THE SEROTYPE-INDEPENDENT BUT CONCENTRATION-DEPENDENT ENHANCING ANTIBODIES AMONG THAI DENGUE PATIENTS

Chai Teerasut[1], Huan-Yao Lei[2], Khuendee Natthakorn[3], Akanitt Jittmittraphap[3], Suwich Thammapalo[4], Prasong Witayathawornwong[5], Ya-Tin Lin[2], Richard G Jarman[6], Pathom Sawanpanyalert[7], Wipawee Jampangern[3] and Kriengsak Limkittikul[8]

[1]Si Sa Ket Provincial Hospital, Office of Permanent Secretary, Ministry of Public Health, Si Sa Ket; [2]Department of Microbiology and Immunology, College of Medicine, National Cheng Kung University, Tainan, Taiwan; [3]Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, Bangkok; [4]Department of Disease Control, Ministry of Public Health, Nonthaburi; [5]Phetchabun Provincial Hospital, Office of Permanent Secretary, Ministry of Public Health, Phetchabun; [6]Armed Force Research Institute of Medicine Science, Bangkok; [7]Department of Medical Sciences, Ministry of Public Health, Nonthaburi; [8]Department of Tropical Pediatrics, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand

Abstract. Antibody-dependent enhancement of infection (ADE) is central to explaining the development of severe disease at the end of post-dengue virus infection. Non-neutralizing anti-dengue antibodies bound to the dengue virion enhances the virus entrance into the target cells via the Fc receptor. The titer of enhancing antibodies in dengue patients is not determined during dengue virus infection. Sensitive flow cytometry detecting dengue virus-infected K562 cells was used to quantitate enhancing activity among Thai DF and DHF patients against four serotypes and the patient's dengue isolate. The titer was defined as the reciprocal of the final dilution that loses enhancing activity. The serum of Thai patients confirmed to have dengue infection were found to have high titers of enhancing antibodies and increased gradually through the convalescent phase of infection. The enhancing antibody titers were not different among the four serotypes or from the infecting isolate. The anti-dengue antibodies from dengue patients can enhance dengue virus infections in a concentration-dependent, serotype-independent manner.

Keywords: anti-dengue antibody, concentration, serotype, DF, DHF

Correspondence: Dr Kriengsak Limkittikul, Department of Tropical Pediatrics, Faculty of Tropical Medicine, Mahidol University, 420/6 Ratchawithi Road, Bangkok 10400, Thailand.
Tel: +66 (0) 2354 9100 ext 1665; Fax: +66 (0) 2354 9163
E-mail: tmklk@mahidol.ac.th
Huan-Yao Lei, Department of Microbiology and Immunology, College of Medicine, National Cheng Kung University, 1 University Road, Tainan, 70101, Taiwan.
Tel : +886 6 2353535 ext 5643; Fax: +886 6 2082705
E-mail: hylei@mail.ncku.edu.tw

INTRODUCTION

Dengue viruses (DENV), mosquito-borne, enveloped, positive-stranded RNA viruses, are the members of the genus *Flavivirus*. There are four antigenically distinct but related serotypes, which cause a spectrum of disease presentations, from undifferentiated febrile illness to dengue fever, dengue hemorrhagic fever and dengue shock syndrome (DHF/DSS) (Bhamarapravati *et al*, 1967; Burke *et al*, 1988; Gubler, 1998; Mackenzie *et al*, 2004). Dengue virus is an important public health problem, especially in tropical and subtropical areas, and had become a major disease burden in Southeast Asia, South and Central America, and the Caribbean. Dengue hemorrhagic fever was first reported in Thailand in 1950. Currently, there are no effective strategies to prevent the progression of severity to DHF/DSS and the pathogenic mechanism is not well understood (Halstead, 1988; Lei *et al*, 2001). The theory of antibody-dependent enhancement (ADE) has been proposed as playing a major role in disease severity (Halstead, 1970; Halstead *et al*, 1980). It explains the finding that the more severe disease manifestations of DHF/DSS occur predominantly, although not exclusively, in children experiencing a secondary dengue virus infection. Infection with one serotype confers lifelong homotypic immunity, but there is only short-term cross-protection against heterotypic serotypes (Sbin, 1952). Non-neutralizing anti-dengue antibodies bound to the dengue virus enhance the virus entrance into target cells via the Fc receptor. Dengue virus of a different serotype can propagate in infected cells, rapidly leading to an increase in viral burden (Kliks *et al*, 1988, 1989). ADE has been reported in children with secondary infection of a different serotype of dengue virus causing more severe DHF/DSS which could be fatal. In infants less than one year old, maternal antibodies may initially protect the infant from clinical disease, but later enhance the dengue infections.

