Studies on functional properties of monoclonal antibodies elicited by DENV EDIII recombinant proteins

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ABSTRACT

Studies on functional properties of monoclonal antibodies elicited by DENV EDIII recombinant proteins

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Approximately 400 million people worldwide are infected annually with dengue virus (DENV). DENV includes four serotypes (1-4). Most DENV infections are asymptomatic, but sometimes severe manifestations, such as dengue hemorrhagic fever (DHF), dengue fever (DF), and dengue shock syndrome (DSS), occur during secondary heterologous infections, due to antibody dependent enhancement (ADE). With the acceleration of global warming, the number of mosquitoes is increasing worldwide. There is no therapeutic agent for DENV, and the recently approved vaccine has limitations due to side effects. Therefore, it is necessary to develop an efficient tetravalent vaccine.

In this study, the functional properties of the monoclonal antibodies (mAbs) elicited by soluble DENV E protein domain III (EDIII) recombinant proteins

were identified. The soluble human RNA-interacting domain (hRID)-DENV EDIII recombinant fusion proteins were expressed as antigens in *E. coli*, and then 29 DENV EDIII mAbs against the four serotypes of DENV were generated. DENV EDIII overlapping peptides were synthesized, and mAb binding activity was confirmed via enzyme-linked immunosorbent assay. Neutralizing activity of the mAbs was then tested using a microneutralization test. Binding activity and neutralizing activity occurred independently of each other. Although binding activity was not shown, neutralizing activity still occurred for various reasons. We confirmed that the interaction between mAb 8G6 and peptides 2-12 and 2-13 represented potential neutralizing activity and binding activity. Furthermore, mAb 12D7 exhibited broad-spectrum antiviral activity.

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Introduction

Dengue virus (DENV) is a flavivirus, a group of viruses that includes yellow fever virus (YFV), tick-borne encephalitis virus (TBEV), Zika virus (ZIKV), and West Nile virus (WNV) [1]. DENV is an arbovirus transmitted by mosquitoes such as *Aedes aegypti* and *Aedes albopictus*. Annually, over 400 million people are infected with DENV in tropical and subtropical countries [2]. Most DENV infections are asymptomatic, but severe manifestations, including dengue hemorrhagic fever (DHF), dengue fever (DF), and dengue shock syndrome (DSS), sometimes occur during secondary heterologous infections.

DENV is an enveloped, positive single-stranded RNA that codes for three structural proteins (capsid protein (C), membrane protein (M), and envelope protein (E)) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). There are four serotypes of DENV (1-4), which share up to 70% sequence homology [3]. The E protein mediates viral attachment and cell membrane fusion during viral entry [4]. It consists of three domains: domain I (EDI), which is the central domain; domain II (EDII), which is the dimerization domain; and domain III (EDIII), which has an immunoglobulin-like structure [5.6]. Among them, EDIII contains host receptor binding sites. Since the binding with

monoclonal antibodies (mAbs) tends to be serotype specific, EDIII is considered the domain that contains the most effective neutralizing antigenic sites [7-9]. Moreover, the NS2B/NS3 viral serine protease is crucial for the polyprotein processing required for DENV replication. Therefore, E protein, NS2B, and NS3 have been studied as promising targets for vaccines and antiviral drugs [10].

As global warming has accelerated, mosquito habitats and populations have increased worldwide [11]. Additionally, the prevalence of arboviral disease is expected to increase gradually in Korea because of climate change [12]. The DENV vaccine Dengvaxia (CYD-TDV), a live-attenuated tetravalent vaccine, was approved by the FDA in 2019. However, due to side effects, the vaccine is only approved for patients aged 9-16 years who have been infected with DENV previously [13]. Accordingly, it remains necessary to develop an effective vaccine or antiviral drug for DENV. This endeavor is difficult because properties of the four DENV serotypes cause antibody-dependent enhancement (ADE) [14,15]. Although infection with the homologous DENV serotype induces long-term immunity, infection with the other serotypes has severe manifestations, like DF, DHF, and DSS [16]. ADE enhances the infection in cells expressing Fc-gamma receptor (FcγR) [17.18].

