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Human monoclonal antibodies to neutralize all dengue virus serotypes using lymphocytes from patients at acute phase of the secondary infection

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ABSTRACT

The global spread of the four dengue virus serotypes (DENV-1 to -4) has made this virus a major and growing public health concern. Generally, pre-existing neutralizing antibodies derived from primary infection play a significant role in protecting against subsequent infection with the same serotype. By contrast, these pre-existing antibodies are believed to mediate a non-protective response to subsequent heterotypic DENV infections, leading to the onset of dengue illness. In this study, we prepared hybridomas producing human monoclonal antibodies (HuMAbs) against DENV using peripheral blood mononuclear cells (PBMCs) from patients in the acute phase (around 1 week after the onset of illness) or the convalescent phase (around 2 weeks after the onset of illness) of secondary infection. Interestingly, a larger number of hybridoma clones was obtained from patients in the acute phase than from those in the convalescent phase. Most HuMAbs from acute-phase infections were cross-reactive with all four DENV serotypes and showed significant neutralization activity to all four DENV serotypes. Thus, secondary DENV infection plays a significant role in stimulating memory cells to transiently increase the number of antibody-secreting plasma cells in patients in the early phase after the secondary infection. These HuMAbs will enable us to better understand the protective and pathogenic effects of DENV infection, which could vary greatly among secondarily-infected individuals.

1. Introduction

Mosquito-borne dengue virus (DENV) infection occurs in tropical and subtropical regions around the world. The spread of this virus, combined with its severe clinical outcome, has made dengue a major and increasing global public health concern.

DENV has a positive-sense, single-stranded RNA genome of approximately 11 kb that encodes a capsid protein (C), a pre-membrane protein (prM), and an envelope glycoprotein (E), in addition to seven nonstructural proteins (NS) such as NS1 [1].

When humans are repeatedly infected with the same virus, pre-existing memory immune cells quickly produce neutralizing antibodies to protect against the current infection [2]. In DENV, pre-existing neutralizing antibodies raised by the primary infection are protective against subsequent infections with the

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same DENV serotype [3]. Severe dengue cases mostly occur among patients secondarily infected with different DENV serotypes [3]. This may be due to antibody-dependent enhancement (ADE), by which the current infecting virus can use pre-existing anti-DENV antibodies raised during the primary infection to gain entry to Fc receptor-positive macrophages [4–6]. However, it is thought-provoking that most DENV infections are asymptomatic [7], even among individuals secondarily infected with heterotypic DENV [8], and some of these cases show a wide spectrum of clinical symptoms, from a mild illness such as dengue fever (DF) to severe illness such as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) [9]. In this study, we comparatively prepared hybridomas producing human monoclonal antibodies (HuMAbs) using peripheral blood mononuclear cells (PBMCs) from dengue patients at the acute and convalescent phases of secondary infection.

2. Materials and methods

2.1. Patients

Patient participants were selected based on clinical diagnosis and the results of a rapid test with immunochromatography (SD BIOLINE Dengue Duo kit, SD, Kyonggi-do, Korea). A total of 9 blood specimens for cell fusion were collected from eight Thai dengue patients at the Hospital for Tropical Diseases, Faculty of Tropical Medicine, Mahidol University: three acute-phase patients (around 1 week after the onset of fever) and four convalescent-phase patients (around 2 weeks after the onset of fever), with one patient for both of the acute (D23) and convalescent phases (D26) (Table 1). PBMCs isolated from peripheral blood as described below were used for cell fusion.

2.2. Cell lines and viruses

SPYMEG cells used as fusion partner cells to develop hybridomas producing HuMAbs, were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 15% fetal bovine serum (FBS) [10]. Vero cells were maintained in a 5% CO_2 incubator at 37 °C in minimum essential medium (MEM) with 10% FBS. The DENVs used in this study were the Mochizuki strain of DENV-1, the 16681 and New Guinea C (NGC) strains of DENV-2, the H87 strain of DENV-3, and the H241 strain of DENV-4. Culture supernatants from C6/36 cells infected with individual strains were used as viral stocks. Infectivity titers were estimated according to the number of focus-forming units (FFU) as described previously [11].