In this study, we evaluated the enhancing activity of antibodies among patients acutely infected with dengue virus shown to be secondarily infected by ELISA. Serum samples collected from patients during the acute, convalescent and recovery phases were analyzed using a sensitive flow cytometric assay for dengue virus-infected K562 cells to determine the titer of enhancing antibodies (Huang *et al*, 2005, 2006).

MATERIALS AND METHODS

Patients and sample collection

Venous blood samples were collected from suspected dengue infected patients hospitalized at Phetchabun and Nakhon Si Thammarat Hospitals from June 2004 to September 2004. Five milliliters blood samples were obtained 3 times from each patient suspected of dengue virus infection and admitted to the hospital. The first blood sample (acute phase) was collected within a day of hospital admission. The second blood sample (convalescent phase) was collected when the patient was discharged from the hospital, and the third blood sample (recovery phase) was collected 15 days after admission. Eleven cases of DF and 14 cases of DHF were included in this study. Blood samples were left at room temperature for 30 minutes to allow complete clotting; then centrifuged at 1,500 rpm for 15 minutes. Serum samples were separated and used for a serological assay for dengue virus detection. Serum samples were subsequently

stored at -70ºC. We used the paired *t*-test for statistical analysis. Statistical significance was set at $p < 0.05$.

Serological and virological assays

Serum samples were assayed for anti-dengue antibodies using a rapid colloidal gold-based immunochromatographic test (Dengue Duo IgM and IgG rapid strip test; PANBIO, Brisbane, Australia) based on the IgM and IgG capture assay (Innis *et al*, 1989). Dengue virus serotypes were identified from acute phase serum samples by a serotype-specific RT-PCR and by virus isolation in C6/36 cell lines (Lanciotti *et al*, 1992). Dengue virus serotype 2 strain PL046, and clinically isolated dengue virus serotypes 1, 2 and 4 from Thai patient serum samples were isolated by cell culture in the C6/36 cell line. Viruses were propagated in C6/36 mosquito cell lines with D-MEM medium containing 2% FBS at 28ºC for 5 days.

Antibody-dependent enhancement assays

K562 cells maintained in RPMI 1640 medium containing 10% FBS, L-glutamine and antibiotic at 37ºC with 5% CO_2 were used. After 2 or 3 days subculture, K562 cells were collected, centrifuged at 400*g* for 6 minutes at 4ºC; then, the medium was discarded. One mililiter of RPMI 1640 medium containing 2% FBS was added to the pellets and vortexed briefly. Cells were then counted in eosin Y in a counting chamber, and the 5×10^4 cell samples were prepared in tubes. Sequential three-fold dilutions were prepared from each serum sample beginning with 1:100 dilution. The virus-antibody complexes were prepared by mixing 50 l of serial three-fold dilutions of patient serum with an equal volume of viruses (m.o.i. = 1) for 30 minutes on ice before being added to the K562 cell suspension (5×10^4 cells per sample) for infection. After infection, cells were washed twice with PBS and centrifuged at 1,000 rpm at 4ºC for 5 minutes. The cells were then resuspended in 200 l of RPMI 1640 medium containing 10% FBS, L-glutamine and antibiotic for further culture on 96 well plates at 37ºC with 5% CO_2. At 48 hours, cells were harvested and analyzed with flow cytometry.

Flow cytometry to detect dengue virus-infected cells

Cells were harvested and fixed with 2% paraformaldehyde in PBS for 20 minutes on ice. After being washed, the cells were stained with anti-dengue NS-1 MAb (0.05 g/5×10^4 cells) in the permeabilization buffer (2% FBS, 0.1% saponin, 0.1% sodium azide) for 30 minutes on ice. After washing, the cells were stained with anti-mouse IgG-FITC in the same buffer for 30 minutes. Finally, these cells were washed and resuspended in staining buffer (2%FBS and 0.1% sodium azide) for FACSCalibur analysis (Becton Dickinson Immunocytometry Systems, San Jose, CA), and analyzed using the software WinMDI, version 2.8 (Huang *et al*, 2005, 2006).

