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Dengue virus neutralization and antibody-dependent enhancement activities of human monoclonal antibodies derived from dengue patients at acute phase of secondary infection

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ABSTRACT

Public health concern about dengue diseases, caused by mosquito-borne infections with four serotypes of dengue virus (DENV-1–DENV-4), is escalating in tropical and subtropical countries. Most of the severe dengue cases occur in patients experiencing a secondary infection with a serotype that is different from the first infection. This is believed to be due to antibody-dependent enhancement (ADE), by which one DENV serotype uses pre-existing anti-DENV antibodies elicited in the primary infection to facilitate entry of a different DENV serotype into the Fc receptor-positive macrophages. Recently, we prepared a number of hybridomas producing human monoclonal antibodies (HuMAbs) by using peripheral blood lymphocytes from Thai patients at acute phase of secondary infection with DENV-2. Here, we characterized 17 HuMAbs prepared from two patients with dengue fever (DF) and one patient with dengue hemorrhagic fever (DHF) that were selected as antibodies recognizing viral envelope protein and showing higher neutralization activity to all serotypes. *In vivo* evaluation using suckling mice revealed near perfect activity to prevent mouse lethality following intracerebral DENV-2 inoculation. In a THP-1 cell assay, these HuMAbs showed ADE activities against DENV-2 at similar levels between HuMAbs derived from DF and DHF patients. However, the $F(ab')_2$ fragment of the HuMAb showed a similar virus neutralization activity as original, with no ADE activity. Thus, these HuMAbs could be one of the therapeutic candidates against DENV infection.

1. Introduction

Dengue diseases caused by mosquito-borne infections by the four serotypes of dengue virus (DENV-1 to -4) are becoming more wide-spread in tropical and subtropical regions, posing increasing global public health concerns. Epidemiologic studies suggest that severe cases dengue illness with the development of hemorrhagic plasma leakages, termed dengue hemorrhagic fever (DHF), occur mostly among patients secondarily infected with different serotypes (Sangkawibha et al., 1984; Guzmán et al., 1990; van der

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Schaar et al., 2009; Rothman, 2010). Although the pre-existing neutralizing antibodies elicited by the primary infection are protective against subsequent infections with the homotypic serotype, the antibodies cannot protect against secondary infections with heterotypic serotypes (van der Schaar et al., 2009; Midgley et al., 2011). This is proposed to be due to antibody-dependent enhancement (ADE), by which one DENV serotype uses pre-existing anti-DENV antibodies elicited in the primary infection to facilitate entry of a different DENV serotype into the Fc receptor-positive macrophages (Halstead and O'Rourke, 1977). However, most DENV infections are either asymptomatic or lead to uncomplicated dengue fever (DF) (Sabin, 1952), even among individuals secondarily infected with a heterotypic serotype (García et al., 2010); and only in 1–5% of infected cases lead to the development of DHF (Midgley et al., 2011). Thus, the ADE might not be the predominant factor to explain the mechanism for the severe cases induced in the secondary infection. An alternative hypothesis is that the neutralizing activities of anti-DENV antibodies can regulate viral infection, strongly in asymptomatic cases, but only partially in symptomatic cases.

Recently, several groups have succeeded in generating panels of DENV-specific human monoclonal antibodies (HuMAbs) (Schieffelin et al., 2010; Dejnirattisai et al., 2010; Beltramello et al., 2010; de Alwis et al., 2011, 2012; Smith et al., 2012; Teoh et al., 2012; Zou et al., 2012; Costin et al., 2013). For the most part, these were generated by EB virus immortalization of peripheral blood mononuclear cells (PBMCs) from patients at the convalescent phase of primary and/or secondary infections with DENV. The envelope (E) and pre-membrane (prM) proteins were shown to be the primary targets for the neutralizing HuMAbs (Dejnirattisai et al., 2010; de Alwis et al., 2011; Smith et al., 2012). To further examine the mechanisms of DENV-antibody-specific neutralization in both the acute and convalescent phases of DENV secondary infection, we prepared a number of hybridoma clones that produce specific HuMAbs against DENV (Setthapramote et al., 2012). More number of specific antibody-producing hybridomas was obtained using PBMCs from patients in the acute phase (121 clones from 4 patients) than those in the convalescent phase (15 clones from 5 patients). Interestingly, HuMAbs recognizing the viral E protein and showing strong neutralization of all four serotypes were only derived from the acute phase. This could be significant because these HuMAbs may be representative of the acute host immune responses that work to combat secondary infections.

In this study, to further examine the mechanisms of DENV neutralization by these acute-phase HuMAbs, we selected 17 hybridoma clones that produce HuMAbs with stronger neutralizing activities against all four serotypes.

2. Materials and methods

2.1. Cell lines and DENV

Seventeeen hybridomas producing anti-DENV HuMAbs (Table 1) were prepared by fusing patients' PBMCs with a fusion partner cell, SPYMEG (Setthapramote et al., 2012). These hybridomas were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Vero cells were cultured in minimum essential medium (MEM) with 10% FBS. C6/36 cells were cultured in a 28 °C incubator in Leibovitz's L-15 medium with 10% FBS and 0.3% tryptose phosphate broth. 293T cells were cultured in DMEM with 10% FBS. THP-1 cells were maintained in RPMI-1640 supplemented with 10% FBS.