2.3. Reverse transcriptase (RT)-polymerase chain reaction (PCR) for DENV serotyping

Total RNA was extracted from patient plasma using a QIAamp Viral RNA kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. This RNA was used as the template for reverse transcription using the Superscript III cDNA synthesis kit (Invitrogen, Carlsbad, CA). Oligonucleotide primer pairs previously reported for serotyping were used for the amplification of the DENV E gene [12].

2.4. Hybridoma preparation

Approximately 10 ml of blood was obtained from individual patients and the PBMCs were isolated by centrifugation through Ficoll-Paque™ PLUS (GE Healthcare, Uppsala, Sweden). The PBMCs were fused with SPYMEG cells at a ratio of 10:1 as described previously [10].

2.5. Indirect immunofluorescence (IF) assay

Vero cells in a 96-well microplate were mock-infected or infected with DENV. After incubation for 16–24 h, the cells were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) and permeabilized with 1% Triton X-100 in PBS. Undiluted hybridoma culture fluids were used for the HuMAbs. As a positive control, cells were incubated with 4G2, anti-flavivirus E mouse MAb [13]. The plate was stained with 4G2 at 4 °C overnight. The bound antibody was visualized by further reaction with an AlexaFluor 488-conjugated anti-mouse antibody (1:1,000; Invitrogen).

2.6. Neutralization assay

The virus neutralization assay was conducted on culture media of individual hybridoma clones, as described previously [14]. Twenty-five microliters of hybridoma culture supernatant or DMEM supplemented with 15% FBS (as a negative control) was mixed with 100 FFU of individual DENV serotypes (25 µl). After incubation for 15 min, the mixture was used to infect Vero cells in a 96-well microplate. After inoculation at 37 °C for 2 h, 100 µl of MEM with 3% FBS was added. After incubation at 37 °C overnight, the cells were fixed with 3.7% formaldehyde in PBS and permeabilized with 1% Triton X-100 in PBS. The plate was stained with 4G2 at 4 °C overnight, as for the IF assay. The assays were performed in duplicate and the results expressed as averages. Neutralization activity of HuMAbs in the culture medium from hybridoma clones was expressed as "−" (<50%) and "+" (50–<90%), or "++" (⩾90% reduction in FFU), compared with the negative control.

Table 1
Summary of patients' background and HuMAbs obtained in this study.

Patient	Gender	Age	Diagnosis	Blood collection[a]		Rapid test[b]		PCR serotyping	Hybridoma clone obtained	Isotyping of HuMAb			
				Days	Phase	IgG	IgM			IgG	IgA	IgM	None[c]
D23[d]	Female	33	DF	5	Acute	+	+	DENV-2	75	70	3	0	2
D30	Female	23	DHF grade 1	8	Acute	+	+	DENV-2	25	22	3	0	0
D32	Male	19	DF	6	Acute	+	+	DENV-2	5	5	0	0	0
D33	Male	31	DF	8	Acute	+	+	DENV-2	16	14	2	0	0
D22	Female	25	DHF grade 3	12	Convalescent	+	+	NT[e]	4	3	0	0	1
D25	Male	27	DF	14	Convalescent	+	+	NT	5	5	0	0	0
D26[d]	Female	33	DF	19	Convalescent	+	+	NT	2	2	0	0	0
D27	Male	21	DHF grade 2	13	Convalescent	+	+	NT	2	2	0	0	0
D28	Female	23	DF	15	Convalescent	+	+	NT	2	1	0	0	1

[a] Blood were collected at days after the onset of fever: 5–8 days for acute and 12–19 days for convalescent phase.
[b] Rapid test for D22, D25–D28 was performed with the plasma from these patients at their acute phase.
[c] HuMAbs not reacted with any of IgG, IgA, nor IgM.
[d] D23 and D26 were derived from the same patient at acute and convalescent phases, respectively.
[e] Not tested, because enough amounts of the plasma from the patients at acute phase for RT-PCR were not available.