The recombinant protein vaccine induces a relatively balanced immune response to the four DENV serotypes and can therefore reduce ADE

rates. This vaccine is cost-effective and more stable than the live-attenuated vaccine, but it has relatively low immunogenicity; therefore, adjuvants and booster shots are required [19,20]. In previous studies, recombinant protein vaccines for DENV have been investigated using insect cell expression systems, because the *E. coli* expression system does not have the capacity for post-translational modifications (PTMs), such as glycosylation and protein folding [21-24].

In this study, we produced soluble hRID-DENV EDIII recombinant fusion protein antigens in an *E. coli* expression system. To ensure proper protein folding and increase the solubility, we fused a target protein with a human RNA interacting domain (hRID) that functioned as a chaperone [25,26]. DENV EDIII mAbs were then produced using the recombinant protein antigens, and DENV EDIII overlapping peptide libraries were synthesized. To identify the properties of the DENV EDIII mAbs, their DENV EDIII peptide binding activity was confirmed via enzyme-linked immunosorbent assay (ELISA), and neutralizing activity was evaluated using a microneutralization test (MNT). The location of the DENV EDIII binding epitopes was mapped onto the crystal structure obtained from the Protein Data Bank (PDB).

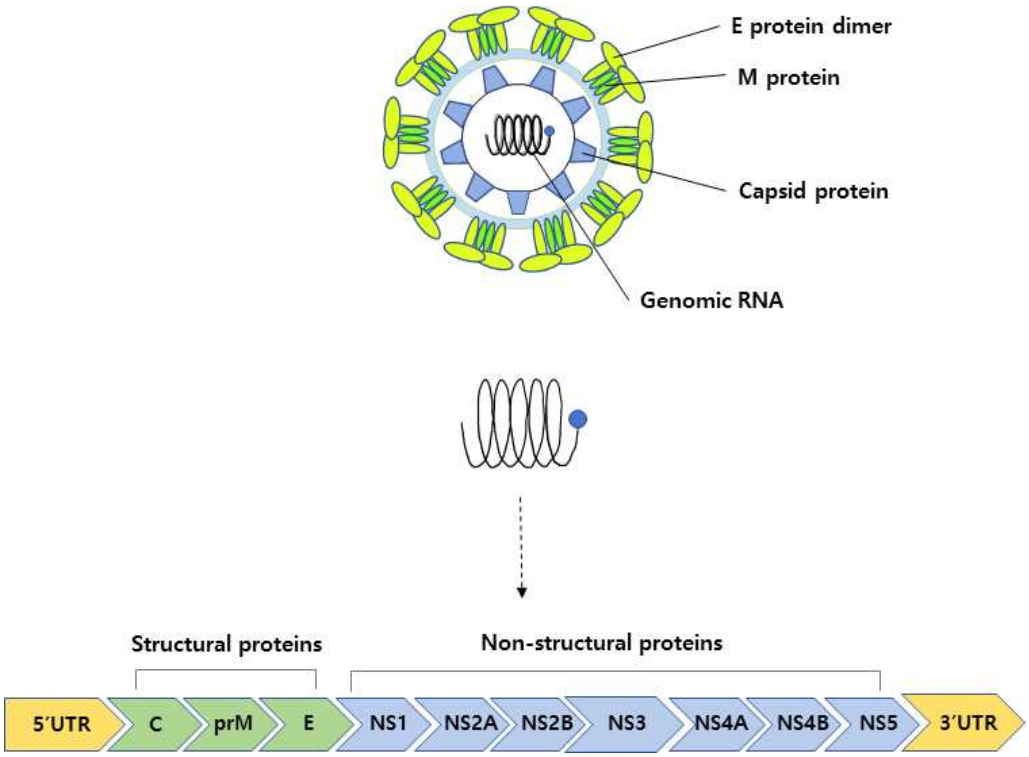

Figure. 1. Structure of dengue virus

The RNA genome is a single stranded positive sense that encodes three structural proteins, capsid (C), membrane (M), envelope (E), and seven non-structural proteins, NS1, NS2a, NS2b, NS3, NS4a, NS4b, NS5. They are required for polyprotein processing by serine protease.