The DENV laboratory strains used 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. In addition, recent clinical isolates for individual serotypes (a total of 20 isolates; 5 isolates per serotype) were obtained from the blood samples of Thai patients between 2007 and 2010 at the Tropical Medicine Hospital, Mahidol University. Culture supernatants from infected C6/36 were used as viral stocks. Infectivity titers were estimated according to the number of focus-forming unit (FFU) as previously (Kurosu et al., 2010).

2.2. IgG subclass detection

IgG subclasses of HuMAbs were determined by ELISA microplates (MaxiSorp; Thermo Fisher Scientific) coated with goat anti-human IgG (Jackson ImmunoResearch Laboratories), and incubated with hybridoma supernatants, then further incubated with HRP-conjugated anti-human IgG_1, IgG_2, IgG_3, or IgG_4 (SouthernBiotech, Birmingham, AL).

2.3. Sequencing of HuMAb IgG variable regions

Total RNA extracted from individual hybridoma clones using an RNeasy Mini kit (Qiagen, Tokyo, Japan) was subjected to RT-PCR using a PrimeScript RT reagent kit (Takara, Shiga, Japan) with an oligo (dT) primer, as described previously (Yasugi et al., 2013).

2.4. Purification of HuMAbs by protein G affinity column chromatography

HuMAbs were purified from hybridomas grown in serum-free medium (Hybridoma SFM, Life Technologies). IgG in the culture fluid was purified by Protein G column chromatography (HiTrap Protein G HP Columns, GE Healthcare UK Ltd., Amersham Place, Little Chalfont, England). The IgG concentration was measured using the Pierce® BCA™ Protein Assay kit (Thermo Scientific).

2.5. Preparation of $F(ab')_2$ from HuMAb IgG

$F(ab')_2$ fragment from purified HuMAb IgG was generated by $F(ab')_2$ Preparation kit (Thermo Scientific), then filtered through a syringe filter (0.2 μm).

2.6. Construction of plasmids expressing truncated forms of DENV E protein

The DENV-2 E gene of the NGC strain was amplified by one-step RT-PCR (Setthapramote et al., 2012) and ligated into the pGEM-T Easy Vector (Promega Corporation, Tokyo, Japan). Truncated forms of the DENV-2 E protein (amino acid residues 1–495, 1–394, 1–296, 1–192, and 1–132) were generated by PCR (Expand High Fidelity^{PLUS} PCR System; Roche, Mannheim, Germany). Each of the plasmids was subcloned into the expression vector pCAGGS/MCSII (Ueda et al., 2010). 293T cells were transfected with the plasmids with lipofectamine 2000 (Invitrogen Life Technologies). In addition, the gene fragment for expression of amino acid residues 52–132 of DENV-2 E protein (1st domain II) was inserted into *Escherichia coli* expression vector pET32b (Merck KGaA, Darmstadt, Germany) and site-directed mutagenic PCR (KOD-PLUS- Mutagenesis kit; TOYOBO Life Science, Tokyo, Japan), and expressed in *E. coli* BL21 (DE3) strain.

2.7. Viral neutralization (VN) assay

The VN assay was conducted as described previously (Okuno et al., 1978). Purified HuMAb with variable concentrations was mixed individually with 100 FFU of each DENV serotype. After incubation at 37 °C for 30 min, the mixture was used to infect Vero cells. After incubation at 37 °C for 2–3 days, the cells were fixed

Table 1
Characteristics of 17 HuMAbs.

HuMAb clone	Patient (gender, age)	Disease (days after illness onset)	Serological testing (DENV RT-PCR serotyping)	IgG subclass	Accession number oflgG variable region (heavy/light chains)	Epitope residues	VN_{50} (μg/ml)				ADE against DENV-2	
							DENV-1	DENV-2	DENV-3	DENV-4	HuMAbs concentration of highest enhancement (μg/ml)	Fold enhancement
D23-1A10H7	D23 (Female, 33 y)	DF (Day 5)	2nd Infection (DENV-2)	IgG_1	AB776831/AB776830	52–132	9.2	2.2	5.7	4.6	0.1	123.4
D23-1B3B9				IgG_1	AB776832/AB776833	52–132	11.0	2.6	4.8	2.6	1.0	75.8
D23-1C1G4				IgG_1	AB776857/AB776858	NI[a]	10.0	4.0	9.2	2.2	1.0	56.2
D23-1C2D2				IgG_1	AB776834/AB776835	52–132	5.6	2.7	4.2	2.7	1.0	62.4
D23-1G7C2				IgG_1	AB776836/AB776837	52–132	9.9	1.2	1.7	2.2	1.0	175.7
D23-1H5A11				IgG_1	AB776838/AB776839	52–132	5.2	1.5	5.3	1.2	1.0	81.1
D23-3A10G12				IgG_1	AB776840/AB776841	52–132	5.1	1.5	5.4	1.5	1.0	59.5
D23-4A6F9				IgG_1	AB776842/AB776843	52–132	13.9	2.5	5.5	4.4	10.0	61.2
D23-4F5E1				IgG_1	AB776846/AB776847	52–132	14.3	4.8	9.7	4.7	10.0	41.1
D23-4H12C8				IgG_1	AB776849/AB776850	52–132	10.6	5.5	11.3	8.4	1.0	33.8
D23-5G2D2				IgG_1	AB776853/AB776854	52–132	5.2	3.9	5.1	2.3	0.1	264.5
D23-5G8E3				IgG_1	AB776855/AB776856	52–132	10.6	8.4	10.5	3.1	1.0	63.1
D30-1E7B8	D30 (Female, 23 y)	DHF Grade 1 (Day 8)	2nd Infection (DENV-2)	IgG_4	AB776859/AB776860	NI	10.1	1.5	2.8	1.9	0.1	35.3
D30-3A1E2				IgG_1	AB776861/AB776862	52–132	4.8	1.4	5.6	0.9	0.1	81.2
D30-3B6C7				IgG_1	AB776863/AB776864	52–132	11.7	4.3	12.2	3.6	1.0	141.4
D32-2D1G5	D32 (Male, 19 y)	DF (Day 6)	2nd Infection (DENV-2)	IgG_1	AB776865/AB776866	NI	18.8	13.9	12.0	16.4	1.0	36.7
D32-2H8G1				IgG_1	AB776867/AB776868	NI	16.8	5.1	25.0	6.6	1.0	24.0

