



Characterization of Monoclonal Antibodies against Dengue Serotype 2

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Background: Dengue virus (DEN), the pathogen behind dengue hemorrhagic fever, remains a public health problem in Asia and South America. **Method:** Monoclonal antibodies (MAbs) specifically against dengue serotype 2 were generated by fusion of immunized mouse spleen cells with NS1/1-Ab4-1 myeloma cells. DEN-2 strain PL046, a local strain isolated from Taiwanese dengue fever patients, was used in the immunization. **Results:** The specificities of MAbs were determined by Western blot and ELISA assays. Six anti-E and twenty-one anti-NS1 MAbs were generated from mice immunized with PL046 strain, two of the six anti-E MAbs strongly inhibited infection by PL046 and 16681 strains. Ten of the twenty-one anti-NS1 MAbs were specifically against dengue serotype 2. Overall, three anti-E and twelve anti-NS1 MAbs were produced and characterized. The isotypes were also determined and described. **Conclusions:** The MAbs generated in this study may provide a powerful tool for antigenic analysis and clinical diagnosis.

Key words: dengue, monoclonal antibodies (MAbs)

INTRODUCTION

Dengue viruses (DENV) are emerging, mosquito-borne flaviviruses and are the causative agents of dengue fever (DF) and dengue hemorrhagic fever (DHF). There are approximately 50 to 100 million cases of dengue infection per year, and 2.5 billion people living in regions where dengue is endemic are at risk of infection. The dengue viruses are enveloped and contain a single, positive-sense RNA genome of about 11 kb that encodes a large polyprotein precursor. Co- and posttranslational processing give rise to three structural and seven nonstructural proteins, encoded by genes in the order C, prM, E, NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5. NS1 is a 46- to 50-kilodalton glycoprotein which is expressed in both membrane-associated (mNS1) and secreted (sNS1) forms.^{1,2} A soluble hexameric form may be released in a glycosylation-dependent fashion from infected mammalian cells but not from vector-derived mosquito cells.^{3,4}

The detection of viral antigens has been proposed, and more attention has recently been focused on nonstructur-

al protein 1 (NS1) of DV. This protein has been identified as a highly conserved glycoprotein expressed in either membrane-associated or secreted forms. It possesses not only group-specific but also type-specific determinants and has been recognized as an important immunogen in DV infections.^{5,6} Therefore, the NS1 protein might be used as a serotyping marker and an early diagnostic marker.⁷⁻¹⁰

In addition to diagnostic kit development, a large body of work has demonstrated that antibodies can neutralize or enhance DENV infection. Investigators have mainly used mouse monoclonal antibodies (MAbs) to study interactions between DENVs and antibodies. The most-potent neutralizing mouse MAbs binding to surface-exposed epitopes on domain III of the dengue envelope (E) protein, which adopts an immunoglobulin-like fold, has been argued to contain a cell surface receptor recognition site.^{11,12}

This study developed and characterized a panel of monoclonal antibodies (MAbs) to serotype-specific epitopes of NS1 from DEN-2. These serotype-specific NS1 MAbs can be employed to develop a convenient, efficient serologic test that identifies DEN serotypes.

Two neutralizing MAbs against DEN-2 were generated in this study. The neutralizing MAbs of DEN-2 may be useful for studying the mechanism of viral entry and may provide information for the development of vaccines.

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METHODS

Cells and viruses. DEN-2 strain PL046, a local Taiwanese strain isolated from DF patients was provided by the National Institute of Preventive Medicine, Taiwan, Republic of China. Four prototype DEN strains DEN-1 (Hawaii), DEN-2 (New Guinea C), DEN-3 (H87) and DEN-4 (H241) were provided by D. J. Gobbler (Centers for Disease Control and Prevention, Fort Collins, USA). These viruses were passaged in *Aedes albopictus* C6/36 cells and grown in RPMI 1640 medium containing 10% heat-inactivated FBS. BHK-21 cells were grown in RPMI 1640 medium containing 5% heat-inactivated FBS.

