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Characterization of two anti-dengue human monoclonal antibodies prepared from PBMCs of patients with dengue illness in Thailand

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Summary. – The global spread of the four dengue virus (DENV) serotypes (dengue-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, two monoclonal antibodies prepared by using peripheral blood mononuclear cells (PBMCs) from patients with dengue fever were characterized. Epitope mapping revealed that amino acid residues 254–278 in domain II of the viral envelope protein E were the target region of these antibodies. A database search revealed that certain sequences in this epitope region showed high conservation among the four serotypes of DENV. These two human monoclonal antibodies could neutralize DENV-2,-4 more effectively than DENV-1,-3. The amino acid sequences could not explain this difference in neutralizing activity. However, the 3D structure results showed that amino acid 274 could be the critical residue for the difference in neutralization. These results may provide basic information for the development of a dengue vaccine.

Keywords: human monoclonal antibody; epitope; dengue virus

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. An estimated 50 million cases of dengue infection occur worldwide each year.

There are four antigenically-distinct dengue virus serotypes (DENV-1 to -4), which share major antigens with each other. DENVs display antibody epitopes that are unique to each serotype and epitopes that are shared between serotypes (Wahala *et al.*, 2011). When humans are repeatedly infected with the same virus, pre-existing memory immune cells quickly produce neutralizing antibodies to protect against the current infection (Braciale *et al.*, 2006). In DENV, pre-existing neutralizing antibodies raised by the primary infection are protective against subsequent infections with the same DENV serotype (van der Schaar *et al.*, 2009). Severe dengue cases mostly occur among patients secondarily infected with different DENV serotypes (van der Schaar *et al.*, 2009). This may be due to antibody-dependent enhancement (ADE), by which the current infecting virus can use pre-existing anti-DENV antibodies raised during

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Abbreviations: ADE = antibody-dependent enhancement; DENV = dengue virus; HuMAbs = human monoclonal antibodies; IF = immunofluorescence

the primary infection to gain entry to Fc receptor-positive macrophages (Halstead and O'Rourke, 1977; Sangkawibha *et al.*, 1984). However, it is thought-provoking that most DENV infections are asymptomatic (Reiter, 2010), even among individuals secondarily infected with heterotypic DENV (Garcia *et al.*, 2010); and 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) (Harris *et al.*, 2000). Therefore, a comparative characterization of humoral immune status in DENV-infected individuals, including secondarily infected patients at the acute and convalescent phases of infection, could provide valuable information for dengue vaccine development.

We previously prepared human monoclonal antibodies (HuMAbs) using the peripheral blood mononuclear cells (PBMCs) from patients in the acute phase of secondary infection with DENV-2 (Setthapramote *et al.*, 2012). Most of them showed cross-reaction with all four serotypes both by immunofluorescence (IF) assay and viral neutralization assay (Setthapramote *et al.*, 2012). In the present study, we focused on two HuMAbs that showed much stronger neutralization against DENV-2 and -4 than against DENV-1 and -3. As is well known, dengue is an enveloped, positive-strand RNA virus that produces a spherical particle with a diameter of approximately 500Å. The viral envelope contains two integral membrane proteins designated the envelope (E) and pre-membrane (prM) proteins. The E protein binds to cellular receptors and mediates the fusion of viral and cellular membranes during viral entry into cells. The E protein is also the main target of neutralizing antibodies. The crystal structures of the E protein of several flaviviruses have been solved (Modis *et al.*, 2003, 2005; Zhang *et al.*, 2004; Kanai *et al.*, 2006; Nybakken *et al.*, 2006). Individual subunits of the E protein consist of three beta-barrel domains designated domains I (EDI), II (EDII) and III (EDIII). EDI is located in the center. EDII, an elongated domain, contains an internal fusion and dimerization domain of the E-protein. EDIII appears to be responsible for the binding to cellular receptors, since several mutations that affect receptor binding are located in this domain (Roehrig, 2003). Epitope mapping revealed that amino acid residues 254-278 in domain II of the envelope protein E were the target region for these two antibodies. A database search revealed that certain sequences in this epitope region showed high conservation among the four serotypes of DENV. Several papers have reported that potent and broadly neutralizing human monoclonal antibodies recognize the epitope that is located on Domain II of the DENV envelope protein E (Lai *et al.*, 2008; Costin *et al.*, 2013; Smith *et al.*, 2013; Rocha *et al.*, 2014). The two HuMAbs characterized here might neutralize DENV -2,-4 more effectively than DENV-1,-3. The amino acid sequences showed candidate residues that may explain the difference in the neutralization activity. Further analysis of the 3D structure results clearly showed that amino acid 274 was likely the critical residue for the observed difference in neutralization. These results may provide basic information for the development of dengue vaccine.