[a] Not identified.

with 3.7% formaldehyde in PBS and permeabilized with 1% Triton X-100 in PBS. The plate was observed by indirect immunofluorescence (IF) assay using 4G2 (Falconar, 1999), as previously (Setthapramote et al., 2012). The assays were performed in triplicate and the results were expressed as averages with standard deviation. The neutralization activity of individual plasma samples was expressed as the concentration showing a 50% reduction in FFU (referred to as the VN_{50}), compared with the negative control that was calculated by Beheren–Karber method.

2.8. ADE assay

The serially diluted purified HuMAbs were incubated with DENV at 37 °C for 30 min. Then, THP-1 cells under the condition without FBS were inoculated with the HuMAb-DENV mixture and incubated at 37 °C for 1 h. After culture at 37 °C for 3 days, total RNA was extracted from the infected cells using TRIzol® reagent (Life technologies) and was subjected to one-step real-time PCR (Modis et al., 2004). The data derived from real-time PCR were analyzed by the △△Ct analysis method (Modis et al., 2005) using GAPDH as an internal control. The assays were performed in triplicate and the results were expressed as averages with standard error.

2.9. In vivo evaluation of HuMAbs in suckling mice

Two-day-old suckling BALB/c mice (Japan SLC, Shizuoka, Japan) were intracerebrally inoculated with a 20 μl culture of a supernatant containing 20,000 FFU of DENV-2 16681 strain, which had been pre-incubated for 30 min on ice with 0.2 or 1.0 μg of purified HuMAb, or PBS as a negative control.

3. Results

3.1. Hybridoma clones producing strong neutralizing antibodies to DENV-1 to -4

We selected 17 hybridoma clones derived from three patients in the acute phase of secondary infection that produce HuMAbs with strong neutralizing antibodies to all four serotypes of DENV (Table 1): >85% inhibition of viral replication for all serotypes by antibodies in the fluids of hybridoma cell cultures (Setthapramote et al., 2012). All of the HuMAbs produced by these hybridomas were IgG_1, except for D301E7B8, which was IgG_4, and they all were shown to recognize the DENV E protein by IF using the cells transfected with DENV E protein gene-expression vector (Setthapramote et al., 2012). The variable regions of IgG in these 17 HuMAbs, including CDR1, CDR2 and CDR3, were determined and their accession numbers are shown in Table 1.

3.2. Epitope mapping of the HuMAbs

The epitope regions recognized by the 17 HuMAbs were determined by Western blotting using truncated forms of DENV-2 E protein. Plasmids expressing full-length (1–495) and truncated forms of DENV-2 E protein in 293T cells were constructed. Under the conditions that anti-FLAG reacted with all of the constructs were reacted with 13 of the 17 HuMAbs (data not shown) indicating that the region (1–132) including the 1st domain I and 1st domain II contains the epitope for these HuMAbs (Table 1). Further analysis by Western blotting using truncated form (52–132) that was expressed in *E. coli* revealed that all the 13 HuMAbs were reacted with this form, indicating that the residues 52–132 of the E protein contains the epitope for the 13 HuMAbs. The remaining four HuMAbs did not react with any of these truncated forms or with full-length (Table 1), indicating that the epitope for these four HuMAbs may be conformation-dependent.