Animals. BALB/c mice used for all the experiments were bred and maintained at the Institute's Animal Housing Facility. Eight-week-old BALB/c female mice were purchased from the National Laboratory Animal Center. The experiments using animals were licensed by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Generation of serotype-specific MAbs against DEN-2. Hybridoma secreting anti-DEN-2 antibodies were generated according to standard procedures (Kohler & Milstein, 1975). Briefly, the spleen of an immunized mouse was removed. Splenocytes were fused with NSI/1-Ab4-1 (NSI) myeloma cells and washed twice with RPMI. Fused cells were then mixed in a 15-ml conical tube and 1 ml 50% (v/v) PEG (invitrogen) was added over 1 min with gentle stirring. The mixture was diluted twice by slow addition (1 min) of 1 ml RPMI, followed by slow addition (2 min) of 8 ml serum-free RPMI. The mixture was then centrifuged at 400 g for 5 min. The fused cell pellet was re-suspended in RPMI supplemented with 15% FBS, HAT medium and hybridoma factor (ICN). Next, 150 μ l/well of resuspension mixture was distributed to 96-well tissue culture plates. Hybridoma colonies were screened by ELISA for MAbs that bound DEN-infected C6/36 cells. Selected clones were sub-cloned by limiting dilution. Final hybridoma clones were isotyped using an isotyping kit from Roche Diagnostics. Ascitic fluids were produced in pristane-primed BALB/c mice. Hybridoma cell lines were grown in RPMI 1640 medium with 10% heat-inactivated FBS. MAbs were purified by protein G-sepharose gel. ELISA and Western blot assays were employed to measure the activity and specificity of the antibodies isolated.

Preparation of DENV antigen. C6/36 were infected with DENVs, pelleted and washed three times with PBS. Cells were then lysed in lysis buffer (25 mM Tris/HCl,

pH 7.4, 150 mM NaCl and 1% Nonidet-P40) in the presence of protease inhibitors (1mM EDTA, 0.1mM PMSF, 10 μ M leupeptin, 10 μ M chymostatin and 1 μ M pepstatin). Cell debris was removed by centrifugation at 3000 g for 10 min at 4°C and protein was quantified using the protein dye-binding method described by Bradford (1976).

ELISA. ELISA revealed C6/36 cells to be infected with DEN-1, -2, -3, and -4. Infected cells were fixed with -20°C methanol/acetone (1:1). After washing with PBS-0.1% Tween 20 (PBST), wells were blocked with 250 μ l of PBS-5% skimmed milk for 1 h at 37°C. The wells were washed and incubated with MAbs against DEN, normal mouse IgG (NM-IgG), and normal mouse serum (NMS) for 1 h at 37°C. The plate were then washed and a 1:5000 dilution of goat anti mouse IgG (H+L) conjugated HRP (Jackson ImmunoResearch Laboratories) was added and incubated for 1 h at 37°C. After further washing, 100 μ l/well of TMB solution was added, and the reaction was stopped after incubation for 10 min with 1 N sulfuric acid, and absorbance was read at 450 nm in a microplate autoreader (Amersham Pharmacia Biotech).

Western blot analysis. Cell lysates or proteins were mixed with equal volumes of sample buffer (50 mM Tris/HCl, PH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue and 10% glycerol), separated by SDS-PAGE and transferred to nitrocellulose membrane (Hybond-C Super, GE Healthcare). Non-specific antibody-binding sites were blocked with 5% skimmed milk in PBS and membranes were incubated with primary antibody. Blots were then treated with HRP-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) and developed with chemiluminescence reagents (ECL, GE Healthcare).

Indirect immunofluorescence assay. BHK-21 cells were mock-infected or infected with DEN-2. Cells were then fixed with -20°C methanol/acetone (1:1) for 10 min and washed three times with PBS. Cells were incubated with 100-fold diluted MAbs or normal ascitic fluid. After 60 min of incubation, cells were washed three times with PBST (PBS plus 0.5% (w/v) Tween-20) for 5 min each time. Cells were then treated with FITC-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories) with 200-fold diluted for 30 min, washed four times with PBST for 5 min each time and mounted for fluorescent microscopic observation.