RESULTS

Flow cytometric assay of enhancing activity in dengue patient serum

Using cell-based flow cytometry with anti-dengue antibody intracellular staining of dengue virus-infected cells is a sensitive assay to detect dengue virus infected cells and can be used to evaluate the effect of enhancing antibody on dengue virus infection (Huang *et al*, 2005, 2006). We evaluated enhancing antibodies among Thai dengue patients during three stages of infection: acute, convalescent, and recovery. Serum, at 3-fold serial dilutions, starting at 1:100, was added to K562

Table 1
Enhancing antibody titers (log_{10}) against PL046 (DENV-2) among DF patients.

DF patient serum (infecting serotype)[a]	Enhancement titer (log_{10}) to DENV-2		
	Acute	Convalescent	Recovery
NS04/138 (DENV-1)	3.47	6.24	5.97
NS04/063 (DENV-1)	4.50	5.75	5.80
NS04/019 (DENV-1)	3.90	5.23	5.32
NS04/086 (DENV-3)	3.98	5.70	5.20
PB04/124 (DENV-4)	4.65	5.65	5.51
NS04/114 (Untypable)	5.13	5.86	5.60
PB04/032 (Untypable)	4.80	5.80	5.70
PB04/038 (Untypable)	4.18	5.24	5.30
PB04/039 (Untypable)	2.83	4.71	5.13
PB04/058 (Untypable)	4.30	4.92	4.55
PB04/071 (Untypable)	3.85	5.40	4.90
Mean ± SD	4.14 ± 0.65	5.50 ± 0.45	5.36 ± 0.41

[a]Infecting serotype determined by nested RT-PCR or isolation of virus on C6/36 cells.

cells at a m.o.i. of 1 to test for enhancing activity. The percentage of infected cells without the addition of serum was approximately 2% at 48 hours, but the serum enhanced dengue-2 virus infection among K562 at 1/900 and 1/2,700 dilutions. The acute phase serum of the DF patient had enhancing activity to a dilution of 1/2,700, the convalescent and recovery phases showed enhancement to a dilution of 1/72,900 (Fig 1a). In a DHF patient acute, convalescent and recovery serum possessed enhancing activity to dilutions of 1/72,900, 1/218,700, 1/218,700, respectively (Fig 1b).

To compare patients samples, we determined their titers. Using sigma plot software, we plotted the percentage of infected cells *versus* the log_{10} serial dilution of the sera (Fig 2). We defined the neutralization titer as the reciprocal of the end point of dilution that could not neutralize the dengue virus infection, whereas the enhancement titer is the reciprocal of the final dilution that loses enhancing activity. Since the condition was set at m.o.i.=1 for 48 hours to observe optimal enhancement in which the neutralizing effect was not so obvious at a low infection rate, we focused only on the enhancing antibody titers. The enhancing titers for the different phases (acute, convalescent and recovery) are shown in Tables 1 and 2. For DF, the mean dilutions were: 1/14,000 (log_{10} = 4.14, acute phase), 1/316,000 (log_{10} = 5.50, convalescent phase) and 1/229,000 (log_{10} = 5.36, recovery phase) whereas the mean dilutions for DHF were: 1/34,000 (log_{10} = 4.53, acute phase), 1/692,000 (log_{10} =5.84, convalescent phase) and 1/417,000 (log_{10} = 5.62, recovery phase). The convalescent phase serum contained a significantly higher ($p < 0.05$) titer of enhancing antibodies than the acute phase serum, but there were no differences between the convalescent and recovery phases. The enhancing titers were not different between the DF and DHF patients.