Materials and Methods

HuMAbs. Two HuMAbs, named 4B9H3 and 5B9C9, were prepared from the same patient by cell fusion between the PBMCs and fusion partner cells, named SPYNEG (Setthapramote *et al.*, 2012).

Viruses and cells. 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. Vero cells were maintained in a 5% CO_2 incubator at 37°C in minimum essential medium (MEM) with 10% FBS. The mosquito-derived cell line C6/36 was maintained in an incubator at 28°C in Leibovitz's L-15 medium with 10% FBS and 0.3% tryptose phosphate broth. 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 (Kurosu *et al.*, 2010).

Construction of plasmids. Truncated DENV-2 envelope protein sequences were cloned into the pFLAG-CMV™-3 Expression Vector (Sigma Aldrich). The four plasmids containing different parts of the envelope sequence were named pCMV-domain I (Dom1), pCMV-domain II (Dom2) and pCMV-domain III (Dom3) and pCMV254-278 (Fig. 1). The expression of all plasmids was confirmed by IF assay by using the anti-Flag antibody (Sigma Aldrich) after transfection into 293T cells.

IF assay. 293T cells, at 2.0×10^6 per well in a 6-well microplate, were transfected with the constructed plasmids or empty plasmid. After incubation for 24 hr, the cells were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) and permeabilized with 1% Triton X-100 in PBS. Vero cells were then incubated with undiluted hybridoma culture fluids. Anti-Flag antibody was used as a positive control. The bound antibody was visualized by reaction with an AlexaFluor 488-conjugated anti-human and anti-mouse secondary antibody (1:1000; Invitrogen).

Western blot analysis. The DENV-infected Vero cells or 293T cells transfected with different plasmids containing a truncated DENV-2 E protein were suspended for 24 hr in a loading buffer containing 2-mercaptoethanol, electrophoresed in 10% sodium dodecyl sulfate polyacrylamide gels, and then blotted onto polyvinylidene fluoride membranes (Millipore). The blots were then incubated with undiluted hybridoma culture fluid at 4°C overnight, followed by incubation with horseradish peroxidase-conjugated anti-human IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) for a further 2 hr at room temperature. The peroxidase reaction was visualized using an ECL Plus Western Blotting Detection System (GE Healthcare UK, Ltd., Little Chalfont, UK).

Neutralization assay. The viral neutralization assay was performed using hybridoma culture supernatant of individual hybridoma clones, as described previously (Okuno *et al*., 1978). Twenty-five microliters of undiluted 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 a 15 min-incubation, the mixture was used to infect Vero cells in a 96-well microplate. Following an inoculation at 37°C for 2 hr, 100 µl of MEM with 3% FBS was added. Cells were incubated at 37°C overnight, fixed with 3.7% formaldehyde in PBS and permeabilized with 1% Triton X-100 in PBS. The plate was stained with 4G2 (Falconar, 1999) at 4°C overnight, as for the IF assay. The bound antibody was visualized by further reaction with an AlexaFluor 488-conjugated anti-mouse antibody (1:1000; Invitrogen). The assays were performed in duplicates and the results expressed as averages. The neutralization activity of HuMAbs in the culture medium from hybridoma clones was expressed as "-" (<50%), "+" (50% to <90%), or "++" (≥90% reduction) in FFU, compared with the negative control.