Plaque reduction neutralization test (PRNT). MAbs were diluted with serum-free RPMI, mixed with an equal volume of virus suspension, and incubated for 1 h at 37°C. The antibody-virus mixture was incubated in duplicate with BHK-21 cells in six-well plates. MAbs

1A

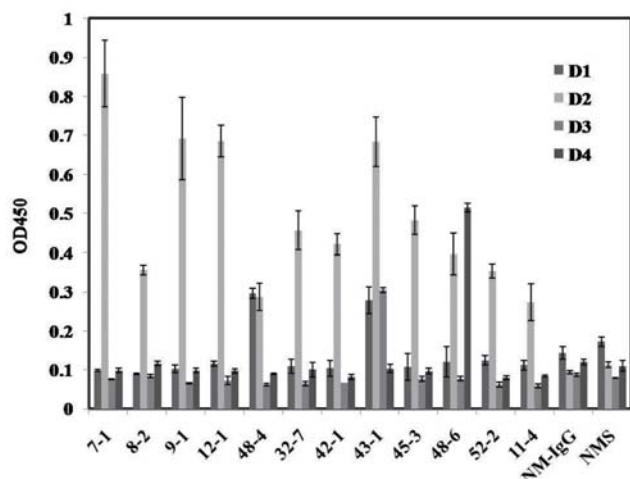


Fig. 1A Identification of serotype-specific MAbs of DEN-2 by ELISA that bound DEN-infected C6/36 cells. Only DEN-2-infected C6/36 cells could be detected by DEN-2 serotype-specific MAbs but not DEN-1, -3 and -4-infected cells. Normal mouse IgG (NM-IgG) and normal mouse serum (NMS) had no such reactivity against four serotypes of DEN. Bars are means of triplicate determinations. Error bars indicate SD.

were tested using ten-fold dilution starting at 1:100. After adsorption of virus for 2 h, 4 ml of medium (RPMI containing 2% FBS, antibiotic, and 1% agarose) was added to each well. Plates were incubated in 5% CO₂ at 37°C for 5 to 7 days. Plaque-forming units were visualized by staining with 5% crystal violet. Each MAb was tested in duplicate and the number of plaque-forming units was recorded as the average of the numbers observed in the two cultures.

RESULTS

Generation of mouse monoclonal antibodies

For production of MAbs, mice were immunized with DEN-2 PL046 strain. After thrice immunization, the mouse that presented the highest antibody titer to the immunogen was selected and given a booster injection so that its spleen cells could be used in cell fusion. All antibodies generated by the hybridomas were initially screened with ELISA. Positive hybridoma supernatants were stably subcloned and used for further characterization. In this study, six anti-E and twenty-one anti-NS1 MAbs were generated.

1B

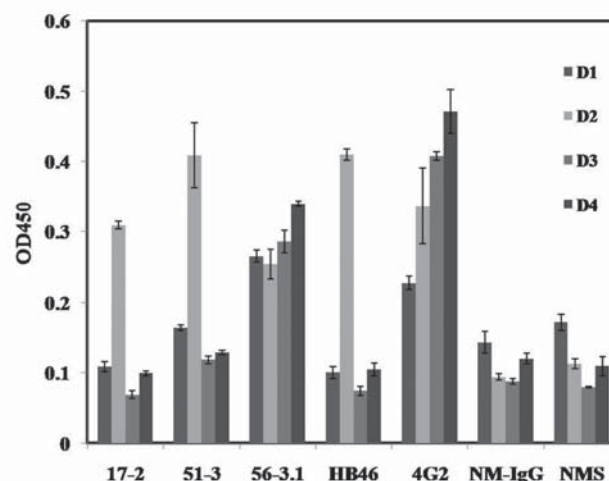


Fig. 1B Identification of serotype-specific MAbs of DEN-2 by ELISA that bound four serotypes of DEN-infected C6/36 cells. Normal mouse IgG (NM-IgG) and normal mouse serum (NMS) had no such reactivity against four serotypes of DEN. Bars are means of triplicate determinations. Error bars indicate SD.

Characterization of antibodies by ELISA

ELISA revealed C6/36 cells to be infected with DEN-1, -2, -3, and -4. Infected cells were fixed and incubated with MAbs against DEN, normal mouse IgG (NM-IgG), and normal mouse serum (NMS). ELISA confirmed the specificity of MAbs (Fig. 1A and Fig. 1B). D₂ 17-2, D₂ 51-3, D₂ 7-1, D₂ 8-2, D₂ 9-1, D₂ 12-1, D₂ 32-7, D₂ 42-1, D₂ 43-1, D₂ 45-3, and D₂ 11-4 specifically detected DEN-2-infected cells but not DEN-1, -3, -4-infected cells. D₂ 48-4 reacted with DEN-1 and DEN-2, while D₂ 48-6 reacted with DEN-2 and DEN-4 (Fig. 1A). D₂ 51-3 and D₂ 17-2 detected specifically DEN-2-infected cells but D₂ 56-3.1 recognized all four serotypes of DEN (Fig. 1B). Normal mouse IgG (NM-IgG) and normal mouse serum (NMS) had no such reactivity with four serotypes of DEN.