3.3. VN activities of the HuMAbs to DENV-1 to -4

The VN_{50} titer of individual HuMAbs for the laboratory strains of DENV-1 to DENV-4 was measured using Vero cells (Fig. 1). We used 4G2 MAb (Falconar, 1999) as a positive control and 5E4 HuMAb, an IgG_1 that was prepared with the PBMCs from an influenza-vaccinated donor and shows strong neutralization against H1N1 2009 (Yasugi et al., 2013 unpublished), as a negative control. Most of the HuMAbs showed strong VN activity towards DENV-2 and DENV-4, with exception of HuMAbs showing lower to all serotypes such as D32-2D1G5; much lower to DENV-1 such as

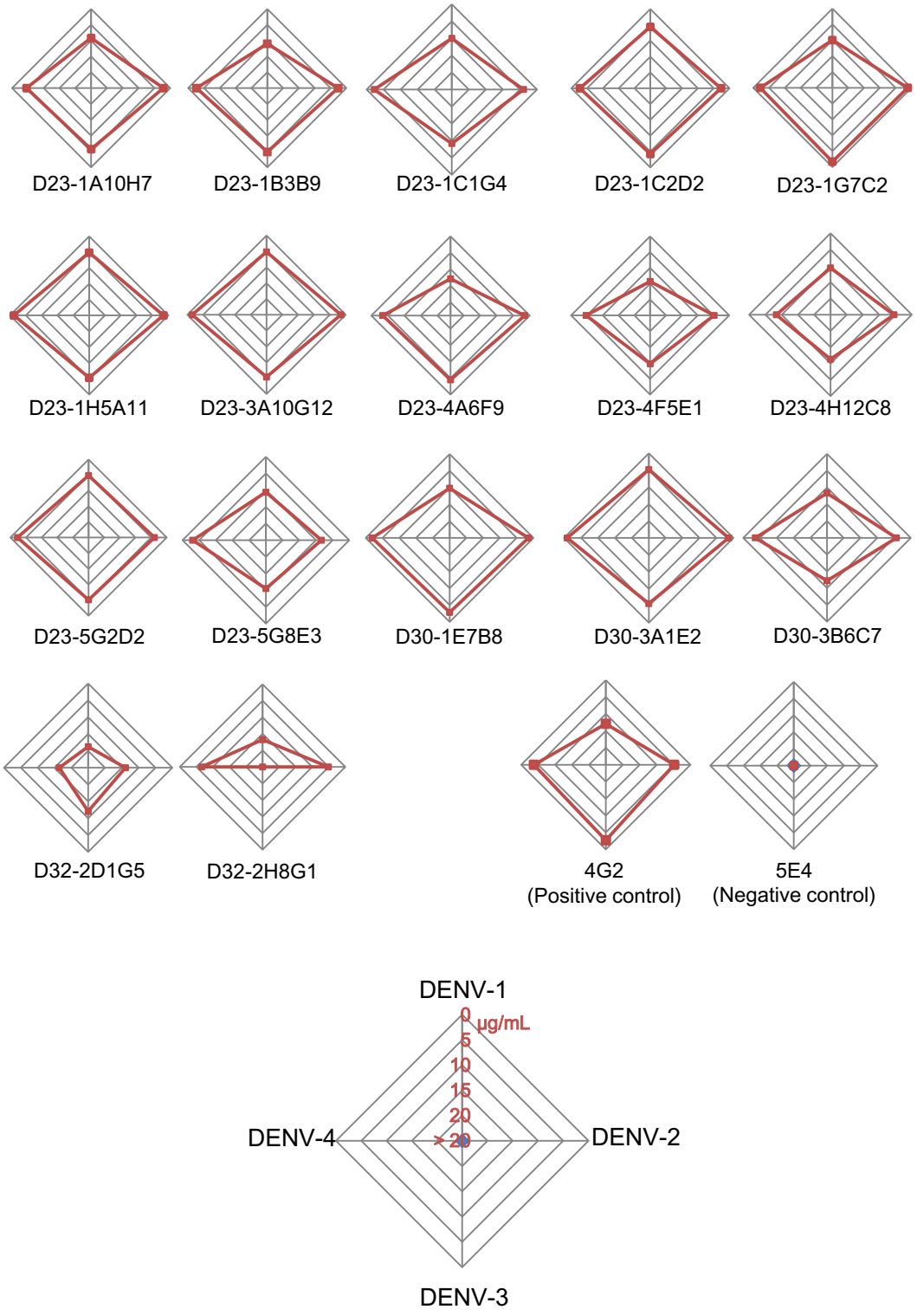

Fig. 1. VN activities of the 17 HuMAbs to four serotypes of DENV. The VN activities of the HuMAbs were performed using DENV-1 Mochizuki strain, DENV-2 16681 strain, DENV-3 H87 strain, and DENV-4 H241 strain. The VN_{50} antibody concentration is shown as the titer for neutralization of individual serotypes of DENV. 4G2 and 5E4 antibodies were used as positive and negative controls, respectively.

D23-4A6F9; and much lower to DENV-3 such as D32-2H8G1. Most of them showed stronger VN activities to DENV-1 to -4 than those of 4G2. To increase the clinical relevance of the study, we further examined the reactivities of these HuMAbs to 20 clinical isolates (five isolates per serotype) by VN_{50} assay. It was confirmed that all clinical isolates reacted well with 17 HuMAbs by indirect IF assay (data not shown). The results of the VN_{50} assay (Fig. 2) showed that three representative HuMAbs (D23-1A10H7, D23-1B3B9, and D23-1G7C2) we examined had stronger activities towards the clinical isolates than towards the laboratory strains.