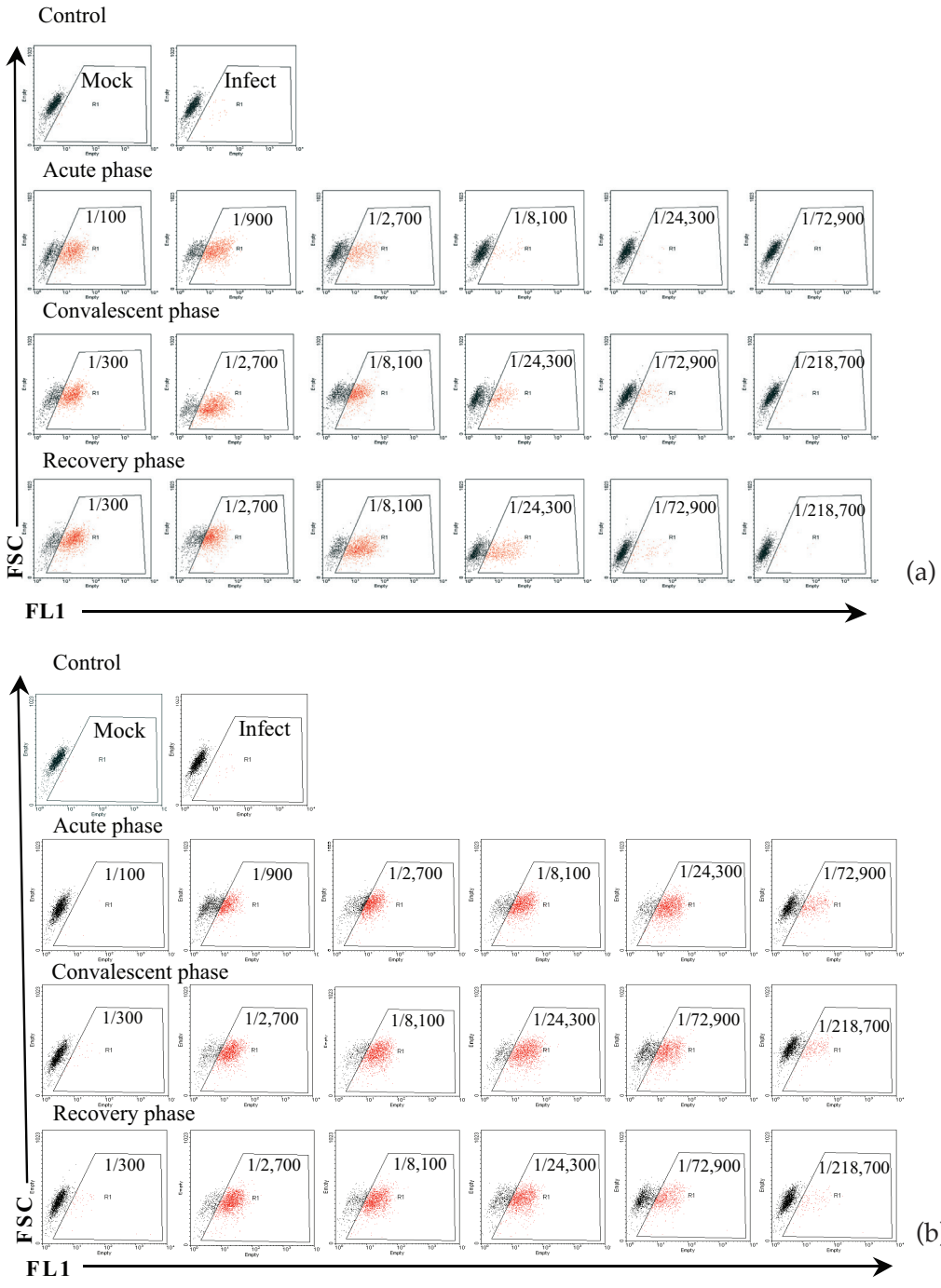

Fig 1–The representative flow cytometric determination of ADE in DF (a) and DHF (b) patient serum in K562 cells. K562 cell lines were infected with DENV-2 PL046 (Taiwan strain) in the presence of a three-fold serial diluted patient's serum. Mock was uninfected with DENV to serve as a negative control. K562 cell infected with DENV-2 PL046 in the absence of serum was used as a positive control.