Western blot analysis

The MAbs obtained were tested to analyze their specificity for immunoblot application. This study generated both group- and serotype-specific MAbs. The specificity of MAbs to E and NS1 proteins were determined by western blotting. D₂ 17-2 and D₂ 51-3 reacted only with E proteins of DEN-2, D₂ 56-3.1 reacted with all four serotypes of DEN (Fig. 2A). D₂ 7-1, D₂ 8-2, D₂ 9-1, D₂ 12-1, D₂ 32-7, D₂ 42-1, D₂ 43-1, D₂ 45-3, D₂ 48-6, and

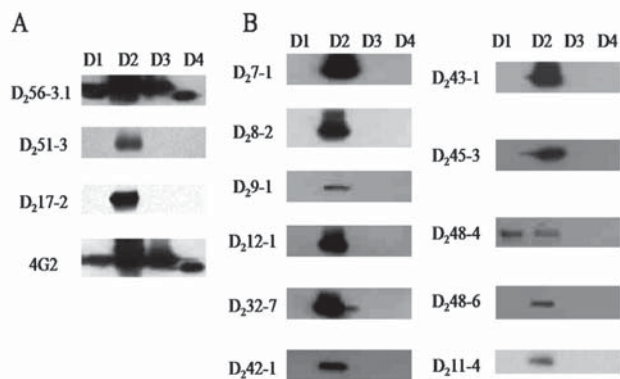


Fig. 2 Identification of MAbs against E and NS1 proteins of DEN by immunoblot analysis. Four serotypes of DEN antigens (D1 to D4) from DEN-infected C6/36 cell lysates were size-fractionated in polyacrylamide gels. The blots were incubated with MAbs. (A). MAbs D₂ 56-3.1, D₂ 51-3, D₂ 17-2, and 4G2, recognizing E proteins (55 kDa) of DEN, were identified by immunoblot analysis using a nonreducing gel. (B). MAbs D₂ 7-1, D₂ 8-2, D₂ 9-1, D₂ 12-1, D₂ 32-7, D₂ 42-1, D₂ 43-1, D₂ 45-3, D₂ 48-4, D₂ 48-6, D₂ 52-2, and D₂ 11-4 against the dimeric form of NS1 proteins (75 kDa) of DEN were identified by immunoblot analysis using a nonreducing gel.

D₂ 11-4 reacted specifically with NS1 proteins of DEN-2, while D₂ 48-4 reacted with NS1 proteins of DEN-1 and DEN-2 (Fig. 2B). The characteristics of MAbs and the classes and subclasses of these MAbs were summarized in Table 1. A majority of the MAbs are of IgG isotype.

Characterization of antibodies by immunofluorescence staining

Whether the generated MAbs against E protein could be available for the application of immunofluorescence staining was assessed. BHK-21 cells were infected by DEN-2 PL046 virus, fixed with -20°C methanol/acetone (1:1), and incubated with D₂ 17-2, D₂ 51-3, D₂ 56-3.1, 4G2, and normal mouse IgG (NM-IgG), as described in the Methods section. Immunofluorescence staining using MAbs generated against E protein showed that the antibodies worked very well in this application (Fig. 3).

Characterization of neutralizing MAbs

(a) Neutralizing potential of MAbs against different DEN-2 virus

To evaluate this, the inhibitory activity of four MAbs against two DEN-2 virus was assessed semi-quantitatively by a standard plaque reduction assay with 10² PFU

Table 1 Summary of anti-DEN MAbs

Hybridoma cell strains	Isotypes	Western blot results				Specificity
		DEN-1	DEN-2	DEN-3	DEN-4	
D ₂ 17-2	IgG1	—	+	—	—	E
D ₂ 51-3	IgG2b	—	+	—	—	E
D ₂ 56-3.1	IgG1	+	+	+	+	E
D ₂ 7-1	IgG2a	—	+	—	—	NS1
D ₂ 8-2	IgG1	—	+	—	—	NS1
D ₂ 9-1	IgG2a	—	+	—	—	NS1
D ₂ 12-1	IgG2a	—	+	—	—	NS1
D ₂ 48-4	IgG1	+	+	—	—	NS1
D ₂ 32-7	IgG1	—	+	—	—	NS1
D ₂ 42-1	IgG1	—	+	—	—	NS1
D ₂ 43-1	IgG1	—	+	—	—	NS1
D ₂ 45-1	IgG1	—	+	—	—	NS1
D ₂ 48-6	IgG1	—	+	—	+	NS1
D ₂ 52-2	IgG1	±	+	—	—	NS1
D ₂ 11-4	IgG3	—	+	—	+	NS1