2.7. Expression vectors for DENV proteins

The CMV4-HA vector was used for the molecular cloning of a fusion form of the E and prM (prM-E) and E DENV genes. On the other hand, the pcDNA3-C-Flag vector was used for the molecular cloning of the prM and C DENV genes. The individual coding regions for these viral proteins derived from DENV-2 NGC strain were amplified and cloned in the above vectors. The NS1 gene was cloned as reported previously [15]. 293T cells transfected with individual plasmids were used as viral antigens for the identification of viral proteins recognized by HuMAbs by IF.

2.8. Isotyping of HuMAbs

HuMAbs were isotyped using the Human IgG ELISA Quantitation set, Human IgM ELISA Quantitation set, and Human IgA ELISA Quantitation set (Bethyl Laboratories, Inc., Montgomery, TX). Fluids from individual hybridoma clone cultures were used for this isotyping.

2.9. Ethics

The research protocols for human samples were approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University and informed consent was obtained from all patients before enrollment.

3. Results

3.1. Patients demographics

In this study, the preparation of hybridomas producing HuMAbs against DENV was examined using specimens from Thai patients. PBMC samples were obtained from patients in the acute and convalescent phases. A total of nine samples from eight Thai patients were used: three patients (D30, D32, and D33) in the acute phase, ranging between 6 and 8 days after the onset of fever; and four patients (D22, D25, D27, and D28) in the convalescent phase, 12–15 days after onset of fever. Samples were collected from one patient during both the acute phase and the convalescent phase of infection [D23 (5 days after the onset of fever) and D26 (19 days after the onset of fever)] (Table 1). All acute-phase four patients, based on the results for both anti-dengue IgG and IgM, as well as RT-PCR for DENV serotyping, were the cases of secondary infection with DENV-2. For patients from whom blood samples were available for hybridoma preparation at the convalescent phase, acute-phase plasma samples were used for rapid tests. These tests were all positive for both anti-dengue IgG and IgM, indicating that these patients were also secondarily infected.

3.2. Hybridoma preparation

PBMCs from four acute-phase patients and five convalescent-phase patients were used to prepare hybridomas by fusion with SPYMEG cells, as described [10]. As summarized in Table 1 (see Supplementary Table S1 for the data on individual HuMAbs), 121 acute-phase and 15 convalescent-phase hybridomas showing stable proliferation and production of anti-DENV MAbs were obtained. Isotyping showed IgG-type in 91.7% (111/121) of HuMAbs from acute-phase cells and 86.7% (13/15) of HuMAbs from convalescent-phase cells. IgA-type was detected only in 6.6% (8/121) of HuMAbs from acute-phase cells. There were no positive cases for IgM-type. Culture fluids of four hybridoma clones did not react for IgG, IgA, or IgM.

3.3. Cross-reactivity of HuMAbs with four DENV serotypes

The HuMAbs obtained as described above were characterized for their serological reactivity to all four DENV serotypes by IF and neutralization assays. HuMAbs in the fluids of individual hybridoma cell cultures were used for these assays. As shown in Fig. 1A, the HuMAbs were classified into groups 1–10 and groups A–X based on their cross-reactivity with the four serotypes of DENV in IF and neutralization assays, respectively: group A showed no neutralization activity to any of four serotypes; groups 1–2 and groups B–E showed specific reactions with a single serotype; groups 3–6 and groups F–H showed cross-reactions with two serotypes; groups 7–9 and groups I–O showed cross-reactions with three serotypes; and group 10 and groups P–X showed cross-reactions with all four serotypes.

