MAJOR ARTICLE

Dengue Virus (DV) Enhancing Antibody Activity in Preillness Plasma Does Not Predict Subsequent Disease Severity or Viremia in Secondary DV Infection

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Background. Dengue hemorrhagic fever, the most severe form of dengue illness, is associated with secondary dengue virus (DV) infection. Preexisting nonneutralizing antibodies to DV that enhance the infection of Fcγ receptor–bearing cells have been implicated in DV pathogenesis.

Methods. We conducted a prospective cohort study in Thai schoolchildren. Enhancing activity (EA) was measured as the percentage of DV-infected K562 cells, and viral titer (infected K562 cell supernatants) was measured in preillness plasma samples from children who subsequently had secondary DV2 or DV3 infection.

Results. Plaque-reduction neutralizing titers to the child's own DV2 or DV3 isolate were detected in 23 of 32 and 8 of 27 of the preillness plasma samples, and EA was detected to a low-passage Thai DV2 or DV3 in 31 of 32 and 26 of 27, respectively, of the samples. EA in undiluted preillness plasma did not correlate with subsequent disease severity or peak viremia levels in either secondary DV2 or DV3 infections.

Conclusions. Preillness plasma enhances DV infection of K562 cells even in the presence of detectable neutralizing antibodies in LLC-MK2 cells. However, levels of preillness plasma EA of DV infection in K562 cells did not correlate with the clinical severity or viral burden of secondary DV infection.

Dengue viruses (DVs) are mosquitoborne RNA viruses of the family *Flaviviridae*. There are 4 closely related serotypes (DV1–4) [1]. It is estimated that at least 80 million DV infections occur worldwide annually [2]. The majority of DV infections are asymptomatic. In symptomatic DV infection, clinical syndromes range from an undifferentiated febrile illness to dengue fever (DF) to dengue hemorrhagic fever (DHF), a plasma leakage syndrome that, in its most severe form—dengue shock syndrome (DSS)—can be life threatening [3]. The immunopathogenesis of DHF/DSS is not well understood. Infection with 1 serotype confers lifelong homotypic immunity, but there is only short-term cross-protection against heterotypic serotypes [4]. Epidemiological evidence has demonstrated that DHF is associated more commonly with secondary DV infection [5–8]. Preexisting nonneutralizing antibodies from prior heterologous DV infection have been proposed to play a major role in disease severity by facilitating virus entry into Fcγ receptor (FcR)–bearing cells, leading to an increase in viral burden and disease severity. This phenomenon is known as "antibody-dependent enhancement" (ADE). The results of an in vivo study showed that monkeys infected with DV2 had higher viremia levels after the

Received 3 December 2004; accepted 26 February 2005; electronically published 5 July 2005.

Presented in part: 52nd Annual Meeting of the American Society of Tropical Medicine and Hygiene, Philadelphia, PA, 3–7 December 2003 (abstract 2057); 7th International Symposium on Positive Strand RNA Viruses, San Francisco, CA, 27 May–1 June 2004 (abstract 286).

Financial support: National Institutes of Health (grant P01-AI-34533); US Army Medical Research and Materiel Command.

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The Journal of Infectious Diseases 2005;192:510–9

0022-1899/2005/19203-0021$15.00

passive transfer of DV2 antibodies than did control monkeys [9]. Infants born to DV antibody–positive mothers can present with DHF/DSS in primary DV infection after maternal levels of IgG to DV decrease to subneutralizing levels [10]. Kliks et al. [11] reported that preillness serum from children with symptomatic secondary DV2 infection had higher levels of enhancing activity (EA) than those from asymptomatic DV-infected children.

We previously demonstrated that peak DV2 and DV3 viremia levels correlated with disease severity [6, 12, 13]. We also found that preillness cross-reactive neutralizing antibodies appear to be associated with less-severe DV disease in symptomatic secondary DV3 infection but not in secondary DV2 or DV1 infection [13]. In the present study, we examined the relationship, in preillness plasma prospectively obtained from children with subsequent secondary DV2 or DV3 infection, between EA and subsequent viremia levels and disease severity.

MATERIALS AND METHODS

Study design and sample collection. The design of the present prospective study has been described elsewhere [13, 14]. More than 2000 primary-school students in Kamphaeng Phet, Thailand were enrolled as part of an ongoing study of dengue pathogenesis. Plasma samples were collected each January. Children were under daily active surveillance for school absences during the peak season of dengue transmission, 1 June–15 November of each year. Children with fever or a history of fever within 7 days of the first day of school absence were evaluated, and serum samples were collected during the acute and convalescent phases of illness. We defined 3 categories of dengue disease severity: (1) not hospitalized, with DF, (2) hospitalized with DF, and (3) hospitalized with DHF. DHF was defined as a febrile illness with laboratory confirmation of acute DV infection and evidence of plasma leakage according to World Health Organization criteria [15]. Signed consent was obtained from the parents or guardians of all children. 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.

Study population. During the first 3 years of the study, 148 children received a diagnosis of acute symptomatic secondary DV infection. The infecting serotype was identified in 107 of them (DV1, $n = 24$; DV2, $n = 37$; DV3, $n = 45$; DV4, $n = 1$) [13, 14]. For the present study, we selected children with symptomatic secondary DV2 and DV3 infection whose acute plasma samples were collected within 3 days of the onset of fever, which approximates the time of peak viremia levels [6, 13]. Among all cases of secondary DV2 and DV3, 32 of 37 and 27 of 45 children fitted these criteria. Clinical and laboratory findings for the study population are shown in table 1. For suspected cases of asymptomatic secondary DV3, we randomly selected 10 asymptomatic children who had serologic evidence of secondary DV infection from a school that had an isolated DV3 outbreak [16].