Collection of DENV sequences corresponding to the epitope amino acid sequence. DENV sequences were downloaded from NCBI using the key words "dengue virus" [porgn:__txid12637] on Dec. 12, 2011. The BLASTx program was used to search for sequences that were corresponding among the four serotypes. The following template amino acid sequences were used for the search:

>Denv_1
GSQEGAMHTALTGATEIQTSGTTTI
>Denv_2
GSQEGAMHTALTGATEIQMSSGNLL
>Denv_3
GSQEGAMHTALTGATEIQNSGGTSI
>Denv_4
GSQEGAMHSALAGATEVDSGDGNHM

3D structure of the epitope region. The crystal structure of the DENV-2 E protein in its post-fusion form (Protein Data Bank ID code 1OK8) was used as a template for homology modeling of DENV-1, -3, and -4. The Mochizuki (DENV-1), H87 (DENV-3), and H241 (DENV-4) strains were used as the modeling sequence. The MMFF94x force field and the generalized Born (GB) solvation model were used for the force field. Default values were used for the other parameters.

Results

Construction of the plasmids and analysis of the epitope mapping

Construction of plasmids: Truncated DENV-2 envelope protein sequences were cloned into the expression vector pFLAG-CMV™-3. The four plasmids containing different parts of the envelope sequence were named pCMV-domain I, pCMV-domain II and pCMV-domain III and pCMV254-278. These plasmids were constructed from the DENV-2 strain NGC (Fig. 1). The expression of all plasmids was confirmed by IF assay by using the anti-Flag antibody after transfection into 293T cells. First, both human antibodies

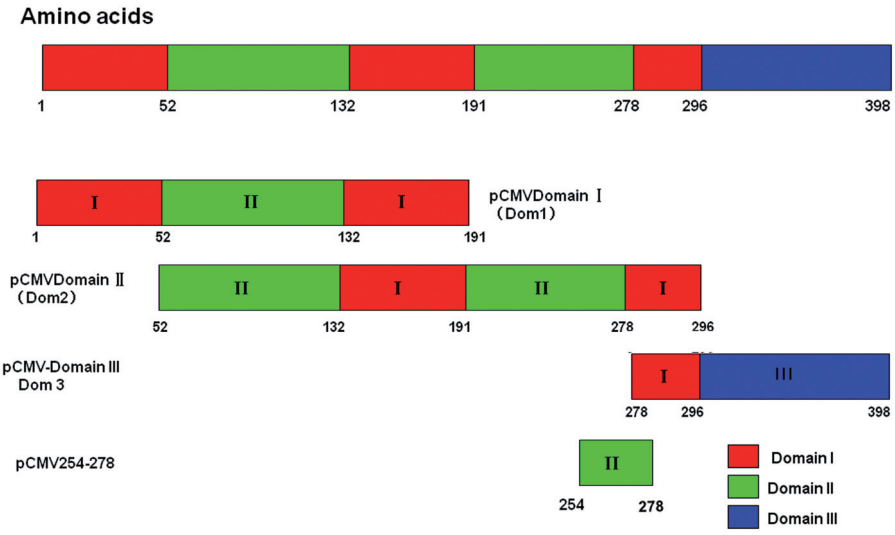

Fig. 1

Schematic representation of the DENV-2 envelope protein sequence and truncated sequence

The protein is composed of three domains (EDI, EDII and EDIII) and a steam-anchor region. The truncated expression plasmids were named pCMV-domain I (Dom1), pCMV-domain II (Dom2), pCMV-domain III (Dom3) and pCMV254-278.