Further, we next examined the *in vivo* therapeutic efficacy of the HuMAbs in neutralizing DENV in suckling mice. The mice in each group were intracerebrally inoculated with 20,000 FFUs of DENV-2, or PBS as a control, that had been incubated with

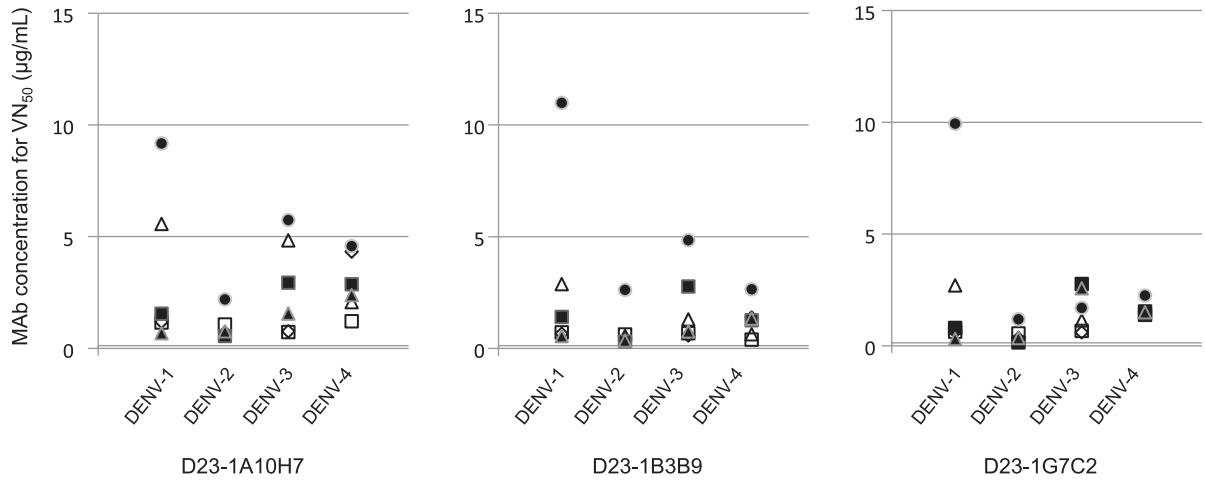

Fig. 2. VN activities of three representative HuMAbs to clinical isolates of DENV. Representative HuMAbs, D23-1A10H7, D23-1B3B9, and D23-1G7C2, were examined for the VN_{50} to a total of 20 clinical isolates (DENV-1–1 to -1–5, DENV-2–1 to -2–5, DENV-3–1 to -3–5, and DENV-4–1 to -4–5 as shown by (◇), (□), (△), (■), and (▲), respectively, that were prepared from the plasma samples from Thai patients between 2007 and 2010) and laboratory strains (●). The data of VN_{50} to the laboratory strains in four serotypes are derived from Fig 1.

D23-1B3B9 and D23-1G7C2 (1 and 0.2 μg per head) for 30 min on ice. Over an 18 day observation period, all the mock-treated mice died. In contrast, perfect survival was observed when the DENV-2 virus was incubated with HuMAb at 1 μg/ml and partial survival was observed when it was incubated with HuMAb at 0.2 μg/ml, before injection into the mouse heads (Fig. 3).

3.4. No apparent differences in the VN and ADE activities against DENV-2 between HuMAbs derived from patients with DF and those with DHF

To compare individual HuMAb activities against DENV-2, VN assay with Vero cells and ADE assay with THP-1 cells were performed. As controls, 4G2 and 5E4 were also similarly used. The results of the VN and ADE assays for individual HuMAbs are shown in Figs. 4 and 5, respectively. Most of the HuMAbs showed similar levels of VN and ADE with several exceptions, i.e., highest VN activities by D23-1G7C2 and relatively higher VN activities by D23-1H5A11, D23-3A10G12, D30-1E7B8 and D30-3A1E2 were observed, while higher ADE activities by D23-1A10H7, D23-5G2D2 and D30-3B6C7 were observed. In Table 1, HuMAb concentrations showing VN_{50} against individual DENV serotypes as well as ADE activities of these HuMAbs were summarized. Thus, there was no apparent difference in the VN and ADE activities against DENV-2 among the HuMAbs derived from patients with DF (D23 and D32) and DHF (D30) in the acute phase with DENV-2 secondary infection.

When we examined the VN and ADE activities between D23-1B3B9 IgG and derived $F(ab')_2$. The result with $F(ab')_2$ revealed the maintenance of similar VN activity, without any ADE activity (Fig. 6).

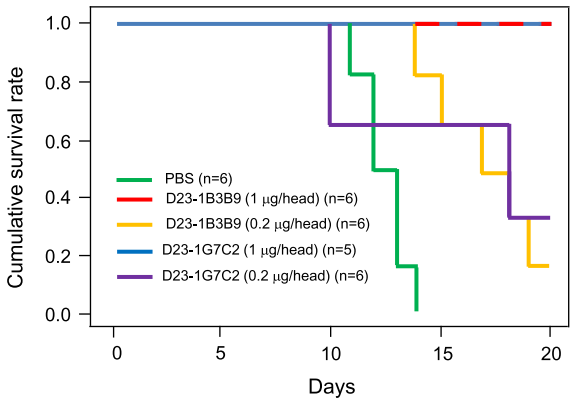

Fig. 3. *In vivo* evaluation of the protection conferred by two representative HuMAbs against death in suckling mice after intracerebral inoculation with DENV-2. BALB/c suckling mice (one-day-old) were intracerebrally inoculated with 20,000 FFU of DENV-2 (16681 strain), which had been incubated with 1.0 or 0.2 μg of purified HuMAbs, D23-1B3B9 and D23-1G7C2, or PBS as a negative control, for 30 min on ice.