Virologic and serologic studies. Viremia levels in acute plasma samples were assessed by quantitative fluorogenic reverse-transcription polymerase chain reaction (RT-PCR) [17]. The infecting DV serotype was identified from acute-phase serum samples by a serotype-specific RT-PCR [18] or by virus isolation in *Toxorhynchites splendens* mosquitoes [19]. Levels of neutralizing antibody (NAb) were measured by use of the 50% plaque-reduction neutralization test ($PRNT_{50}$) in LLC-MK2 cells against prototype strains of all 4 DVs (DV1 16007, DV2 16681, DV3 16562, and DV4 1036), Japanese encephalitis virus (JEV; Nakayama strain), and the patient's own DV isolate [20]. Symptomatic secondary DV infection was defined as a dengue IgM:IgG EIA ratio ≤ 1.8 [21]. The DV-specific IgG1 subclass assay was determined by EIA [22]. Asymptomatic secondary DV infection was defined as a $\geq$4-fold increase in the hemagglutination inhibition titer [21, 23]. We defined 3 patterns of preillness plasma DV $PRNT_{50}$ results: undetectable ($PRNT_{50}$ <10 to all 4 DVs), monotypic ($PRNT_{50}$ >10 to only 1 DV serotype or >10 to more than 1 DV serotype but with a $PRNT_{50} \geq 80$ to only 1 DV serotype), and multitypic $PRNT_{50}$ pattern ($PRNT_{50}$ >10 to more than 1 DV serotype with a $PRNT_{50} \geq 80$ to more than 1 DV serotype) [13]. The presence or absence of $PRNT_{50}$ to JEV was determined but was not used for this classification.

Virus stocks. DV2 strain C0167/96 and DV3 strain C0360/94 are low-passage viruses that were isolated in 1996 and 1994, respectively, from the plasma of 2 children with DHF who were enrolled in an ongoing prospective study of DHF in Bangkok at the Queen Sirikit Institute of Child Health [6].

Fluorescent focus assay. Serial 10-fold dilutions of DV-infected K562 cell supernatants were added to duplicate chambers of CV-1 cells on chamber slides (Nalge Nunc). Mouse anti–DV2 antibody (3H5) [24] or mouse anti–DV3 antibody (American Type Culture Collection) was added 24 h later and then incubated at 37°C for 30 min. After washing, fluorescein isothiocyanate (FITC)–conjugated sheep anti–mouse IgG (ICN/Cappel) was added and incubated for 30 min at 37°C. Fluorescent foci of CV-1 cells were counted, and titers were expressed as fluorescent focus units (ffu) per milliliter. The limit of detection was 100 ffu/mL.

Plaque assay. Serial 10-fold dilutions of DV-infected K562 cell supernatants were adsorbed for 2 h onto duplicate wells of Vero cells and then cultured for 7 days. Cells were fixed and stained with crystal violet. Plaques were counted, and titers were expressed as plaque-forming units per milliliter. The limit of detection was 100 pfu/mL [20].

Antibody-dependent infection with DV. Heat-inactivated (for 30 min at 56°C) plasma obtained 6–9 months before sec-

Table 1. Characteristics of the study population: disease severity, preinfection plaque-reduction neutralizing titer ($PRNT_{50}$), and peak viremia level.

Type of infection, group, case no.	Preinfection $PRNT_{50}$[a]							Peak viremia level, log_{10}[b]
	DV1[c]	DV2[c]	DV2[d]	DV3[c]	DV3[d]	DV4[c]	JEV[c]	
Symptomatic secondary DV2								
nhDF								
1	310	153	ND	25		<10	<10	8.15
2	2560	127	186	2560		51	<10	9.01
3[e]	<10	<10	<10	<10		<10	718	8.68
4	<10	<10	<10	<10		17	<10	8.18
5	290	<10	<10	<10		<10	25	6.88
6[e]	<10	<10	<10	<10		<10	47	6.23
7	352	475	404	2532		11	<10	8.03
8	181	10	<10	236		<10	<10	6.86
9	<10	<10	<10	162		<10	233	9.09
10	136	13	47	65		<10	31	8.26
11[f]	<10	<10	<10	<10		<10	16	7.90
12	152	<10	<10	2560		<10	80	7.18
13	365	424	40	899		<10	42	6.21
14	660	43	21	87		<10	<10	6.56
15	434	280	1676	2560		<10	1600	6.22
16	1317	107	110	364		<10	<10	6.83
17	544	96	82	185		<10	541	7.82
18	2470	62	39	93		61	<10	7.29
19	<10	13	11	<10		<10	<10	6.34
20	76	34	129	44		<10	<10	8.01
21	<10	69	234	335		<10	333	7.58
22	<10	<10	121	<10		<10	12	7.48
23	217	83	95	409		<10	49	9.11
24	2560	1316	5120	1954		19	37	6.77
25	<10	<10	68	1518		<10	<10	6.79
hDF								
26	<10	<10	14	2560		<10	113	9.37
27	22	ND	41	837		<10	44	9.22
28	709	63	91	23		12	64	6.93
hDHF								
29	276	57	242	40		<10	30	9.29
30	349	30	20	2560		<10	40	8.06
31	ND	36	72	ND		ND	ND	7.92
32	106	59	107	361		<10	<10	8.35
Symptomatic and asymptomatic secondary DV3								
nhDF								
1	253	<10		<10	<10	<10	<10	6.79
2[e]	<10	<10		<10	<10	<10	51	8.67
3	<10	294		25	<10	<10	<10	8.09
4	745	21		57	<10	56	<10	5.47
5[e]	<10	<10		<10	<10	<10	<10	8.00
6	9914	536		439	61	<10	34	6.39
7[e]	<10	<10		<10	<10	<10	98	4.59
8	7443	635		271	102	<10	<10	4.94
9	<10	116		<10	<10	<10	<10	8.44
10	211	898		449	67	<10	79	7.00
11	884	27		<10	15	<10	43	5.71
12	60	1893		932	75	<10	24	6.54
13	1108	331		<10	112	<10	25	5.37
14[e]	<10	<10		<10	<10	<10	35	7.35
15	<10	377		<10	<10	<10	21	7.54
16[e]	<10	<10		<10	<10	<10	30	7.83
17	<10	161		<10	<10	<10	26	8.46
18	152	620		139	<10	21	96	7.37
19	282	717		298	59	<10	608	5.45