of virus in BHK-21 cells. A single dose (25 μ g/ml) of neutralizing MAbs against DENV-2 was evaluated by single endpoint plaque reduction assay on BHK-21. Two MAbs (D₂ 17-2 and D₂ 56-3.1) had 100% neutralizing activity but MAb D₂ 51-3 had no neutralizing activity even at the high (~25 μ g/ml) concentration of MAbs used in the assay (Table 2).

(b) PRNT₅₀

Plaque-reduction neutralization endpoints were calculated using probit analysis as described previously.¹³ Briefly, a reduction in plaque count of 50% (PRNT₅₀) was used as the neutralizing endpoint. Plaques generated by test MAbs at varying dilutions and the control preparation were counted. The percentage of plaques counted in test MAbs were compared with that in the control preparation. Log dilutions of the test MAb preparations were plotted along the X axis, whereas percent reduction in plaque count was detailed along the Y axis.¹⁴ To more rigorously characterize the neutralizing potency, MAbs were purified and their inhibitory activity against DEN-2 16681 and DEN-2 PL046 strains in cell culture was assessed by performing a dose-response curve and determining the concentration of MAbs (PRNT₅₀, expressed here as μ g/ml of antibodies) that blocked plaque formation by 50% (Table 3). MAb D₂ 56-3.1 potently neutralized infection with PRNT₅₀ values below 0.035 μ g/ml. This value is significant as it is lower than MAb 4G2 (PRNT₅₀ value of ~0.51 μ g/ml).