The IF assay revealed that 109 of 121 clones (90.1%) derived from acute-phase patients were cross-reactive with all four serotypes (Fig. 1A): 65 of 75 clones (86.7%) from D23, 23 of 25 clones (92.0%) from D30, five of five clones (100%) from D32, and 16 of 16 clones (100%) from D33 (Supplementary Table S1). By contrast, only seven of 15 clones (46.7%) derived from convalescent-phase patients were shown to be cross-reactive with all four serotypes (Fig. 1A): three of four clones (75.0%) from D22, two of five clones (40.0%) from D25, one of two clones (50.0%) from D26, one of two clones (50.0%) from D27, and neither of the two clones from D28 (0%) (Supplementary Table S1). Thus, obtaining HuMAbs cross-reactive with all four serotypes was significantly more efficient using PBMCs from acute-phase patients, as compared to convalescent-phase patients ($P = 0.008$). The IF profiles of several representative HuMAbs by IF are shown in Fig. 1B.

Next, we examined the neutralization activity of HuMAbs. The culture fluids from individual hybridoma clones were reacted with DENV-1 to -4. Under these conditions, the control 4G2 showed a $\geqslant$90% reduction in FFU compared with the negative control (DMEM with 15% FBS) in all four serotypes of DENV and, therefore, this MAb was classified into group X. On the other hand, 103 of 121 acute-phase clones (85.1%) and four of 15 convalescent-phase clones (26.7%) showed a $\geqslant$50% reduction in viral replication (Fig. 1A). A $\geqslant$90% reduction in viral replication was detected in 62 of 121 acute-phase clones (51.2%) and one of 15 convalescent-phase clones (6.7%) (Fig. 1A). A total of 70 acute-phase clones (57.9%) and one convalescent-phase clone (6.7%) showed neutralization activity (a $\geqslant$50% reduction in viral replication) against all four serotypes, while only 11 acute-phase (9.1%) and no convalescent-phase clones (0%) showed neutralization activity (a $\geqslant$90% reduction in viral replication) against all four serotypes (Fig. 1A and Supplementary Table S1).

There were inconsistencies between the IF and neutralization data regarding the four HuMAbs: one from patient D23 belonging to group 4-C (in the IF and neutralization assays, respectively), one from patient D23 belonging to group 5-K, one from patient D23 belonging to group 8-U, and one from patient D30 belonging to group 7-N (Fig. 1A).

3.4. Viral protein recognized by HuMAbs

293T cells transfected with expression vectors for the DENV-2 prM, E, NS1, and C proteins, or for the prM-E fusion protein, were used as targets for the identification of viral proteins recognized by individual HuMAbs by IF. Summarized data on viral proteins recognized by individual HuMAbs classified in groups 1–10 by IF assay and in groups A–X by neutralization assay are shown in Tables 2 and 3, respectively (the results from individual HuMAbs are shown in Supplementary Table S1). Of the acute-phase HuMAbs, 99 were reactive with E, eight with prM, four with NS1, and none with C. Culture fluid from the remaining 10 hybridoma clones