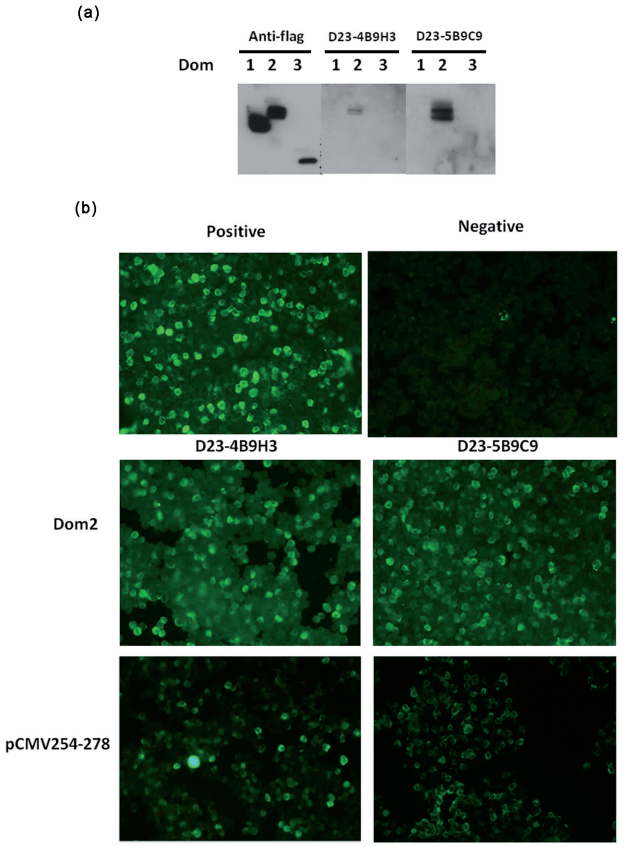

Fig. 2

Epitope localization of the two tested antibodies to region of 254–278
(a) 293T cells were transfected with plasmids Dom1, Dom2 and Dom3. After 24 hr, cells were collected and analyzed by Western blot to confirm the expression of Dom1, Dom2 and Dom3 and the epitope localization of these two antibodies in Dom 2. Anti-Flag antibody was used as control. (b) 293T cells were transfected with plasmid Dom2 and 254–278 for 24 hr, then collected and analyzed by IFA assay to localize the epitope in the region of 254–278. Anti-Flag antibody was used as a positive control.

Table 1. The IFA results of two antibodies recognized different parts of E protein

	D23-4B9H3	D23-5B9C9
Domain 1	–	–
Domain 2	+	+
Domain 3	–	–
pCMV254-278	+	+

recognized the region in Dom 2 as confirmed both by Western blotting and IFA, but not the region in Dom 1 and Dom3 (Fig. 2a and 2b, Table 1). Second, the epitope localization was found in the region of 254–278 by IF assay (Fig. 2), which revealed the positive reaction in 293T cells transfected by the plasmid pCMV254–278 (Fig. 2, Table 2). These results indicated that the epitope recognized by these two antibodies was located in the 254–278 amino acid residue region. This epitope was reported here for the first time.

Neutralizing potential of the two HuMAbs against different genotypes of DENV

The two HuMAbs were characterized for their serological reactivity to all four DENV serotypes by IF and neutralization assays. HuMAbs in the supernatant of hybridoma cell cultures were used for these assays. The supernatant of hybridoma was directly used for this assay. These two antibodies reacted with all four serotypes of DENV, but showed different levels of neutralization activity for the different serotypes of DENV, i.e., nearly 90% neutralization activity against DENV-2 and DENV-4, versus about 60% and 80% neutralization activity for DENV-1 and DENV-3, respectively (Table 2). The sequence of this region was compared among the four DENVs in order to explain the different levels of neutralization activity.

Collection of DENV sequences corresponding to the epitope amino acid sequence.

DENV sequences were downloaded from NCBI. The DENV-1 2119, DENV-2 2032, DENV-3 1184 and DENV-4 544 sequences were examined. Most of the amino acids at positions 254–278, except for the residues 272 in DENV-3 and 265 in DENV-4, were conserved within each serotype and 14 of 25 residues were conserved among all dengue serotypes. There was greater heterogeneity among different DENV serotypes at the residue 272 than at the residue 265 (T vs A). The residues at the position 272 (T, M, N, and S) were highly diverse in all serotypes (Fig. 3.) These two differences could not explain the differences in the neutralization activity between DENV-1,-3, and DENV-2,-4. On the other hand, residue 274 was Glycine and residue 276 was Threonine only in DENV-1,-3, while in DENV-2,-4 residue 276 was conserved as Asparagine. Therefore, residues 274 and/or 276 may explain the difference in the neutralization.

