughn DW, Kanesathasan N. Development of a fluorogenic RT-PCR system for quantitative identification of dengue virus serotypes 1, 2, 3 and 4, using conserved and serotype-specific 3′ non-coding sequences. J Virol Methods **2001**; 95:19–32.
25. Hoke CH, Nisalak A, Sangawhipa N, et al. Protection against Japanese encephalitis by inactivated vaccines. N Engl J Med **1988**; 319:608–14.
26. Vaughn DW, Green S, Kalayanarooj S, et al. Dengue viremia titer, antibody response pattern, and virus serotype correlate with disease severity. J Infect Dis **2000**; 181:2–9.
27. Libraty DH, Endy TP, Houng HS, et al. Differing influences of virus burden and immune activation on disease severity in secondary dengue-3 virus infections. J Infect Dis **2002**; 185:1213–21.
28. Libraty DH, Young PR, Pickering D, et al. High circulating levels of the dengue virus nonstructural protein NS1 early in dengue illness correlate with the development of dengue hemorrhagic fever. J Infect Dis **2002**; 186:1165–8.
29. Ecology of arboviruses in Thailand. Annual progress report SEATO. Bangkok, Thailand: US Army–SEATO Medical Research Unit, **1968**: 43–71.
30. Tick-borne viruses in Thailand. Annual progress report SEATO. Bang-

kok, Thailand: US Army–SEATO Medical Research Unit, **1975**:57–68.
31. Burton DR. Antibodies, viruses, and vaccines. Nat Rev Immunol **2002**; 2:706–13.
32. Parren PWHI, Burton DR. The anti-viral activity of antibodies *in vitro* and *in vivo*. Adv Immunol **2001**; 77:195–262.
33. Vazquez S, Guzman MG, Guillen G, et al. Immune responses to synthetic peptides of dengue prM protein. Vaccine **2002**; 20:1823–30.
34. Thullier P, Demangel C, Bedouelle H, et al. Mapping of a dengue virus neutralizing epitope critical for the infectivity of all serotypes: insight into the neutralization mechanism. J Gen Virol **2001**; 82:1885–92.
35. Mangada MM, Endy TP, Nisalak A, et al. Dengue-specific T cell responses in peripheral blood mononuclear cells obtained prior to secondary dengue virus infections in Thai schoolchildren. J Infect Dis **2002**; 185:1697–703.
36. Gagnon SJ, Ennis FA, Rothman AL. Bystander target cell lysis and cytokine production by dengue virus–specific human $CD4^+$ cytotoxic T-lymphocyte clones. J Virol **1999**; 73:3623–9.
37. Sabchareon A, Lang J, Yoksan S, et al. Immunogenicity of a booster dose of two tetravalent live attenuated dengue vaccines in Thai children. Am J Trop Med Hyg **2002**; 67(Suppl 2):167–8.