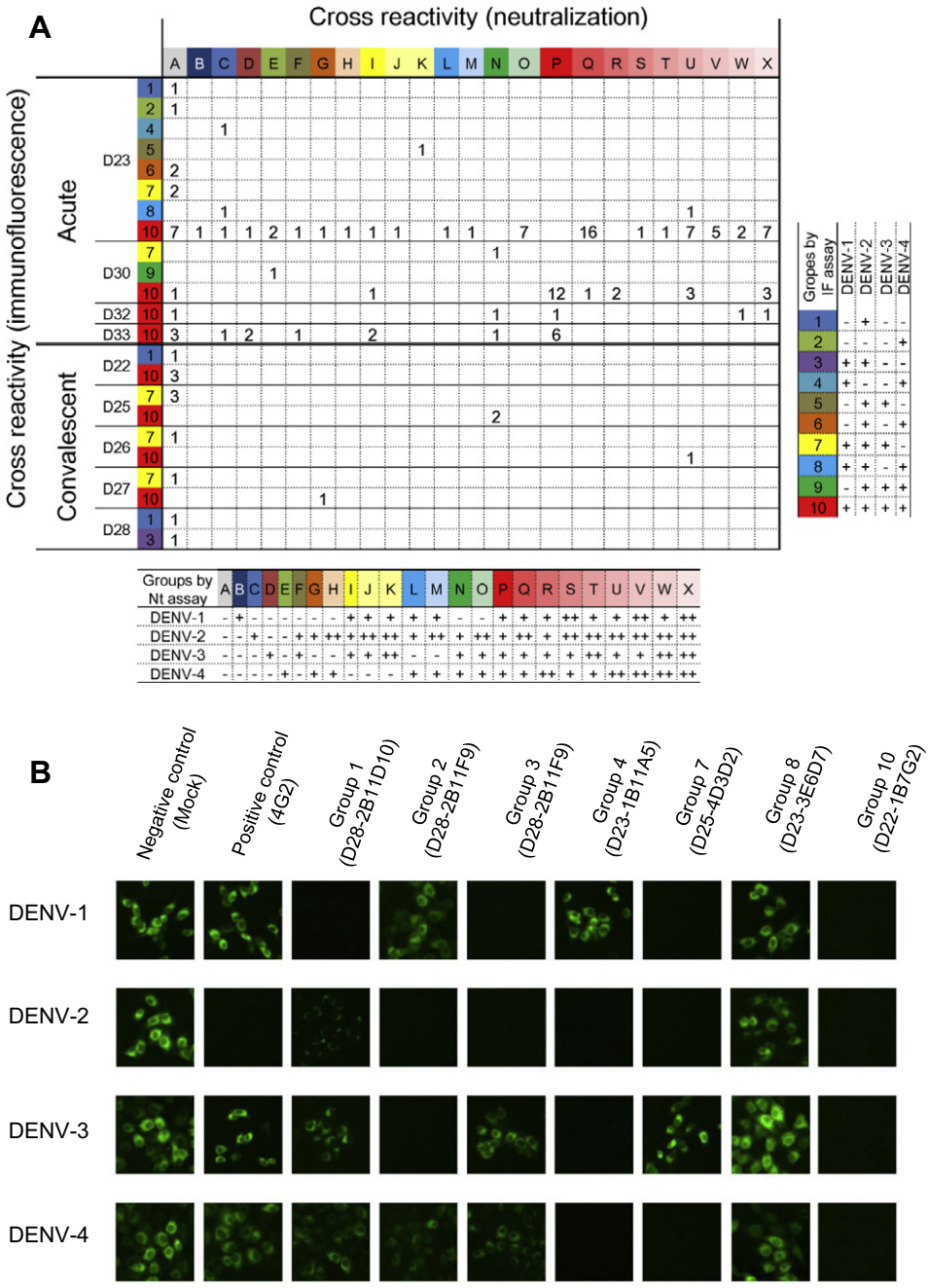

Fig. 1. Correlation between IF and neutralization assay results. (A), A total of 121 acute-phase HuMAbs and 15 convalescent-phase HuMAbs are shown separately to highlight the correlation between IF and neutralization assay ("-",<50%; "+", 50–<90%; and "++", ⩾90% neutralization) according to their cross-reactivity with different DENV serotypes (groups 1–10 according to the IF assay and groups A–X according to the neutralization assay). Culture fluids of HuMAb-producing hybridoma clones were used. Individual groups are shown by different colors. Vero cells individually infected with DENV-1–4 were used as target cells in these assays. (B), The HuMAbs in the culture fluids of hybridoma clones producing DENV serotype-specific (D28–2B11D10 in group 1 and D23–4A7D6 in group 2), cross-reactive with two serotypes (D28–2B11F9 in group 3 and D23–1B11A5 in group 4), and cross-reactive with three serotypes (D25–4D3D2 in group 7 and D23–3E6D7 in group 8), and cross-reactive with all four serotypes (D22–1B7G2 in group 10) antibodies were used for IF. Vero cells mock-infected with PBS or individually infected with DENV-1–4 were used as target cells. As a positive control, 4G2 anti-flavivirus E mouse MAb [13] was used.