Table 2. Two antibodies recognized 254–278 in IF and neutralization assay for 4 types DV

	IF	IF (infected cells)				Neutralization			
	E-254-278	DV1	DV2	DV3	DV4	DV1	DV2	DV3	DV4
D23-4B9H3	+	+	+	+	+	+ 59	++ 99.4	+ 78.8	++ 91.5
D23-5B9C9	+	+	+	+	+	+ 66.7	++ 99.4	+ 79.6	+ 82.9

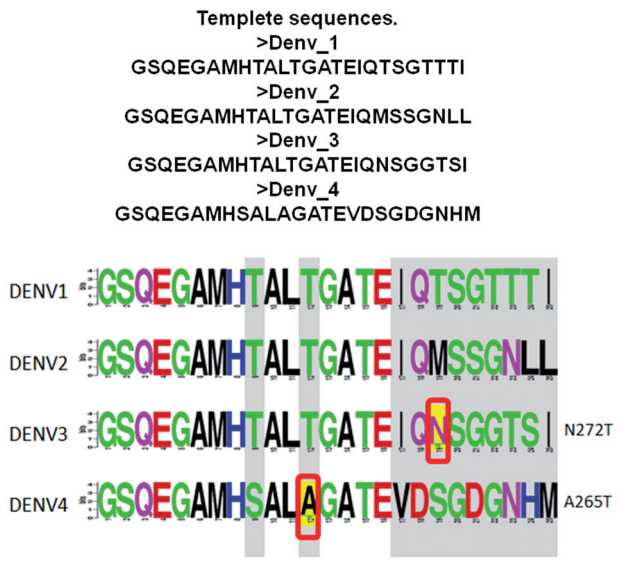

Fig. 3

Template sequence and sequence logos of the epitope regions
Sequence logos were generated using the region of aa 254–278 of 2119, 2032, 1184 and 544 DENV-1, -2, -3 and -4 sequences, respectively. Only 2 sites have high variations; all the others are conserved within each serotype. 14/25 aaare conserved among serotypes. This figure shows that most of the residues in the epitope region that are conserved at position 272 in DENV-3 and at position 265 in DENV-4 have some variation.