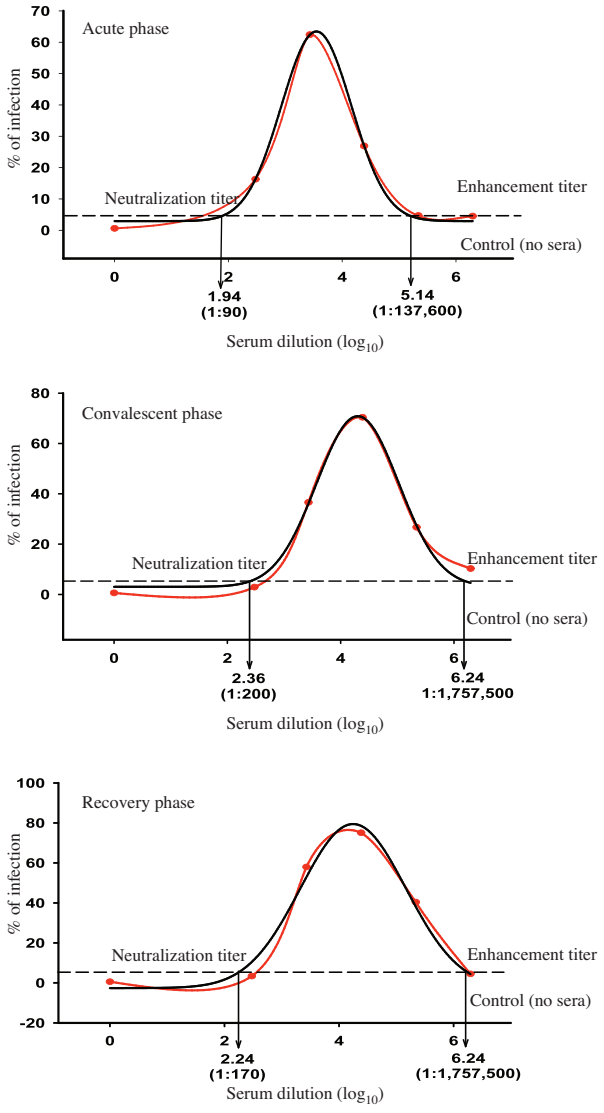

Fig 2–Representative example of curves obtained after plotting the percentage of infected cells *versus* the $\log_{10}$ serial dilution of sera. The red line is the experimental curve (the curve from experimentally observed values) while the black line is the theoretical curve (the curve derived from calculation). The dotted line is the cut-off point for a positive control of dengue virus infection in the absence of patient serum. The neutralization titer is defined as the reciprocal of the end point of dilution that cannot neutralize the dengue virus infection. The enhancement titer is defined as the reciprocal of final dilution that loses the enhancing activity.

Enhancing antibody titers among dengue patient serum against the four serotypes of dengue virus

We used dengue serotype 2 PL046, a laboratory strain, for the assay virus. Dengue virus clinical isolates from three dengue patients were also used: serotype 1 from the NS04/138 DF patient, serotype 2 from the PB04/318 DHF patient and serotype 4 from the PB04/055 DHF patient. For serotype 3, we used a Taiwanese isolate. Seven dengue patient serum samples were tested against these four serotypes of dengue virus. The clinical isolates had a slow replication rate compared to the laboratory strain (PL046) but enhancing activity was still detected by flow cytometry (data not shown). Using the same method, enhancing antibody titers were determined using a representative clinical isolate from a patient in this study with the exception of dengue 3, which was isolated in Taiwan (Table 3). No significant differences in enhancing antibodies were found among the four dengue virus serotypes. Although convalescent phase serum still contained a significantly higher ($p < 0.05$) titer of enhancing antibodies than the acute phase, there were no differences among the four serotypes of dengue virus tested. The enhancing titer of serum from the dengue serotype 1-infected NS04/138 patient was not higher than the titers against serotypes 2, 3 or 4. In the other serotype 1-infected PB04/315 patient the enhancing titer was also not higher than against serotypes 2, 3 and 4. The

Table 2
Enhancing antibody titers (log_{10}) against PL046 (DENV-2) in DHF patients.