was not reactive with the E, prM, NS1, or C proteins ("Other"). Of the convalescent-phase HuMAbs, two were reactive with E, two with prM, eight with NS1, and none with C, and the remaining three were not reactive with any of the proteins assayed. Interestingly, five HuMAbs obtained from D25 in the convalescent phase were all reactive against NS1 (Supplementary Table S1).

Tables 2 and 3 summarize the viral proteins recognized by HuMAbs broken down according to reactivity group. The 98 HuMAbs recognizing E (96 of 99 HuMAbs from the acute-phase and two of two HuMAbs from the convalescent-phase) were all in group 10 (cross-reactive with all four serotypes) according to the IF assay (Table 2). Of these, 70 acute-phase and one convalescent-phase HuMAbs showed ⩾50% neutralization activity against all four DENV serotypes (groups P to X). Of the 70 acute-phase HuMAbs, 11 also showed ⩾90% neutralization activity against all four DENV serotypes (group X).

Table 2
Target viral proteins of HuMAbs categorized by immunofluorescence assay results.

	DENV serotype				Immunofluorescence assay							
					HuMAb from acute phase				HuMAb from convalescent phase			
	1	2	3	4	E	prM	NS1	Other[a]	E	prM	NS1	Other[a]
1	-	+	-	-				1				2
2	-	-	-	+				1				
3	+	+	-	-							1	
4	+	-	-	+				1				
5	-	+	+	-	1							
6	-	+	-	+				2				
7	+	+	+	-			3[b]				5	
8	+	+	-	+	2							
9	-	+	+	+				1				
10	+	+	+	+	96	8	1	4	2	2	2[c]	1

[a] No reaction at least with prM-E, E, prM, and NS1.
[b] The HuMAb (D30–2B1G5) is also reactive with E weakly.
[c] The HuMAb (D25–2B11G11) is also reactive with E and prM weakly, while the HuMAb (D25–4D4F10) is also reactive with prM weakly.

Table 3
Target viral proteins categorized by neutralization assay results.

	DENV serotype				Neutralization assay							
					HuMAb from acute phase				HuMAb from convalescent phase			
	1	2	3	4	E	prM	NS1	Other[a]	E	prM	NS1	Other[a]
A[b]	-	-	-	-	5	5	3	5	1	1	6	3
B	+	-	-	-		1						
C	-	+	-	-	3			1				
D	-	-	+	-	1			2				
E	-	-	-	+		2		1				
F	-	+	+	-	2							
G	-	+	-	+	1					1		
H	-	++	-	+	1							
I	+	+	+	-	4							
J	+	++	+	-	1							
K	+	++	++	-	1							
L	+	+	-	+				1				
M	+	++	-	+	1							
N	-	+	+	+	2		1[c]				2[d]	
O	-	++	+	+	7							
P	+	+	+	+	19							
Q	+	++	+	+	17							
R	+	+	+	++	2							
S	++	++	+	+	1							
T	+	++	++	+	1							
U	+	++	+	++	11				1			
V	++	++	+	++	5							
W	+	++	++	++	3							
X	++	++	++	++	11							

[a] No reaction at least with prM-E, E, prM, and NS1.
[b] HuMAbs showing positive reactions with DENV by immunofluorescence assay, but no neutralization activity to any serotypes of DENV.
[c] The HuMAb (D30–2B1G5) is also reactive with E weakly.
[d] The HuMAb (D25–2B11G11) is also reactive with E and prM weakly, while the HuMAb (D25-4D4F10) is also reactive with prM weakly.