4. Discussion

Seventeen HuMAbs derived from acute patients infected secondarily with DENV-2 showed similar characteristics, i.e., strong VN activity to all four serotypes of DENV. Similar levels of ADE activities were observed in most of the HuMAbs derived from DF and DHF. Epitope mapping revealed that most of the HuMAbs recognized the epitope(s) within the residues 52–132 of E protein.

The 17 HuMAbs were derived from three patients at around 1 week after the onset of illness following the secondary infection with DENV-2. No such HuMAbs with strong VN activities to all four serotypes were obtained from any of the five patients at around 2 weeks after the onset of illness by the secondary infection (Setthapramote et al., 2012). Therefore, such antibodies may be generated only transiently in the patients at the acute phase of secondary infection through rapid activation of memory B cells that had been produced during the primary infection with heterotypic serotype. Most of HuMAbs in this study showed rather stronger VN activities against DENV than 4G2 murine MAb (Falconar, 1999) we used as a control, although ADE activities were also observed in all of the HuMAbs, as in 4G2. We could not observe any significant difference in VN and/or ADE activities between HuMAbs derived from patients with DF and DHF. Further studies of HuMAbs derived from more numbers of patients with asymptomatic/mild to severe symptoms are highly important to understand the DENV pathogenesis, i.e., the possible significance of ADE *in vivo* to induce severe dengue illness.

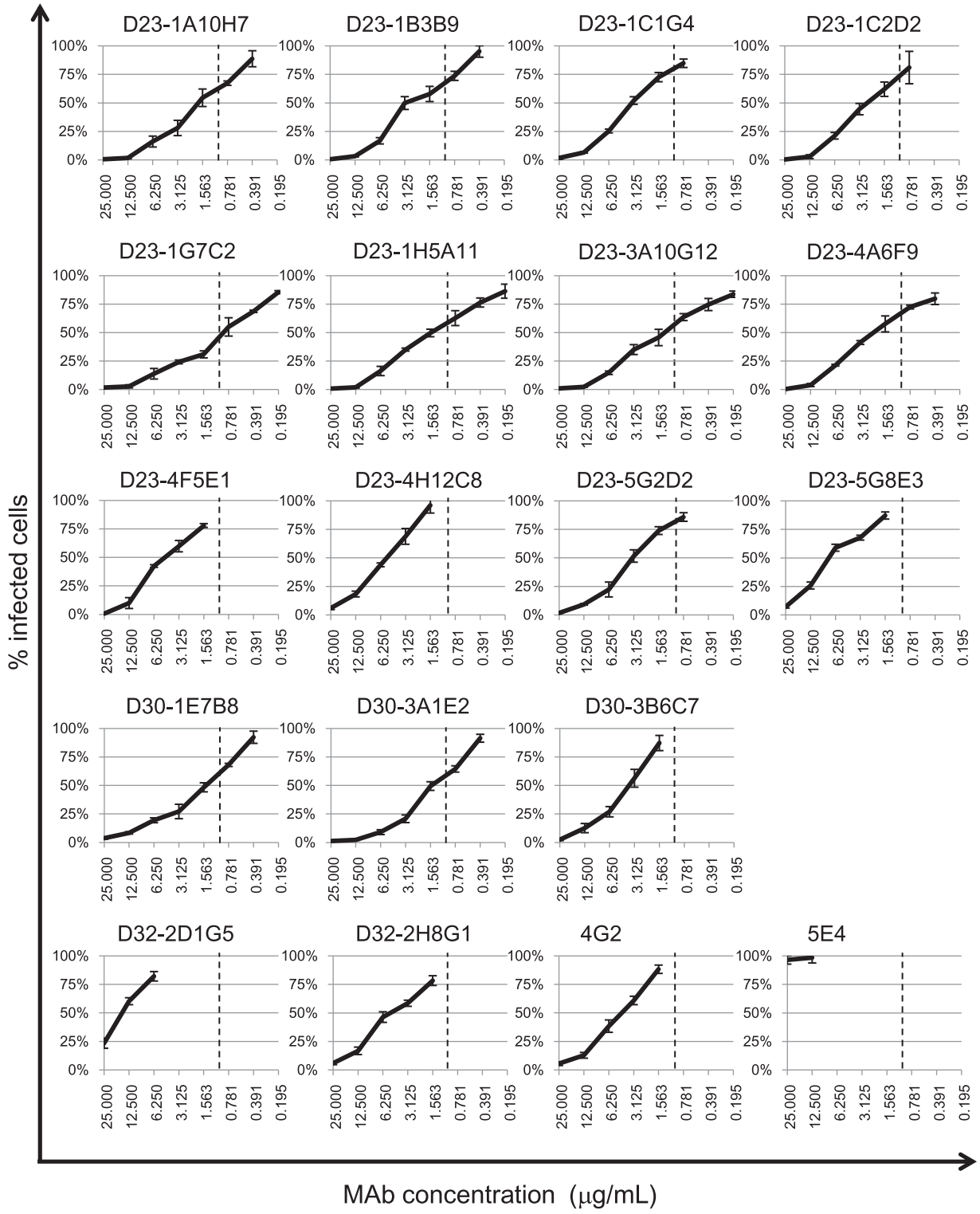

Fig. 4. VN activities of 17 HuMAbs against DENV-2. The VN activities of the HuMAbs were performed using DENV-2 16681 strain. The vertical dotted line in each figure indicates 1 μg/ml of HuMAb.