(continued)

Table 1. ***(Continued.)***

Type of infection, group, case no.	Preinfection $PRNT_{50}$[a]							Peak viremia level, log_{10}[b]
	DV1[c]	DV2[c]	DV2[d]	DV3[c]	DV3[d]	DV4[c]	JEV[c]	
hDF								
20	<10	<10		<10	ND	<10	<10	8.29
21	1125	62		<10	<10	<10	<10	7.82
22	3999	<10		<10	<10	<10	<10	7.83
23	269	173		875	150	<10	187	6.61
24[f]	<10	<10		<10	<10	<10	<10	9.27
hDHF								
25	324	<10		<10	<10	<10	<10	9.00
26	3701	<10		24	<10	<10	<10	7.03
27	<10	95		<10	<10	<10	<10	9.04
Asymptomatic								
28	10,240	211		465	ND	15	<10	ND
29	2031	<10		90	ND	<10	<10	ND
30	5502	<10		48	ND	<10	<10	ND
31[f]	<10	<10		<10	ND	<10	<10	ND
32	1703	504		361	ND	82	<10	ND
33	2628	91		136	ND	<10	<10	ND
34	1524	124		70	ND	<10	<10	ND
35[f]	<10	<10		70	ND	<10	<10	ND
36	746	121		163	ND	36	73	ND
37	334	405		79	ND	23	<10	ND

NOTE. DV, dengue virus; hDF, hospitalized with dengue fever (moderate illness); hDHF, hospitalized with dengue hemorrhagic fever (severe illness); JEV, Japanese encephalitis virus; ND, not determined; nhDF, not hospitalized with dengue fever (mild illness).

[a] Values <10 were undetectable.

[b] Peak viremia levels were determined in patients with symptomatic DV within 3 days of fever by use of serotype-specific fluorogenic reverse-transcription polymerase chain reaction [13]. Viremia levels are expressed as DV genome–equivalent cDNA copies per milliliter.

[c] Reference virus strains DV1 16007, DV2 16681, DV3 16562, DV4 1036, and JEV Nakayama.

[d] DV2 and DV3 from child's own serum.

[e] Enhancing activity detected in the absence of detectable anti–DV neutralizing antibodies.

[f] Enhancing activity not detected.

ondary DV2 and DV3 infection and 4–7 months after secondary DV2 and DV3 infection (undiluted and diluted 1:5) were mixed with DV (100 μL of plasma:50 μL of virus) and incubated on ice for 30 min. Virus-antibody complexes were added to 250 μL of K562 cells in RPMI 1640 that contained 2% fetal calf serum (FCS) at an MOI of 0.5 for DV2 and of 0.3 for DV3 and then were incubated for 2 h at 37°C. Cells were washed to remove residual virus and then cultured at 3×10^5 cells/mL in RPMI 1640 that contained 10% FCS, for 3 days at 37°C, in 24-well plates [25]. Cells were harvested for flow-cytometry studies to determine the percentage of DV-infected cells, and cell supernatants were collected for viral titration. To minimize the effect of interassay variability [9], we performed ADE on all patient samples concurrently. Viral titrations of all infected cell supernatants were also performed simultaneously. DV3 immune plasma was used as a positive control, and flavivirus-naive plasma ($PRNT_{50}$ <1:20 to DV1–DV4, JEV, and West Nile virus) was used as a negative control. EA was defined as an increase in either the percentage of DV-infected K562 cells or viral titers in supernatant that was greater than the mean $\pm$ 3 SD of negative control samples.

Flow-cytometric analysis. K562 cells were fixed in PBS with 4% paraformaldehyde, washed twice in PBS, and permeabilized in Hanks' balanced salt solution that contained 10 mmol/L HEPES (pH 7.3), 0.1% saponin, and 0.02% sodium azide. An FITC-labeled anti–DV complex mouse IgG (Fitzgerald) was added at 1:20 dilution and incubated for 1 h at 4°C. Cells were washed and fixed for analysis on a FACScan flow cytometer. Data were analyzed by use of FlowJo (version 6.1; Tree Star).

Statistical analysis. Statistical analyses were performed by use of SPSS software for Windows (version 11.0; SPSS). Spearman rank correlation estimates measured the association of continuous variables with skewed distribution. Analysis of variance (ANOVA) was used to test the mean EA among disease-severity groups and the $PRNT_{50}$ pattern. Student's t test was used to compare the EA between 2 groups. The F test was used to test equal variance. The Sidak test was used for comparison of preillness plasma EA among the neutralizing-antibody pattern groups. McNemar's test was used to test the number of detectable cases of EA between pre- and postillness plasma samples. In all analyses, $P < .05$ was considered to be significant, $.05 < P < .10$ was considered to be a nonsignificant trend, and $P > .1$ was considered to be not significant.