Table 1. Four serotypes of DENV we used in this study

Serotype	Source	GenBank accession no.
1	DenKor-07	KP406803
2	KBPV-VR-29	KP406804
3	KBPV-VR-30	KP406805
4	KBPV-VR-31	KP406806

Table 2. The sequences of DENV EDIII proteins for overlapping peptide libraries

Source	Sequence of protein
DENV-1	KGVSYVMCTGSFKLEKEVAETQHGTVLVQVKYEGTDA PCKIPFSSQDEKGVTQNGRLITANPIVTDKEKPVNIEAE PPFGESYLVVGAGEKALKLSWFKKG
DENV-2	KGMSYSMCTGKFKVVKEIAETQHGTIVIRVQYEGDGS PCKIPFEIMDLEKRHVLGRLITVNPIVTEKDRPVNIEAE PPFGDSYIIIGVEPGQLKLNWFKKG
DENV-3	KGMSYAMCLNTFVLKKEVSETQHGTILIKVEYKGKDAP CKIPFSTEDGQGKAHNGRLITANPVVTKEEEPVNIEAE PPFGESNIVIGIGDKALKINWYRKG
DENV-4	KGMSYTMCSGKFSIDKEMAETQHGTTVVRVKYEGAG APCKVPIEIRDVNKEKVVGRIISSTPFAEYTDSVTNIELE PPFGDSYIVIGVGDSALTLHWFRKG

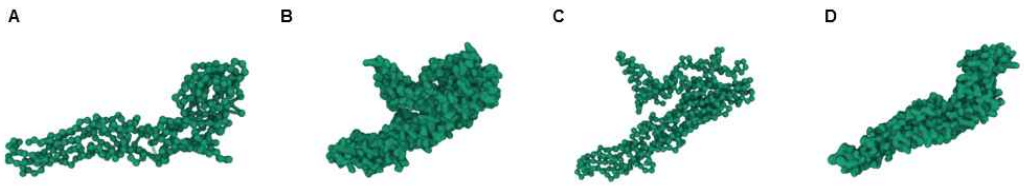

Figure. 2. Crystal structures of DENV E proteins

Crystal structures of four serotypes DENV E proteins were retrieved from PDB. (A) DENV-1 (PDB ID: 3J05) (B) DENV-2 (PDB ID: 3J27) (C) DENV-3 (PDB ID: 3J6U) (D) DENV-4 (PDB ID: 3UAJ).

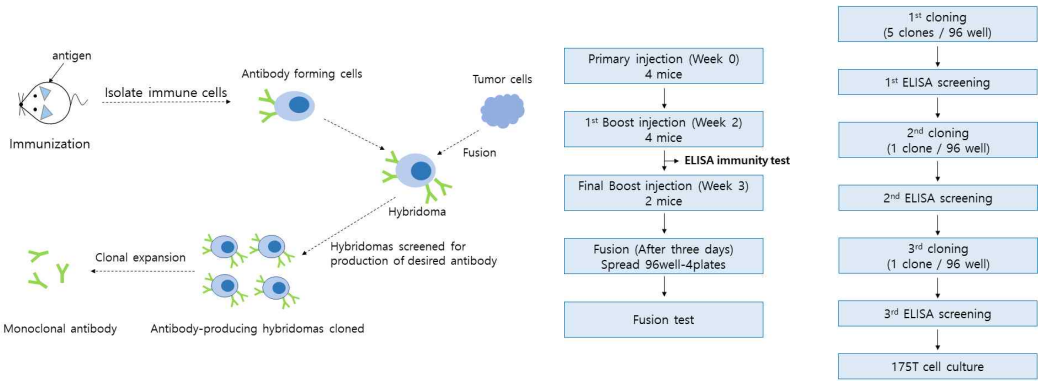

Figure. 3. Procedure of monoclonal antibodies production

Balb/c mice were immunized with the recombinant proteins. B-lymphocytes were collected from spleen and fusion was performed with myeloma cells Sp2/0. The fused cells were selected using HAT medium and hybridomas were screened three times by ELISA.