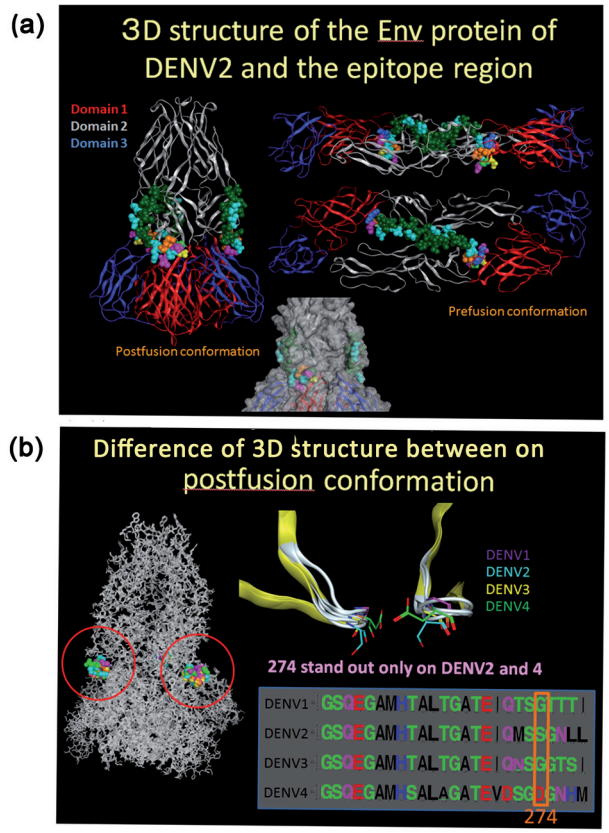

Fig. 4

3D structure of the Env protein and the epitope region
(a) Ribbon model of DENV-2 E proteins. The "before" model is obtained from 1OAN.pdb and the "after" one is obtained from 1OA8.pdb. Red is domain 1, gray is domain 2, and blue is domain 3. Atoms of the 254–278 region are represented by a space-filling model. Dark green means the amino acid was conserved among all the serotypes. Light blue means the amino acid was conserved among all the serotypes except for DENV-4. Yellow means the amino acid was conserved in only DENV-2 and DENV-4. Purple means the amino acid was conserved in only DENV-1 and DENV-3. Orange means the amino acid was not conserved among all serotypes. (b) (Left) Superposed structures of DENV1-4 E protein after fusion of the fusion complex. The amino acids at the position 274 (Glycine in DENV-1 and -3, serine is in DENV-2, and aspartic acid is DENV-4) are represented with a space-filling model and the other residues are shown in a gray stick model. (Right) Superposed structures of the DENV-1,-4 ribbon model. Only aa 274 is shown as a stick model. DENV-1 is purple, DENV-2 is light blue, DENV-3 is yellow, and DENV-4 is light green.

3D structure analyses of the epitope region

We compared tertiary structures around aa 274 and 276 prepared by homology modeling. Although the amino acid sequence shows a little difference among serotypes, most of the 3D structures are conserved in this region except for the residue 274. Residue 274 was Ser in DENV-2, and Asp in DENV-4. Ser and Asp are larger and more hydrophilic than Gly. Therefore, there are structural changes on the surface, which in turn would cause the different neutralization activities.

Discussion

The DENV complex consists of four distinct but related viruses designated as serotypes. The members of the DENV complex display antibody epitopes that are unique to each serotype as well as epitopes that are shared between serotypes. Many studies in different regions of the world have documented that individuals exposed to secondary infections are at greater risk of developing severe disease compared to individuals exposed to primary infections. The leading theory proposed to explain the increased risk of severe disease in secondary cases of ADE postulates that weakly neutralizing antibodies from the first infection bind to the second serotype and enhance infection of Fc gamma receptor-bearing myeloid cells such as monocytes and macrophages (Halstead, 2003). MAbs against DENV have served as powerful research tools for antiviral development and pathological investigations. Smith reported that the human monoclonal antibodies from transformed memory B cells from people naturally infected by dengue virus target diverse regions of the E protein, including epitopes present only on