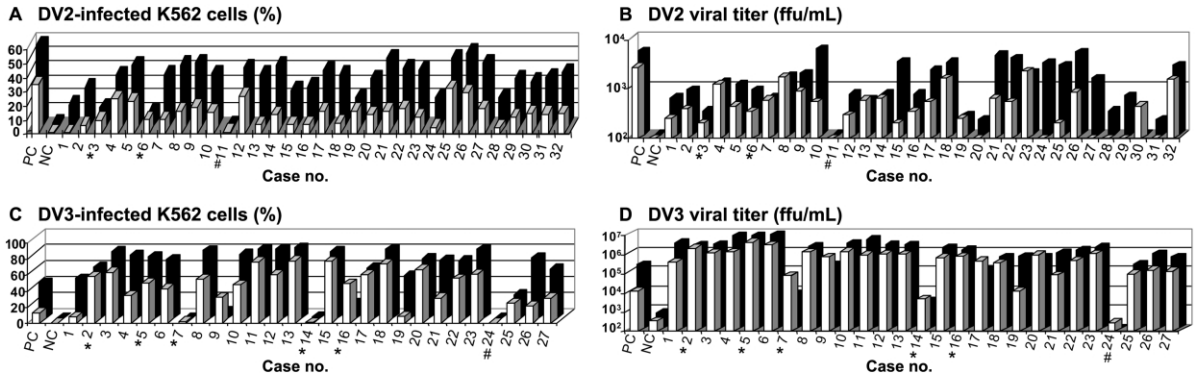

Figure 1. Enhancing activity (EA) of preillness plasma from children with subsequent symptomatic secondary Dengue virus (DV) 2 infection. Enhancing activity was determined by the percentage of infected K562 cells and by $\log_{10}$ viral titer in infected K562 cell supernatants (in fluorescent focus units [ffu] per milliliter) in DV2 *(A* and *B)* and DV3 *(C* and *D)* infection. *White bar,* Undiluted plasma; *black bar,* plasma diluted 1:5; *EA detected in the absence of detectable anti–DV neutralizing antibodies; #EA not detected. NC, negative control (flavivirus-naive plasma); PC, positive control (DV3-immune plasma).

RESULTS

EA of preillness plasma from children with subsequent symptomatic secondary DV infection. Preillness plasma samples from children with secondary DV2 infection demonstrated enhancement of DV2 infection of K562 cells in undiluted plasma and in plasma diluted 1:5 in 30 (94%) of 32 and 31 (97%) of 32 samples, respectively (figure 1*A* and 1*B*). A similar pattern of EA was seen in preillness plasma samples from children with secondary DV3 infection; 26 (96%) of 27 samples demonstrated EA in both undiluted plasma and plasma diluted 1:5 (figure 1*C* and 1*D*). Although EA in samples with undiluted plasma was lower than that in plasma diluted 1:5 ($P < .05$, Student's *t* test), EA in diluted and undiluted plasma correlated with each other (Spearman's $r = 0.36$ and $r = 0.79$, for the percentage of infected cell comparisons in DV2 and DV3, respectively; $r = 0.47$ and $r = 0.82$, for log-log viral titer comparisons in DV2 and DV3, respectively; all $P < .05$) (data not shown).

Preillness plasma EA and subsequent plasma viremia levels. We have previously shown that, in secondary DV2 and DV3 infection, severe disease (DHF) was significantly associated with higher viremia levels in plasma [13]. These results suggested that ADE may play a role in increasing viremia levels by enhancing DV infection of FcR-bearing cells.

In secondary DV2 infection, we found no correlation between viremia levels and EA in preillness plasma samples that were undiluted or diluted 1:5 ($P > .1$, Spearman's rank correlation) (figure 2*A*–2*D*). In secondary DV3 infection, we found no correlation between viremia levels and EA in undiluted plasma ($P > .1$, Spearman's rank correlation) (figure 2*E* and 2*F*). However, in plasma diluted 1:5, EA showed a significant negative correlation with plasma viremia levels ($r = -0.41$; $P < .05$, Spearman's rank correlation) (figure 2*G* and 2*H*).

Preillness plasma EA and subsequent dengue disease severity. We examined whether EA in plasma obtained before documented symptomatic secondary DV2 and DV3 infection or asymptomatic secondary DV seroconversion (presumed DV3 infection) correlated with subsequent disease severity. Preillness plasma EA, as determined by both the percentage of infected K562 cells and by supernatant viral titers, did not correlate with disease severity in documented secondary DV2 or DV3 infection ($P > .1$, ANOVA) in either undiluted plasma (figure 3) or plasma diluted 1:5 (data not shown).

Because it had previously been found that EA was lower in preillness plasma samples from asymptomatic than symptomatic presumed secondary DV2 infection [11], we compared EA between preillness plasma samples from children with asymptomatic presumed DV3 infection and hospitalized children with symptomatic secondary DV3 infection. Surprisingly, the percentage of infected cells in undiluted, but not diluted, preillness plasma was significantly higher in plasma from children with asymptomatic secondary DV3 infection ($P < .05$, Student's *t* test) (figure 4). However, viral titers in infected K562 cell supernatants did not differ between symptomatic and asymptomatic samples in either undiluted or diluted plasma ($P > .1$, Student's *t*-test) (figure 4).