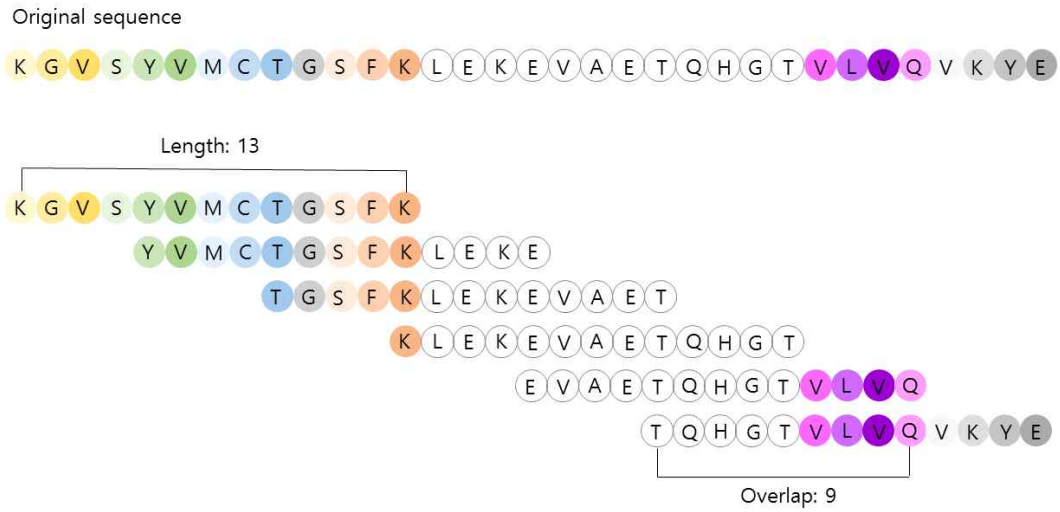

Figure. 4. Overlapping peptide library

A total of 92 overlapping peptides were synthesized (13 amino acids in length, offset by 4 and overlapping by 9 amino acids). There are 23 peptides for each DENV serotype. These peptides were used for epitope mapping with DENV EDIII mAbs.

Table 3. The sequences of DENV EDIII overlapping peptides for epitope mapping (Length: 13, Overlap: 9, Offset: 4)

No.	DENV-1	Sequence	No.	DENV-2	Sequence
1-1	$DV1_{1-13}$	KGVSYVMCTGSFK	2-1	$DV2_{1-13}$	KGMSYSMCTGKFK
1-2	$DV1_{5-17}$	YVMCTGSFKLEKE	2-2	$DV2_{5-17}$	YSMCTGKFKVVKE
1-3	$DV1_{9-21}$	TGSFKLEKEVAET	2-3	$DV2_{9-21}$	TGKFKVVKEIAET
1-4	$DV1_{13-25}$	KLEKEVAETQHGT	2-4	$DV2_{13-25}$	KVVKEIAETQHGT
1-5	$DV1_{17-29}$	EVAETQHGTVLVQ	2-5	$DV2_{17-29}$	EIAETQHGTIVIR
1-6	$DV1_{21-33}$	TQHGTVLVQVKYE	2-6	$DV2_{21-33}$	TQHGTIVIRVQYE
1-7	$DV1_{25-37}$	TVLVQVKYEGTDA	2-7	$DV2_{25-37}$	TIVIRVQYEGDGS
1-8	$DV1_{29-41}$	QVKYEGTDAPCKI	2-8	$DV2_{29-41}$	RVQYEGDGSPCKI
1-9	$DV1_{33-45}$	EGTDAPCKIPFSS	2-9	$DV2_{33-45}$	EGDGSPCKIPFEI
1-10	$DV1_{37-49}$	APCKIPFSSQDEK	2-10	$DV2_{37-49}$	SPCKIPFEIMDLE
1-11	$DV1_{41-53}$	IPFSSQDEKGVTQ	2-11	$DV2_{41-53}$	IPFEIMDLEKRHV
1-12	$DV1_{45-57}$	SQDEKGVTQNGRL	2-12	$DV2_{45-57}$	IMDLEKRHVLGRL
1-13	$DV1_{49-61}$	KGVTQNGRLITAN	2-13	$DV2_{49-61}$	EKRHVLGRLITVN
1-14	$DV1_{53-65}$	QNGRLITANPIVT	2-14	$DV2_{53-65}$	VLGRLITVNPIVT
1-15	$DV1_{57-69}$	LITANPIVTDKEK	2-15	$DV2_{57-69}$	LITVNPIVTEKDR
1-16	$DV1_{61-73}$	NPIVTDKEKPVNI	2-16	$DV2_{61-73}$	NPIVTEKDRPVNI
1-17	$DV1_{65-77}$	TDKEKPVNIEAEP	2-17	$DV2_{65-77}$	TEKDRPVNIEAEP
1-18	$DV1_{69-81}$	KPVNIEAEPPFGE	2-18	$DV2_{69-81}$	RPVNIEAEPPFGD
1-19	$DV1_{73-85}$	IEAEPPFGESYLV	2-19	$DV2_{73-85}$	IEAEPPFGDSYII
1-20	$DV1_{77-89}$	PPFGESYLVVGAG	2-20	$DV2_{77-89}$	PPFGDSYIIIGVE
1-21	$DV1_{81-93}$	ESYLVVGAGEKAL	2-21	$DV2_{81-93}$	DSYIIIGVEPGQL
1-22	$DV1_{85-97}$	VVGAGEKALKLSW	2-22	$DV2_{85-97}$	IIGVEPGQLKLNW
1-23	$DV1_{89-101}$	GEKALKLSWFKKG	2-23	$DV2_{89-101}$	EPGQLKLNWFKKG