Epitope mapping revealed that at least 13 of the 17 HuMAbs recognized the residues 52–132 (1st domain II) and the other four did not, indicating that recognition of these four HuMAbs may be conformation-dependent. Very recently, Costin et al. (2013) also mapped the epitope of three broadly neutralizing anti-DENV HuMAbs (isolated from three convalescent patients with distinct histories of DENV infection) to the fusion loop within the domain II by significant reduction of these antibody binding to E proteins by mutations in this domain. Previous studies using sera from dengue patients also showed that the majority of anti-E antibodies from patients with a primary infection were mostly cross-reactive and type-specific in only a minor proportion, and that the most predominant cross-reactive anti-E antibodies recognized epitopes with highly conserved residues in the fusion loop of domain II

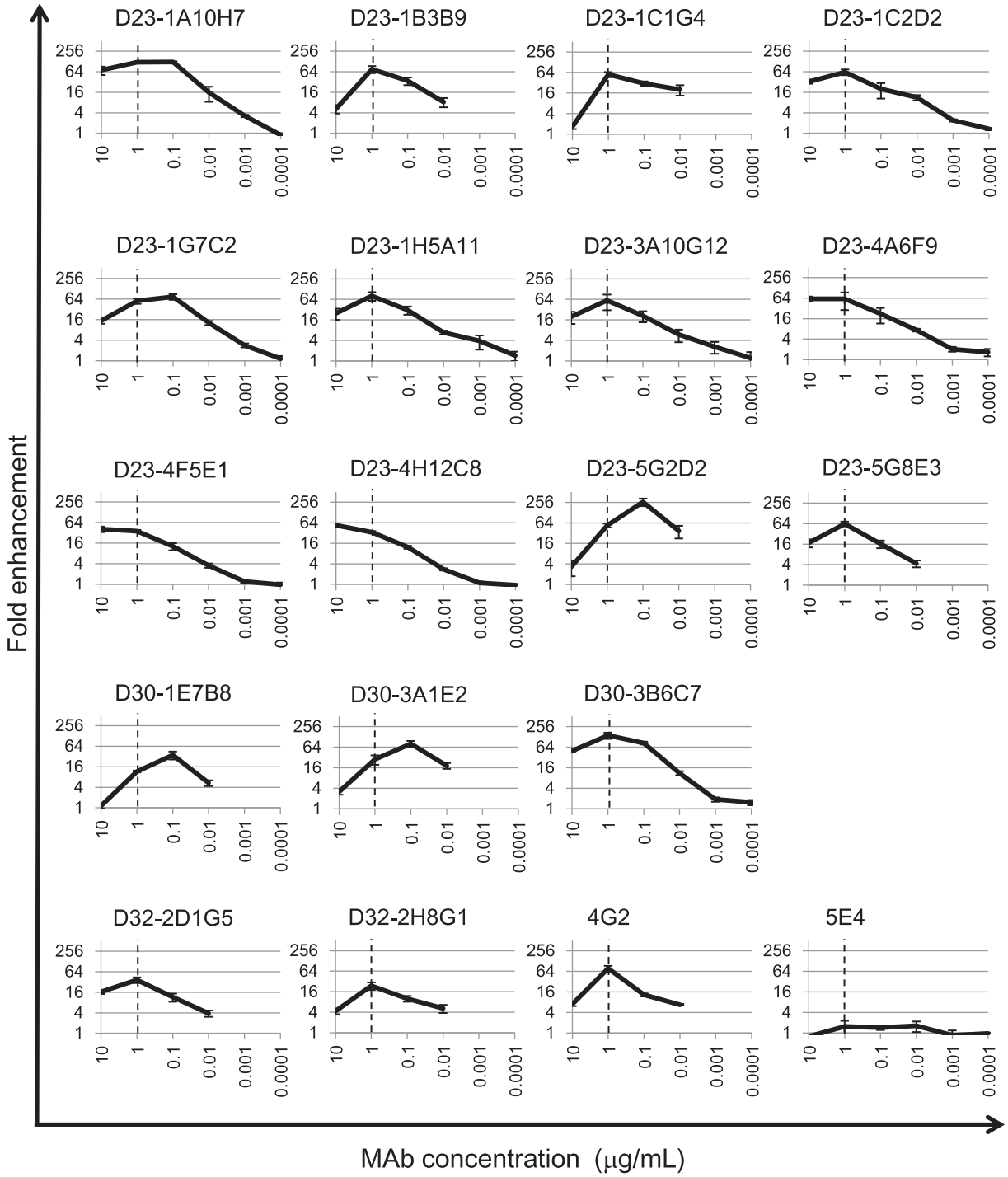

Fig. 5. ADE activities of 17 HuMAbs against DENV-2. The ADE assay was performed using THP-1 cells were infected at an MOI of 0.5 with DENV-2 (16681 strain) that had been incubated with serial 10-fold dilutions of the purified HuMAbs. Total RNA was extracted from the infected cells and used for quantitative amplification of DENV and GAPDH sequences by one-step real-time PCR. The results were analyzed by △△Ct method using Ct value of GAPDH as a control. All values of horizontal line indicate fold enhancement based on the basal level (x1) that was derived from the control (PBS). The vertical dotted line in each figure indicates 1 μg/ml of HuMAb.