Preillness plasma EA and NAb levels. Previous studies have suggested that antibodies in dengue immune serum compete for either a neutralizing or an enhancing effect on DV infection in FcR-bearing cells [11]. We examined whether levels of preexisting anti–DV NAb (measured in LLC-MK2 cells, which do not have FcR) correlated with EA (measured in FcR-bearing K562 cells). Preexisting NAb against a patient's DV isolate was detected in both secondary DV2 (23/32 samples [72%]) and DV3 (8/27 samples [30%]) plasma samples (table

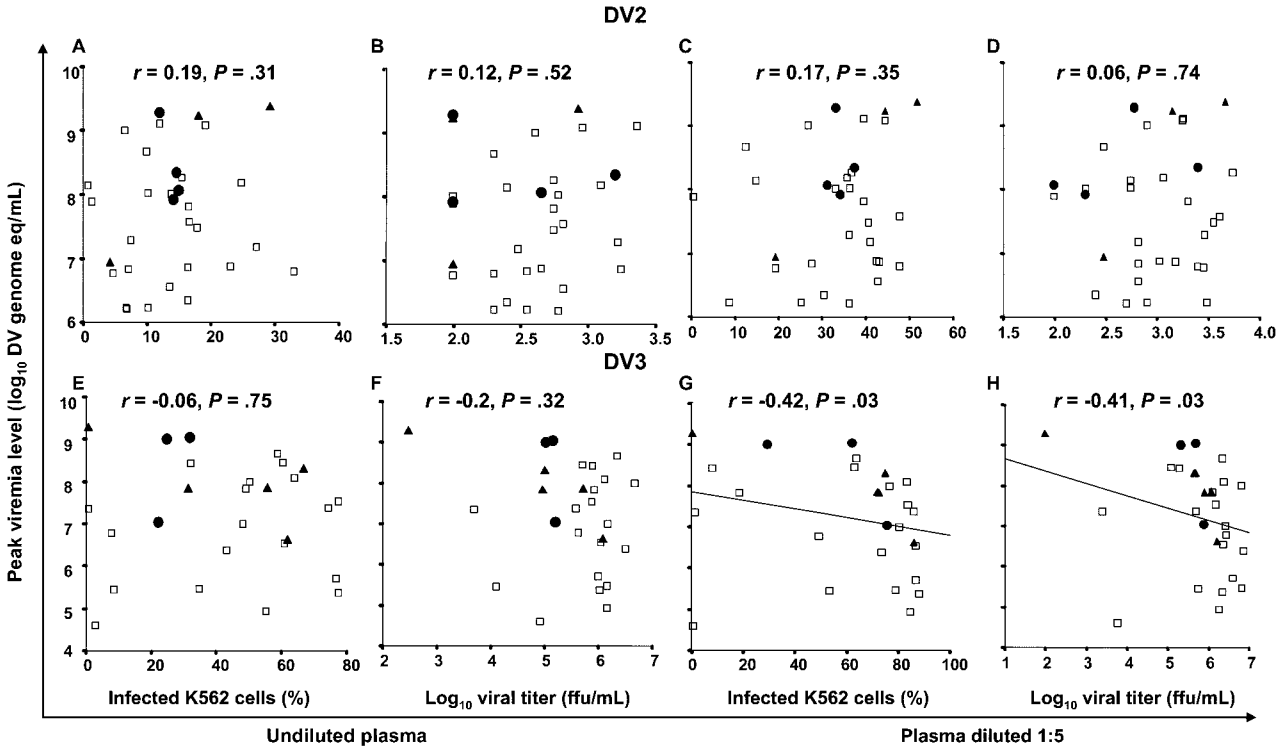

Figure 2. Correlation between preillness plasma enhancing activity (EA) and subsequent peak plasma viremia levels. EA was determined by the percentage of infected K562 cells and $\log_{10}$ viral titer by use of undiluted plasma *(left panels)* and plasma diluted 1:5 *(right panels.)* Plasma samples from children with subsequent secondary Dengue virus (DV) 2 infection are shown in top panels *(A–D),* and those with subsequent secondary DV3 infection are shown in bottom panels *(E–H).* Spearman's rank correlation was used to analyze data; significance was set at $P < .05$. *White squares,* not hospitalized with Dengue fever (DF; mild illness); *black triangles,* hospitalized with DF (moderate illness); *black circles,* hospitalized with Dengue hemorrhagic fever (severe illness). ffu, fluorescent focus units.

1). EA was detected in the majority of plasma samples (31/32 in DV2 [97%] and 26/27 in DV3 [96%]) (figure 1). In 2 children with undetectable EA, preexisting NAb to all DV serotypes were undetectable and the NAb titer to JEV was low (figure 1 and table 1). In children with detectable EA in the absence of DV NAb, all but 1 (DV3, subject 5) had detectable NAb to JEV (figure 1*C* and 1*D* and table 1).

An earlier study involving this cohort demonstrated that higher levels of preexisting NAb to DV3 but not to DV2 was associated with lower viremia levels and milder disease severity [13]. This suggested that in vivo preexisting NAb to DV3 were more effective in inhibiting viral replication in secondary DV3 infection. We would therefore have expected that preexisting NAb to DV2 would not inhibit DV2 infection of K562 cells. However, preexisting NAb to the child's own DV2 isolate negatively correlated with the percentage of infected cells in undiluted plasma samples but not in those diluted 1:5 (figure 5).

In contrast, we expected that preexisting NAb to DV3 would inhibit the infection of K562 cells. However, preexisting NAb to the children's own DV3 isolate positively correlated with EA of plasma diluted 1:5 (figure 5). If only cases with detectable $PRNT_{50}$ to the children's own DV3 isolates (8/26 [31%]) were analyzed, the correlation was no longer detectable.

We also determined the relationship among NAb levels, EA, and anti–dengue IgG1 levels. NAb levels tightly correlated with anti–dengue IgG1 levels in preillness plasma from children with subsequent secondary DV2 and DV3 infection ($r = 0.59$ and $r = 0.49$, respectively; $P < .05$, Spearman rank correlation). All correlations of EA to anti–dengue IgG1 were similar to those demonstrated to NAb (data not shown).

Preillness plasma EA and NAb pattern. We have previously shown that, in secondary DV3 infection, a preexisting monotypic $PRNT_{50}$ pattern reflecting previous DV1 or DV2 infection correlated with DHF [13]. Earlier studies found that secondary DV2 infection after primary infection with DV1 was a risk factor for DSS [8, 26].