No.	DENV-3	Sequence	No.	DENV-4	Sequence
3-1	$DV3_{1-13}$	KGMSYAMCLNTFV	4-1	$DV4_{1-13}$	KGMSYTMCSGKFS
3-2	$DV3_{5-17}$	YAMCLNTFVLKKE	4-2	$DV4_{5-17}$	YTMCSGKFSIDKE
3-3	$DV3_{9-21}$	LNTFVLKKEVSET	4-3	$DV4_{9-21}$	SGKFSIDKEMAET
3-4	$DV3_{13-25}$	VLKKEVSETQHGT	4-4	$DV4_{13-25}$	SIDKEMAETQHGT
3-5	$DV3_{17-29}$	EVSETQHGTILIK	4-5	$DV4_{17-29}$	EMAETQHGTTVVR
3-6	$DV3_{21-33}$	TQHGTILIKVEYK	4-6	$DV4_{21-33}$	TQHGTTVVRVKYE
3-7	$DV3_{25-37}$	TILIKVEYKGKDA	4-7	$DV4_{25-37}$	TTVVRVKYEGAGA
3-8	$DV3_{29-41}$	KVEYKGKDAPCKI	4-8	$DV4_{29-41}$	RVKYEGAGAPCKV
3-9	$DV3_{33-45}$	KGKDAPCKIPFST	4-9	$DV4_{33-45}$	EGAGAPCKVPIEI
3-10	$DV3_{37-49}$	APCKIPFSTEDGQ	4-10	$DV4_{37-49}$	APCKVPIEIRDVN
3-11	$DV3_{41-53}$	IPFSTEDGQGKAH	4-11	$DV4_{41-53}$	VPIEIRDVNKEKV
3-12	$DV3_{45-57}$	TEDGQGKAHNGRL	4-12	$DV4_{45-57}$	IRDVNKEKVVGRI
3-13	$DV3_{49-61}$	QGKAHNGRLITAN	4-13	$DV4_{49-61}$	NKEKVVGRIISST
3-14	$DV3_{53-65}$	HNGRLITANPVVT	4-14	$DV4_{53-65}$	VVGRIISSTPFAE
3-15	$DV3_{57-69}$	LITANPVVTKEEE	4-15	$DV4_{57-69}$	IISSTPFAEYTDS
3-16	$DV3_{61-73}$	NPVVTKEEEPVNI	4-16	$DV4_{61-73}$	TPFAEYTDSVTNI
3-17	$DV3_{65-77}$	TKEEEPVNIEAEP	4-17	$DV4_{65-77}$	EYTDSVTNIELEP
3-18	$DV3_{69-81}$	EPVNIEAEPPFGE	4-18	$DV4_{69-81}$	SVTNIELEPPFGD
3-19	$DV3_{73-85}$	IEAEPPFGESNIV	4-19	$DV4_{73-85}$	IELEPPFGDSYIV
3-20	$DV3_{77-89}$	PPFGESNIVIGIG	4-20	$DV4_{77-89}$	PPFGDSYIVIGVG
3-21	$DV3_{81-93}$	ESNIVIGIGDKAL	4-21	$DV4_{81-93}$	DSYIVIGVGDSAL
3-22	$DV3_{85-97}$	VIGIGDKALKINW	4-22	$DV4_{85-97}$	VIGVGDSALTLHW
3-23	$DV3_{89-101}$	GDKALKINWYRKG	4-23	$DV4_{89-101}$	GDSALTLHWFRKG

Materials and Methods

Viruses and cells

Vero cells, an African green monkey kidney-derived epithelial cell line, were obtained from National Culture Collection for pathogens (NCCP, South Korea) and Korea bank for pathogenic viruses (KBPV). Vero cells were maintained at 37°C with 5% CO_2 in Dulbecco's modified essential medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin.