DHF patient serum (infecting serotype)[a]	Enhancement titer (log_{10}) to DENV-2		
	Acute	Convalescent	Recovery
PB04/315 (DENV-1)	5.14	6.24	6.23
PB04/188 (DENV-2)	4.25	5.83	6.50
PB04/239 (DENV-2)	4.97	5.97	5.68
PB04/318 (DENV-2)	4.35	5.46	5.53
PB04/037 (DENV-4)	5.70	6.10	5.60
PB04/049 (DENV-4)	4.24	5.70	4.78
PB04/055 (DENV-4)	4.25	5.70	5.70
PB04/065 (DENV-4)	4.50	6.50	6.50
PB04/078 (DENV-4)	3.73	5.80	5.30
PB04/088 (DENV-4)	3.59	5.30	5.50
PB04/116 (DENV-4)	4.26	5.70	4.96
PB04/072 (Untypable)	3.41	5.30	5.50
PB04/099 (Untypable)	5.43	6.15	5.38
PB04/151 (Untypable)	5.61	6.00	5.56
Mean ± SD	4.53 ± 0.74	5.84 ± 0.35	5.62 ± 0.50

[a]Infecting serotype determined by nested RT-PCR or isolation of virus in C6/36 cells.

Table 3
Enhancing antibody titers (log_{10}) against four different serotypes of dengue virus.

Dengue patient serum	NS04/138 (DENV-1)			PB04/318 (DENV-2)			Taiwan strain (DENV-3)			PB04/055 (DENV-4)		
	A	C	R	A	C	R	A	C	R	A	C	R
NS04/138(D1)	3.40	5.85	5.93	3.49	4.25	5.06	3.54	4.92	4.99	4.08	5.88	5.81
PB04/315(D1)	4.11	5.98	5.79	4.43	5.06	5.36	3.93	5.51	5.58	4.33	5.14	5.65
PB04/318(D2)	3.96	6.10	6.40	3.93	5.16	5.70	3.74	5.58	6.15	5.58	6.24	6.30
PB04/188(D2)	3.94	5.63	5.55	3.66	5.34	5.48	3.89	5.99	6.12	3.52	5.53	5.78
NS04/086(D3)	3.29	5.11	5.49	3.81	5.48	5.90	4.35	4.25	5.21	3.17	4.92	4.75
PB04/055(D4)	3.90	5.31	4.97	3.27	4.70	4.52	4.57	6.40	5.21	4.47	6.15	5.85
PB04/197(D4)	5.00	7.80	6.20	3.88	5.04	5.51	4.57	5.75	5.16	4.08	5.53	5.29
Mean ± SD	3.94 ±0.52	5.97 ±0.82	5.76 ±0.44	3.78 ±0.34	5.00 ±0.38	5.36 ±0.42	4.08 ±0.38	5.49 ±0.66	5.49 ±0.44	4.18 ±0.71	5.63 ±0.46	5.63 ±0.45

A, acute; C, convalescent; R, recovery

same pattern applied to serotype 2 and serotype 4-infected patients. The serotype 2 (PB04/318) and serotype 4 (PB04/055) dengue patients also had no higher levels of enhancing titers than against the other serotypes. The enhancing titers of 3 dengue patients (NS04/138, PB04/318, and PB04/055) were compared with the

four serotypes and PL046 (Table 3). The enhancing titers were similar irrespective of the serotype of dengue virus used for the assay. This suggests the ADE effect on dengue virus infection among dengue patient serum was not serotype-dependent, but concentration-dependent.

DISCUSSION

Using the flow cytometric assay to detect dengue virus infection in K562 cells bearing the Fc receptor, enabled us to determine the titer of neutralizing and enhancing antibodies. The enhancing activity observed after neutralizing was diluted out. The enhancing antibody titer was extremely high; we could detect enhancing activity among the serum samples of DHF patients at an average dilution of 1/34,000 (acute phase), 1/692,000 (convalescent phase) and 1/417,000 (recovery phase). This might be due to the fact these samples were collected from patients recently secondarily infected with dengue virus, and there was a clonal expansion of memory B cells from the onset of illness. Huang *et al* (2006) previously reported that monoclonal anti-E Ab and anti-prM Ab are enhancing antibodies, and their enhancement is concentration-dependent, but dengue serotype-independent. This was confirmed using the dengue patient serum in this study, where anti-dengue antibodies enhanced dengue virus infections among all four serotypes, in addition to the patient's own infecting virus serotype, in a concentration-dependent manner.