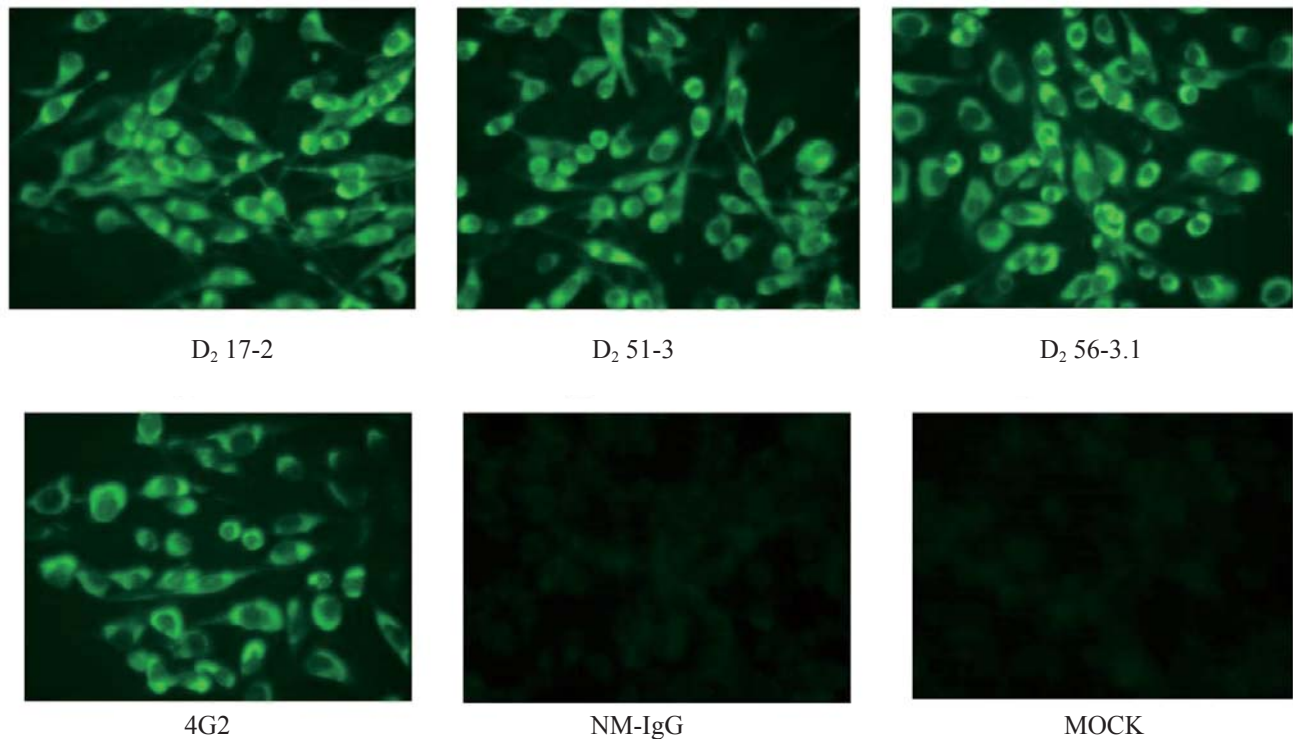


Fig. 3 Immunofluorescence staining analysis. DEN-2 virus was infected to BHK21 cells and stained with each indicated MAb ascitic fluid generated against E proteins. 4G2 was the positive control and normal mouse IgG (NM-IgG) was the negative control.

Table 2 MAb neutralization of different DEN-2 viruses

MAbs	Neutralization (%)	
	16681	PL046
D ₂ 17-2	100	100
D ₂ 51-3	0	0
D ₂ 56-3.1	100	100
4G2	100	100

Neutralization activity was determined semi-quantitatively by single endpoint plaque reduction assay on BHK-21 with purified MAbs (25 µg/ml) and 10² PFU of the indicated DEN-2 virus. The data were derived from two independent assays performed in duplicate.

DISCUSSION

One the most powerful tools in the revelation of protein properties represent the use of MAbs, which have been successfully applied to antigenic analysis, clinical diagnosis, and treatments.^{15,16} This study developed sev-

Table 3 PRNT values of MAbs against DEN-2 strain

MAbs	PRNT ₅₀ (µg/ml)	
	16681	D ₂ 17-2
D ₂ 17-2	0.5779	D ₂ 17-2
D ₂ 56-3.1	0.0245	D ₂ 56-3.1
4G2	0.51	4G2

Neutralization activity was determined by plaque reduction assay on BHK-21 with increasing concentrations of purified MAbs and 10² PFU of the indicated DEN-2 virus. The data were derived from two independent experiments performed in duplicate.

eral mouse lymphocyte hybridoma clones which produce antibodies that bind to dengue serotype 2 antigen. These MAbs were further characterized for their specific reactivity against all four dengue serotypes by various methods. Six anti-E and twenty-one anti-NS1 MAbs were generated from mice immunized with PL046 strain. Ten of the twenty-one anti-NS1 MAbs were specifically

against dengue serotype 2. Two of the six anti-E MAbs strongly inhibited infection by PL046 and 16681 strains. The isotyping study revealed that all fifty MAbs were of IgG type.

NS1 is a relatively conserved 45-50kDa glycoprotein that is highly expressed in DENV-infected cells. The production of epitope-specific MAbs holds potential for development of either group- or serotype-specific NS1 antigen assays.^{17,18} The detection of NS1 protein has recently become a promising diagnosis assay for the early stage of dengue infection.^{7,8} This study selected ten anti-NS1 MAbs highly specific for dengue serotype 2. According to the results, anti-NS1 MAbs can be employed to develop a rapid field-based immunoassay for routine surveillance of dengue-infected case.

The E protein consists of three structural domains (D), DI, DII, and DIII. At one end of the molecule is the fusion loop within DII, and at the other end is DIII, which is involved in host cell binding.^{16,19} Several epitopes of MAbs against DENV E protein have been reported, but the possibility of interdomain epitopes and the relationship between epitopes and neutralizing potency remain largely unexplored. In this study, the neutralizing potential of MAb D₂ 56-3.1 (PRNT₅₀ value $\leq 0.035 \mu\text{g/ml}$) is significantly higher than that of MAb 4G2 (PRNT₅₀ value of $\sim 0.51 \mu\text{g/ml}$). Hence, further investigation on MAb D₂ 56-3.1 may provide information on the mechanism of viral entry.

In this study, three anti-E and twelve anti-NS1 MAbs of dengue fever were characterized, demonstrating that ten of the twenty-one anti-NS1 MAbs were highly specific for dengue serotype 2 and two of the six anti-E MAbs were neutralizing MAbs. The MAbs generated in this study may be useful for clinical application in the diagnosis and therapeutic control of dengue fever.

DISCLOSURE

The authors have no conflict of interest to declare.

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