(Lai et al., 2008). Thus, this region could be a central domain for the transient production of common neutralizing antibodies against DENV.

Finally, we demonstrated that the HuMAbs we examined protected suckling mice from death caused by DENV injection. Previously, murine MAbs recognizing the domain II of West Nile virus were shown to protect mice against this virus (Oliphant et al., 2006). Furthermore, a recombinant HuMAb, mAb11, was recently developed by fusing a human single-chain variable region antibody fragment to an IgG_1 Fc domain (scFvFc) against the West Nile virus E protein using a phage display library screen. mAb11 protected mice from death when injected before West Nile virus infection and afforded substantial protection when administered to mice after viral challenge (Gould et al., 2005); for this reason, it is considered a candidate for the prevention and treatment of West Nile disease. Interestingly, mAb11 cross-reacts with all four serotypes of DENV and provides similar protection against both of the serotypes examined here, DENV-2 and DENV-4 (Gould et al., 2005). Further studies are needed to characterize the positive and negative effects of our HuMAbs in *in vivo* model systems, as previously

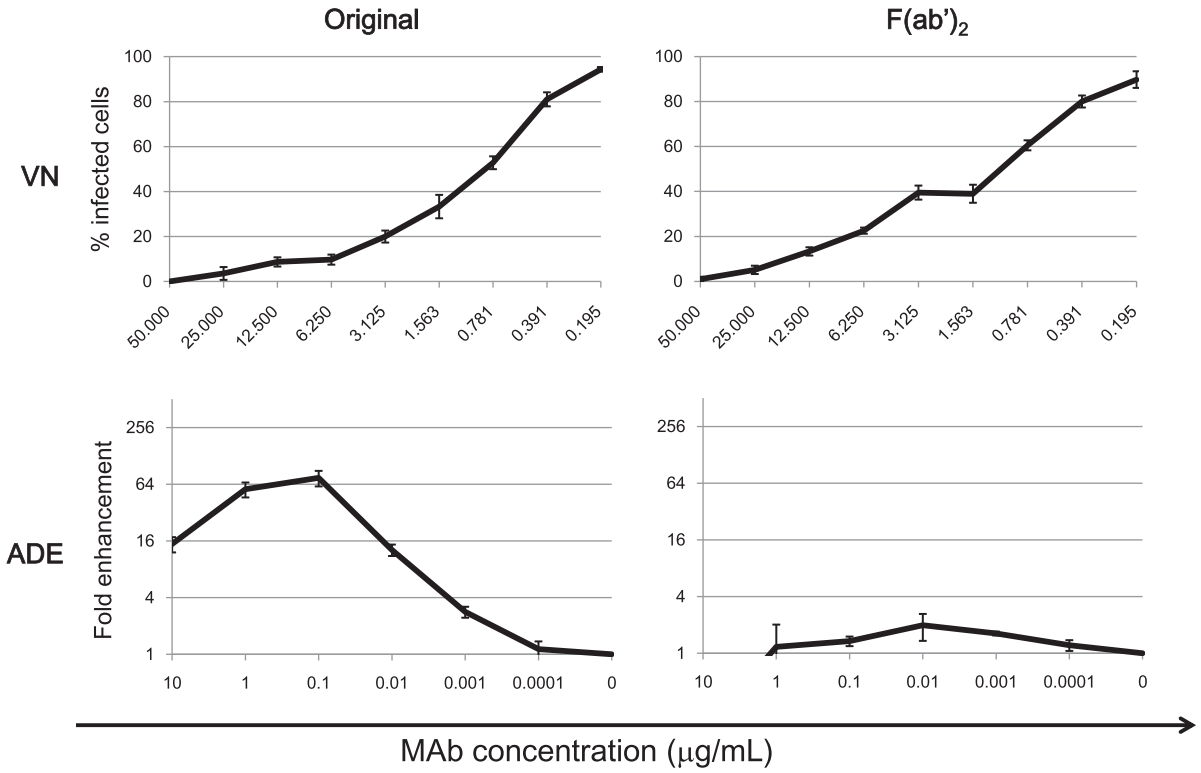

Fig. 6. Comparative VN and ADE activities between IgG and F(ab')$_2$ of HuMAb. IgG and F(ab')$_2$ of D23-1B3B9 comparatively examined for their VN and ADE activities against DENV-2 (16681 strain). F(ab')$_2$ preparation from D23-1B3B9 was performed as described in Section 2. VN and ADE assays were performed as in Figs. 4 and 5, respectively.

including macaque monkeys, marmosets, and mice (Bernardo et al., 2009; Sukupolvi-Petty et al., 2010; Omatsu et al., 2011; Zompi and Harris, 2012), before they can be considered as therapeutic antibodies against DENV.

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