4. Discussion

A total of 136 hybridoma clones producing specific HuMAbs against DENV were obtained using PBMCs from nine blood samples from eight patients. The samples from the four acute-phase patients secondarily infected with DENV-2 efficiently generated hybridomas producing specific and robust HuMAbs, compared with those from the five convalescent-phase patients. In addition, most of the acute-phase HuMAb clones were cross-reactive with all four serotypes of DENV by IF. Further, most of these cross-reactive HuMAb clones recognized the viral E protein and were able to neutralize all four serotypes of DENV. Thus, humoral immune status in patients seems to be dynamically changing between the acute and convalescent phases of secondary DENV infection. Antibodies at the acute phase showed complex cross-reactivity with all four DENV serotypes, with much stronger neutralization activity not only against DENV-2, which was replicating in the patient, but also against the other serotypes of DENV.

PBMC samples in this study were collected from patients at the acute phase (5–8 days after the onset of fever) or at the convalescent phase (12–19 days for convalescent phase) of secondary infection. This study enabled us to compare the efficiency of obtaining HuMAbs at each stage. From the acute-phase PBMCs, 81.8% anti-E, 6.6% anti-prM, and 3.3% anti-NS1 HuMAbs were obtained, while 13.3% anti-E, 13.3% anti-prM, and 53.3% anti-NS1 HuMAbs were obtained from convalescent-phase PBMCs. Several groups have used PBMCs from convalescent-phase, but not acute-phase, patients to prepare HuMAbs by immortalizing patient-derived B cells with EB virus. Dejnirattisai et al. [16] observed that 89% of anti-E HuMAbs were cross-reactive with all four serotypes. Surprisingly,

their studies resulted in the preparation of more anti-prM than anti-E HuMAbs. Beltramello et al. [17] performed a large screen to gain insights into the domain specificity and cross-reactivity of E domain III-specific antibodies. A study by de Alwis et al. [18] showed that the efficiency of preparation of DENV complex cross-reactive neutralizing HuMAbs was significantly higher in secondary infection cases. Indeed, Beltramello et al. [17] differentiated their HuMAbs into two categories: those that recognized the DENV E domain III and showed complex cross-reactive neutralization activity, and those that recognized domain I/domain II and were more broadly cross-reactive but showed lower neutralization activity. Furthermore, our data in this study is the first to report the efficient preparation of HuMAbs with strong neutralization activity against all four DENV serotypes, using PBMCs from acute-phase patients secondarily infected with DENV.

It was an unexpected finding that acute-phase PBMCs were more efficient in the production of DENV-specific HuMAbs than convalescent-phase PBMCs, as neutralizing antibody titers tended to be slightly higher in convalescent-phase patients. This finding is similar to the findings of Wrammert et al. [19], who demonstrated a similar phenomenon for HuMAbs against the influenza virus in vaccinated donors. That study found a rapid and robust induction of influenza-specific IgG^+ antibody-secreting plasma cells, which accounted for up to 6% of the peripheral blood B cells at the peak of the response, approximately 7 days after vaccination. However, the influenza-specific IgG^+ memory B cells fell to an average of 1% of all B cells by 14–21 days after vaccination. Generally, reports show a difference in the B cell phenotype between acute- and convalescent-phase patients with infectious diseases [20]. Consequently, many HuMAbs showing neutralizing activity could be obtained in the acute phase. In addition, neutralization assay of the HuMAbs obtained in this study classified them into heterogeneous groups: serotype-specific HuMAbs and cross-reactive HuMAbs with two, three, and all four serotypes of DENV. These HuMAbs will also be highly useful as probes to understand the complex mechanisms through which the same antibodies mediate neutralization and ADE of heterologous DENV serotypes. Further epitope mapping studies of these HuMAbs would help shed light on this important issue.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.06.057.

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