Using flow cytometry to detect the dengue-infected cells is a very sensitive method, and can quantitate the titer of the enhancing antibody (Huang *et al,* 2005, 2006). The traditional assay to detect ADE is done by plaque assay, which is time consuming and influenced by various parameters such as the virus strains, cell type and the level of infection. Diamond *et al* (2000) reported the *in vitro* infection with dengue virus is modulated by different cell types, viral strains, and m.o.i. We used an m.o.i. of 1 in K562 cells with 48 hours of culture because it is a sensitive cell line and is generally used for enhancing activity assays. If using a lower m.o.i., for example 0.5 or 0.1, a longer culture time than 48 hours is needed. For less sensitive cell lines, a higher m.o.i. can be used (Huang *et al,* 2006). Flow cytometric analysis can quantitate the percentage of dengue virus-infected cell mass used to distinguish the enhancement from the infected control, and can simultaneously measure neutralizing and enhancing Abs. Enhancing activities were revealed only when neutralizing Abs were diluted out. However, as our study was primarily designed to determine the enhancing antibody titer, the m.o.i. was set at 1, which makes it easier to detect the enhancement above the basic level of infection of <2% in the absence of patient serum. However, it is difficult to see the inhibition effect with such a low infected control level. The neutralized titer was derived from the calculation of Sigma plot software. It was presumed to be a "projected titer", not an experimentally determined titer. For quantitation of the neutralization titer, the basic level of dengue virus infected cells has to be increased to 30 ~ 40% so it can be easily suppressed. This can be done by increasing the m.o.i. and using longer infection culture time.

ADE assays typically use laboratory adapted strains with patient serum to assess enhancing activity (Laoprasopwattana *et al*, 2005). In this study, laboratory-adapted PL046 strain was used and representative clinical isolates from

study patients were also used. Although the laboratory adapted virus exhibited higher replication kinetics than the clinical isolates, enhancing activity was not significantly different from what was seen using the laboratory strain. There was also no difference observed in enhancing activity using clinical isolates *versus* isolates from donor serum or non-synonymous patient serum. It is thought the enhancing activity of anti-dengue antibodies against the different serotypes was due to low affinity attributed to cross-reactivity of heterotypic antibodies; therefore, enhancing, rather than neutralizing, the dengue virus infection. Our study using an *in vitro* assay in K562 cells did not show an affect on viral infection. The enhancing titer increased post-dengue virus infection; the convalescent-phase had a higher titer of enhancing antibodies than the acute-phase, because the immune response progressed to generate more anti-dengue antibodies after antigen stimulation.

Dengue disease has a broad spectrum of clinical presentations, from mild DF to severe or even fatal DHF/DSS. Severe DHF is associated with a secondary infection by a different serotype of dengue virus. ADE is a central hypothesis to explain the increased dengue virus-infected diseases (Halstead, 1970; Halstead *et al*, 1980). Babies less than 1 year of age with maternal anti-dengue IgG antibodies are known to be susceptible to DHF/DSS post-primary infection (Kliks *et al*, 1988, 1989). Endy *et al* (2004) reported levels of preexisting neutralizing Ab were associated with a lower viremia levels and milder disease in secondary infection due to DENV-3, but not DENV-2 or DENV-1 infections. Laoprasopwattana *et al* (2005) reported levels of pre-illness plasma enhancing activity of DENV infection in K562 cells did not correlate with the clinical severity or viral burden of secondary DENV infection.

Dengue patients can generate anti-E and anti-prM antibodies post-infection (Cardosa *et al*, 2002), but the relative ratio or concentration between these two dengue virion-binding antibodies has not been determined. The neutralizing activity of monoclonal anti-E Ab, especially anti-domain III of E, is higher than the anti-prM Ab. At low concentrations, anti-E antibodies became enhancing antibodies (Huang *et al*, 2006). In this study, DF and DHF patients characterized as having a secondary infection had enhancing titers not significantly different. However, the dengue patient serum during a primary infection had much lower enhancing antibody titers (unpublished observation). This is due to the lower titer of anti-dengue antibodies post-primary infection. In conclusion, anti-dengue antibodies in dengue patients can enhance dengue virus infections in a concentration-dependent, serotype-independent manner.

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