Dengue viruses (DENVs) were obtained from the National Culture Collection for pathogens (NCCP, South Korea) and International Vaccine Institute (South Korea). DENV serotypes used in this study are DENV-1 (Denkor-7), DENV-2 (KBPV-VR-29), DENV-3 (KBPV-VR-30) and DENV-4 (KBPV-VR-31). Propagation of the parental DENV (1-4) was performed in Vero cells grown at 37°C with 5% CO_2..

Expression of EDIII recombinant proteins

DENV EDIII DNA sequences obtained from GenBank (accession number KP406803, KP406804, KP406805 and KP406806) and the sequences were codon optimized and synthesized. The synthesized EDIII genes were cloned into pGE-RID vector with 6x his-tag at C-terminus end as

described previously [25-27]. After the recombinant plasmids were transformed into *E. coli* BL21 (DE3) cells, pre-culture was prepared by single colony in LB medium for 18h at 37°C and 200 rpm. When the OD600 reached 0.6, Isopropyl β-d-1-thiogalactopyranoside (IPTG) was added at a final concentration of 1 mM and incubated at 20°C overnight. The supernatant and cell debris were centrifuged for 10 min at 6000 rpm, and discarded supernatant. After the cell debris washed using washing buffer (50 mM Tris-HCl and 1 mM EDTA, pH 8.0) two-times, centrifuged, and resuspended in 50 mM Tris-HCl and 0.2 M NaCl. The cells were disrupted by sonicator, and purification was performed using 6x his-tag and Ni-NTA affinity column with wash buffer (50 mM Tris-HCl, 0.2 M NaCl, 10% glycerol, pH 7.4) and elution buffer (50 mM Tris-HCl, 0.2 M NaCl, 10% glycerol, pH 7.4, 0.5 M Imidazole). These protein samples were analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on a 17% acrylamide gel using a Hoefer minigel system at 120 V for 1.5 h, and stained with Coomassie Brilliant Blue.

EDIII monoclonal antibodies

Eight-weeks-old Balb/c mice were immunized with 100 μl of recombinant proteins with Freund's adjuvant. Boost injections were given twice with the same dose. Three days after final boosting, B-lymphocytes were collected from spleen and the fusion was performed with myeloma cells Sp2/0. The fused cells were selected using HAT medium and the

hybridomas were screened three times by ELISA. Selected clones were cultivated in T175 flasks and supernatant was harvested (Fig. 3).

EDIII overlapping peptide libraries

DENV EDIII overlapping peptide libraries (13 amino acids in length, overlapping by 9 amino acids) that cover the entire length of the four serotypes DENV EDIII proteins were synthesized based on the amino acid sequence from GenBank (accession number ALI16135, ALI16136, ALI16137and ALI16138), (Residue 575-675, 575-675, 573-673 and 574-674), (Gen Script, China). These peptides were used to perform the epitope mapping (Fig. 4).

Enzyme-linked immunosorbent assay (ELISA)

Microplates were coated with 100 µl hRID-EDIII proteins or EDIII peptides diluted in PBS (100-10000 ng/well) and incubated at 4°C overnight. Plates were washed and blocked with 3% Bovine serum albumin (BSA) in PBS at 37°C for 1h 30min. Plates were washed with PBST (PBS, 0.05% Tween20) and treated with DENV EDIII mAbs (diluted 1:4-1:1000 in PBS) at 37°C for 2h. After washing the plates, horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG was added and incubated at 37°C for 1h. Finally, the plates were washed six-times and treated with TMB substrate solution for 10min. The reaction was

terminated with the addition of stop solution and the absorbance was measured at 450 nm (Fig. 5).