In secondary DV2 infection, EA did not differ between plasma with either a DV1 or DV3 monotypic $PRNT_{50}$ pattern. However, the percentage of infected cells was significantly higher in plasma samples with a DV3 monotypic $PRNT_{50}$ pattern, compared with samples with an undetectable or multitypic pattern, in either undiluted or diluted plasma (figure 6). In sec-

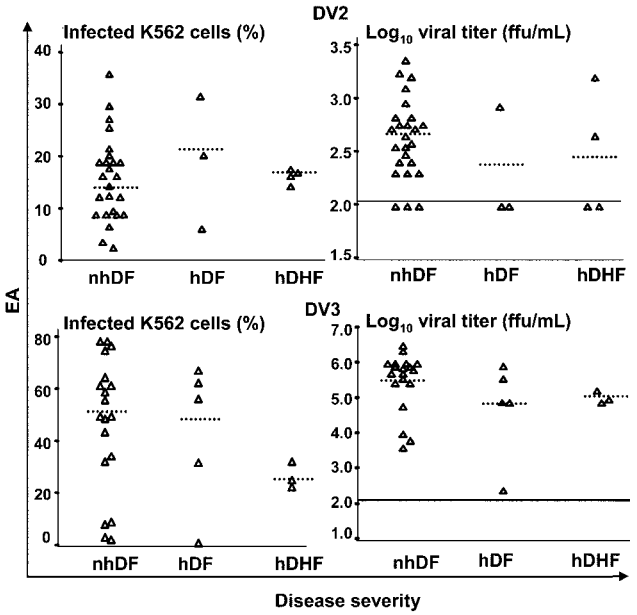

Figure 3. Relationship of preillness plasma enhancing activity (EA) and subsequent disease severity. EA in undiluted plasma, as determined by the percentage of infected K562 cells, is shown in left panels; the $\log_{10}$ viral titer is shown in right panels. Plasma samples from children with subsequent secondary Dengue virus (DV) 2 infection are shown in top panels; samples from children with subsequent secondary DV3 infection are shown in bottom panels. Analysis of variance was used to analyze data; significance was set at $P < .05$. The dotted line represents the mean values; the solid line represents the limit of detection (100 fluorescent focus units [ffu]/mL). hDF, hospitalized with Dengue fever; hDHF, hospitalized with Dengue hemorrhagic fever; nhDF, not hospitalized with Dengue fever.

ondary DV3 infection, EA did not differ between plasma with either a DV1 or DV2 monotypic $PRNT_{50}$ pattern. The percentage of infected cells in plasma diluted 1:5 with an undetectable $PRNT_{50}$ pattern was significantly lower than that of samples with a multitypic $PRNT_{50}$ pattern (figure 6).

Postsecondary DV infection plasma EA. After DV infection, individuals are not reinfected with the same DV serotype because of the presence of long-lived NAb and are protected from infection with heterologous DV serotypes for up to 6 months [4]. We investigated whether plasma samples collected 4–7 months after secondary DV2 and DV3 infection were able to enhance DV infection of K562 cells. In 10 randomly chosen plasma samples, EA was not detected in undiluted plasma but was detected in plasma diluted 1:5 in 2 of 5 and 4 of 5 DV2 and DV3 samples, respectively (figure 7). In both secondary DV2 and DV3 infection, detectable EA in preillness undiluted plasma samples was higher than that in postillness plasma samples (10/10 vs. 0/10; $P = .02$, McNemar's test).

DISCUSSION

In the present study, we measured EA in preillness plasma samples from children with subsequent secondary DV2 or DV3 infection. We did not find a correlation between EA and disease severity. Our results do not support those of a previous study by Kliks et al. [11] that found that EA in presecondary DV2 serum was associated with disease severity. However, these 2 studies differed in several key factors. Although both studies used a low-passage DV2 Thai strain, the DV2 strain used by the previous study infected monocytes efficiently, whereas our DV2 strain did not. In our experience, as well as that of others, an undetectable or very low percentage of human monocytes are infected when low-passage DV isolates are used (data not shown) [27]. In our study, K562 cells, used as a model for FcR-bearing cells to examine ADE, showed more consistent results than did monocytes. In addition, K562 cells were not efficiently infected with DV in the presence of flavivirus-naive plasma. Both studies compared preillness plasma EA between asymptomatic and symptomatic secondary DV infection. However, we did not find higher EA in plasma from asymptomatic children, as did Kliks et al. [11]. This may be related to the different serotypes studied (DV3 in our study and DV2 in the Kliks et al. study). In addition, all of the cases (both asymptomatic and symptomatic) in the Kliks et al. study were presumed to be DV2, whereas, in our study, a presumptive diagnosis of DV3 was made in only the asymptomatic group. In the Kliks et al. study, secondary DV2 infection was presumed on the basis of the fact that 73% of virus isolates from Bangkok Children's Hospital were DV2 [11]. Our

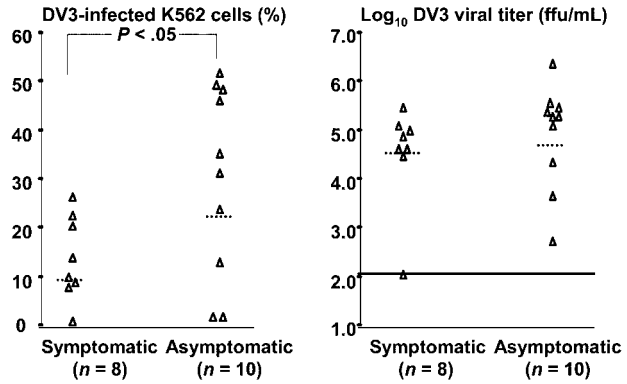

Figure 4. Relationship of enhancing activity (EA) in preillness plasma from children with symptomatic (hospitalized with Dengue fever and hospitalized with Dengue hemorrhagic fever) secondary Dengue virus (DV) 3 infection and asymptomatic presumed secondary DV3 infection. EA in undiluted plasma, as determined by the percentage of infected K562 cells, is shown in left panels; the $\log_{10}$ viral titer is shown in right panels. Student's *t* test was used to analyze data; significance was set at $P < .05$. The dotted line represents the mean values; the solid line represents the limit of detection (100 fluorescent focus units [ffu]/mL).