the intact regions of the E protein with serotype-specific, but the others mostly recognized the domain I/II region containing the fusion loop with serotype-cross-neutralizing activity (Smith *et al.*, 2014). Because they used Epstein-Barr virus to transform the B cells, the antibody from the transformed B cells is difficult to be used for the clinical treatment. Here, we newly generated and characterized two HuMAbs. These two antibodies recognized the same epitope region in domain II of the E protein, region 254–278, which is not the fusion loop. This epitope has not been reported previously. The E protein is the major protein present on the surface of virus particles, and it contains several epitopes that elicit neutralizing antibodies against DENV (Henchal *et al.*, 1985). While it had previously been assumed that the E protein was the main target of antibodies, the HuMAbs indicate a more complex picture. Both E and prM are common targets of human antibodies (Dejnirattisai *et al.*, 2010; de Alwis *et al.*, 2011). The individual subunits of the E protein consist of three beta-barrel domains designated as domains I (EDI), II (EDII) and III (EDIII). ED III appears to be responsible for the binding to cellular receptors, since several mutations that affect receptor binding are located in this domain (Roehrig, 2003). In the present study, the two antibodies showed stronger neutralization activity against DENV-2 and -4 than against DENV-1 and -3. The neutralizing epitope was identified in the region 254–278 of EDII. This is the first report on this epitope located in EDII. The EDII region contains the fusion loop, and thus antibodies against this region may interfere with fusion between the virion and cell membrane. de Alwis reported that humans produce Abs that neutralize DENV infection by binding a complex, i.e., a quaternary structure epitope that is expressed only when E proteins are assembled on a virus particle. Their study indicated that the epitope has a footprint that spans adjacent E protein dimers and includes residues at the hinge between EDI and EDII. EDII may be an alternative to EDIII as a target for vaccine development (de Alwis *et al.*, 2011). The results of the 3D structure may explain the difference in neutralizing activity between the DENV-1,-3 and DENV-2,-4 serotypes. Amino acid 274 only stands out in DENV-2,-4. Amino acid 274 would thus be the critical residue that contributes to the higher neutralizing activity against DENV-2,-4 compared to DENV-1,-3. Investigation of the neutralizing epitopes on E proteins may provide a framework for a detailed understanding of both the specific mechanisms of viral infection as well as for identification of the specific DENV domain that attaches to cellular receptors. Most of the previously reported epitopes are located in EDIII and the fusion region of EDII. For example, several groups have obtained antibodies from convalescent-phase patients by immortalizing patient-derived B cells with Epstein-Barr virus (Traggiai *et al.*, 2004; Dejnirattisai *et al.*, 2010). They observed that 89% of anti-E human antibodies were cross-reactive with all four serotypes. Beltramello and colleagues (2010) performed a large screening to gain insight into the domain-specificity and cross-reactivity of EDIII-specific antibodies. In addition, based on previous epitope mapping results, several epitopes have been shown to elicit strong neutralizing response against individual flaviviruses situated in EDIII (Roehrig *et al.*, 1998; Oliphant *et al.*, 2005). However, the two antibodies reported herein recognized epitopes located in EDII and showed neutralization ability against all the serotypes of DENV, even though they neutralized DENV-2,-4 more strongly than DENV-1,-3. Most of the vaccine targets are against the regions of fusion loop, but those vaccines easily cause ADE, which may cause severe disease. Our results may provide useful information for the development of a vaccine for this region of EDII, which may prevent DENV infection without causing ADE. In fact, most vaccines being developed against DENV are based on the induction of immune responses directed to the E protein from all four serotypes (Whitehead *et al.*, 2007). In the absence of an effective dengue vaccine, neutralizing antibodies can be used as a passive immunotherapeutic strategy for treating dengue. Over the past three years, several panels of HuMAbs have been characterized by multiple groups, showing that the human response to dengue virus targets both the E and prM proteins, and comprises largely serotype cross-reactive and weakly neutralizing antibodies (Beltramello *et al.*, 2010; Dejnirattisai *et al.*, 2010; de Alwis *et al.*, 2011, 2012; Smith *et al.*, 2012). DENV infection remains a serious health threat despite the availability of supportive care in modern medicine. A previous study of mouse MAbs reported that anti-EDIII MAbs primarily block virus at the attachment step and anti EDI-II also block attachment. As far as we know, the antibody response to DENV infection consists of a major population of strongly neutralizing antibodies and a major population of cross-reactive, non-neutralizing antibodies with a potential for enhancement of virus infection and disease. So, if these antibodies only block the virus infection without causing ADE, they would be helpful for the treatment and antiviral development by using humanized monoclonal antibody. Sasaki also showed that the antibodies prepared by our method had higher neutralization activity to all serotypes and no ADE activity was observed (Sasaki *et al.*, 2013). So these HuMAbs could be one of the therapeutic candidates for treatment of DENV infection. In addition, MAbs against DENV would be a powerful research tool for antiviral development. The serotype-specific antibody could also be used for the diagnosis. Because severe disease could be caused by the secondary infection due to the presence of antibody after the first-time infection, the antibody could be used for the investigations of the pathogenesis. The new epitope and the critical amino acid in the epitope found in this study may provide new strategy to develop universal vaccine, which can neutralize different DENV serotypes without causing ADE. A further study on this epitope is needed.

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