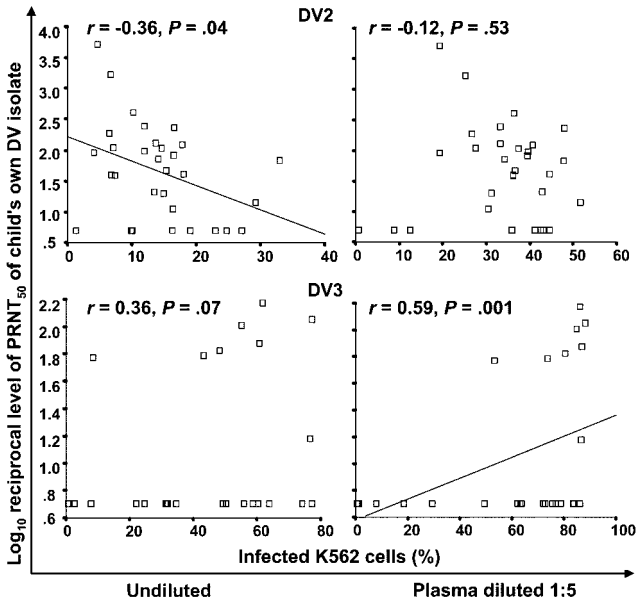

Figure 5. Correlation between preillness plasma enhancing activity (EA) and plaque-reduction neutralizing titer ($PRNT_{50}$) to patient's own Dengue virus (DV) isolate. EA was determined by the percentage of infected K562 cells in both undiluted plasma *(left panels)* and plasma diluted 1:5 *(right panels). Top,* Plasma samples from children with subsequent secondary DV2 infection; *bottom,* children with subsequent secondary DV3 infection. Spearman's rank correlation was used to analyze data; significance was set at $P < .05$.

asymptomatic group was less likely to be as heterogeneous as the previous study cohort, given that our children were selected from a school in which 32 (97%) of 33 of viral isolates were DV3. We did not compare EA in symptomatic and asymptomatic presecondary DV2 infection plasma because there was no insular DV2 outbreak similar to that of DV3. Our small sample size could be an additional factor, although it was comparable to that used in the previous study.

Interestingly, in undiluted plasma, the percentage of infected cells was significantly higher in asymptomatic than in symptomatic children with secondary DV3 infection (figure 4). This could be explained by the fact that the majority of preillness plasma samples (7/10) from asymptomatic children, compared with symptomatic children (1/8), showed a multitypic $PRNT_{50}$ pattern, which we found to be associated with higher EA (figure 6).

We were not able to detect a correlation between EA and subsequent plasma viremia levels in DV2-infected children. The EA of plasma diluted 1:5 from patients with presecondary DV3 infection positively correlated with reciprocal levels of $PRNT_{50}$ to the patient's DV3 isolate (figure 5). In addition, higher $PRNT_{50}$ levels were associated with lower DV3 viremia levels [13]. These results may explain the finding that higher EA correlated with lower plasma viremia levels among DV3-infected children (figure 2*G* and 2*H*). If the analysis was limited to only those children with detectable $PRNT_{50}$ to DV3, this correlation was no longer detected.

In our study, the majority of preillness plasma samples with detectable $PRNT_{50}$ to any of 4 DV serotypes or JEV had EA. EA was also detected in a presecondary DV3 infection plasma with an undetectable $PRNT_{50}$ to all the DV serotypes and to JEV (subject 5). This could be explained by either an exposure to another flavivirus circulating in the region, which was not measured [28, 29], or the presence of DV or JEV antibodies that were below the $PRNT_{50}$ assay's limit of detection. This also suggests, as in previous studies [30, 31], that not only serotype-specific but also serotype and JEV cross-reactive antibodies can enhance DV infection of FcR-bearing cells.

In undiluted plasma, EA was detected in preillness plasma, even when high $PRNT_{50}$ levels to DV were detected, but not in postsecondary DV infection plasma samples. This suggests that preillness plasma NAb is less effective than postillness plasma NAb in protecting against DV infection and that undiluted

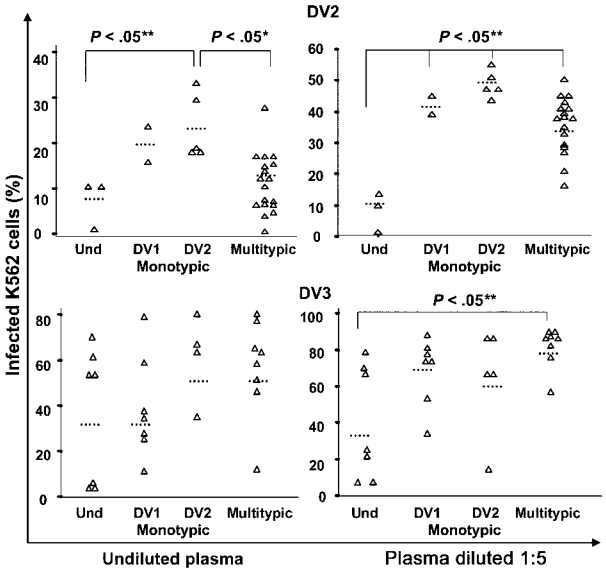

Figure 6. Relationship of preillness plasma enhancing activity (EA) and neutralizing antibody patterns. EA was determined by the percentage of infected K562 cells by use of both undiluted plasma *(left panels)* and plasma diluted 1:5 *(right panels). Top,* Plasma samples from children with subsequent secondary Dengue virus (DV) 2 infection; *bottom,* plasma samples from children with subsequent secondary DV3 infection. We defined 3 patterns of preillness DV plaque-reduction neutralizing titer ($PRNT_{50}$) results: undetectable (Und; $PRNT_{50}$ <10 to all 4 DVs), monotypic ($PRNT_{50}$ >10 to only 1 DV serotype or $PRNT_{50}$ >10 to more than 1 DV serotype but $PRNT_{50}$ ⩾80 to only 1 DV serotype), and multitypic ($PRNT_{50}$ >10 to more than 1 DV serotype and $PRNT_{50}$ ⩾80 to more than 1 DV serotype). The Sidak test was used for comparison of preillness plasma EA among the neutralizing antibody pattern groups. $*P < .05$, EA for plasma samples with DV monotypic vs. multitypic $PRNT_{50}$ patterns. $**P < .05$, EA for plasma samples with undetectable vs. either monotypic or multitypic $PRNT_{50}$ patterns. Und, undetectable $PRNT_{50}$ pattern.

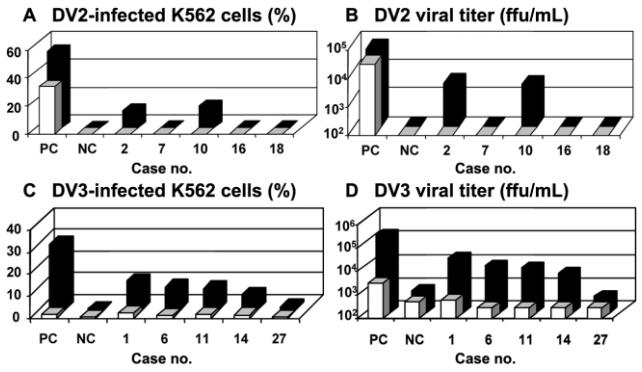

Figure 7. Postsecondary Dengue virus (DV) infection plasma enhancing activity (EA). EA was determined by the percentage of infected K562 cells and by $\log_{10}$ viral titer in infected K562 cell supernatant (in fluorescent focus units [ffu] per milliliter) in DV2 *(A* and *B)* and DV3 *(C* and *D)* infection. *White bar,* Undiluted plasma; *black bars,* plasma diluted 1:5. NC, negative control (flavivirus-naive plasma); PC, positive control (DV3-immunized plasma).

plasma may be more physiologically relevant than diluted plasma in the measurement of EA [11].

Taken together, our data suggest that preexisting DV antibodies may not be necessary for effective DV infection of certain target cells. This is supported by the work of others that has shown that preexisting DV antibodies are not able to enhance the infection of either immature dendritic cells or Langerhans cells, which are the most permissive cell types for DV infection and may be early targets for DV infection [32, 33]. Data from clinical and epidemiological studies have also suggested that preexisting antibodies are not the only factor involved in DV disease severity. Clinically, DHF and DSS have been shown to occur with primary DV infections in children >1 year old and in adults [6, 34–36]. Epidemiological studies have not shown an increased incidence of DHF after either JEV vaccination [37] or infection [38] in a region were dengue is endemic, although our study and others [30, 39] have detected an enhancement of DV infection in vitro by use of JEV-immune plasma. This suggests that other factors in addition to ADE are important in the immunopathogenesis of DV infection.

To our knowledge, this is the first study to have examined the relationship between presecondary DV infection plasma EA and both subsequent DV disease severity and viremia levels. The majority of preillness plasma samples from children with subsequent secondary DV2 and DV3 infection were able to enhance the DV infection of K562 cells. Importantly, the levels of preillness plasma EA of DV infection in K562 cells do not predict subsequent clinical severity or viral burden in secondary DV infections.

Acknowledgments

We thank the entire staff at the Department of Virology, Armed Forces Research Institute of Medical Sciences (AFRIMS, Bangkok, Thailand), for their performance of serologic testing; virus detection, isolation, and quantification; and data collection and entry; the Office of the Director, Provincial Public Health, Kamphaeng Phet Province; the clinical research nurses at AFRIMS; the support staff at Kamphaeng Phet field station; Mary Co, for her critical reading of the manuscript; Jurand Janus, for DV propagation; and especially the study participants and their families.

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In the 1 August 2005 issue of the *Journal,* in the article by Laoprasopwattana et al. (Laoprasopwattana K, Libraty DH, Endy TP, et al. Dengue virus [DV] enhancing antibody activity in preillness plasma does not predict subsequent disease severity or viremia in secondary DV infection. J Infect Dis 2005; 192:510–9), there are two errors. The "Financial support" footnote at the bottom of the left-hand column of the title page should include two additional National Institutes of Health grants: K08 AI01729 (to S.G.) and U19 AI057319. Also, the tenth sentence in the first paragraph of the Discussion section should read as follows: "However, we did not find a higher EA in the plasma from symptomatic children than in the plasma from children with asymptomatic dengue infection, as did the study by Kliks et al." (*not* as "However, we did not find higher EA in the plasma from asymptomatic children, as did the study by Kliks et al."). The authors regret these errors.

The Journal of Infectious Diseases 2005; 192:1863

0022-1899/2005/19210-0030$15.00