Microneutralization test (MNT)

Vero cells were cultured in 96-well plates until 90% confluency at 37°C with 5% CO_2. Dengue virus solutions (DENV-1 to DENV-4) were mixed with an equal volume of mAbs or PBS (negative control) and incubated at room temperature (RT) for 1h. After washing the plates, the mixtures were added to each well and incubated at 37°C with 5% CO_2 for 2h. Plates were washed two-times and DMEM containing 2% FBS and 1% penicillin-streptomycin was added to each well. After incubation at 37°C with 5% CO_2 for 10 days, Vero cells were fixed with 4% formaldehyde in PBS and stained with crystal violet solution for 15min. The plates were completely dried for 2 days and crystal violet was then dissolved in 30% acetic acid at RT for 30 min. The absorbance was read at 560nm (Fig. 6), [28].

Statistics

Statistical analyses were performed using GraphPad Prism software version 4. All experiments were performed in triplicate and error bars indicate standard deviation (SD).

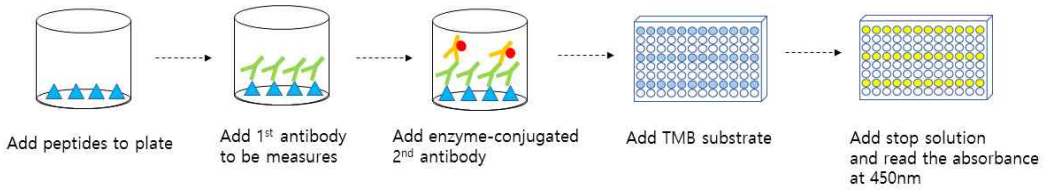

Figure. 5. Procedure of enzyme-linked immunosorbent assay

Microplates (96-well) were coated with hRID-EDIII proteins or EDIII peptides and blocked with blocking buffer the next day. EDIII mAbs were added to the plates as specific antibody that binds to antigen and HRP-conjugated goat anti-mouse IgG was added to the plates. TMB substrate solution was added and the reaction was terminated with stop solution. The optical density was measured at 450 nm.

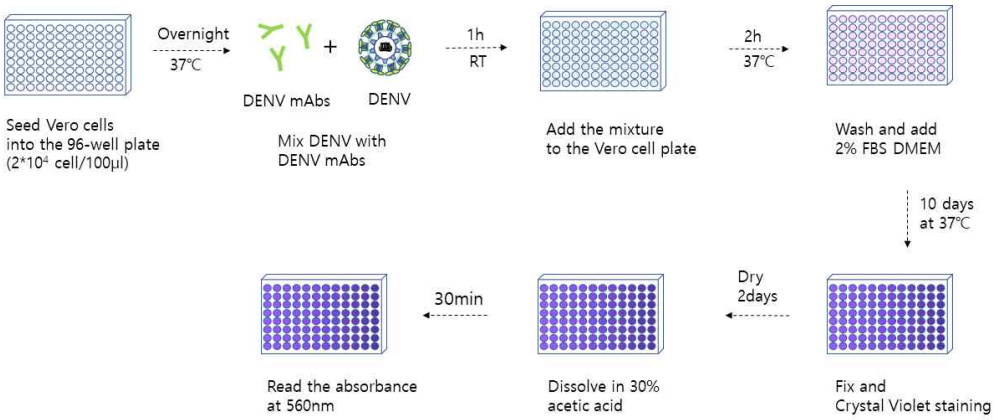

Figure. 6. Procedure of microneutralization test

Virus solutions were mixed with an equal volume of mAb or PBS (negative control) and incubated. The mixtures were added to Vero cells and incubated. After washing, DMEM with 2% FBS was added and incubated 10 days. After fixing and staining, the staining was dissolved in 30% acetic acid solution and the optical density was measured at 560 nm.

Results

Production of soluble recombinant proteins

The soluble hRID-DENV EDIII recombinant fusion proteins were produced in the *E. coli* expression system (Fig. 7). After purification and concentration, recombinant proteins were obtained (Fig. 8). These proteins were used as antigens to produce DENV EDIII monoclonal antibodies [29].

Production of DENV EDIII mAbs

We produced a total of 29 DENV EDIII monoclonal antibodies (Table 4). The soluble hRID-DENV EDIII recombinant fusion proteins were used as antigens. Balb/c mice were immunized with the recombinant proteins and hybridoma technology was used to produce the mAbs.

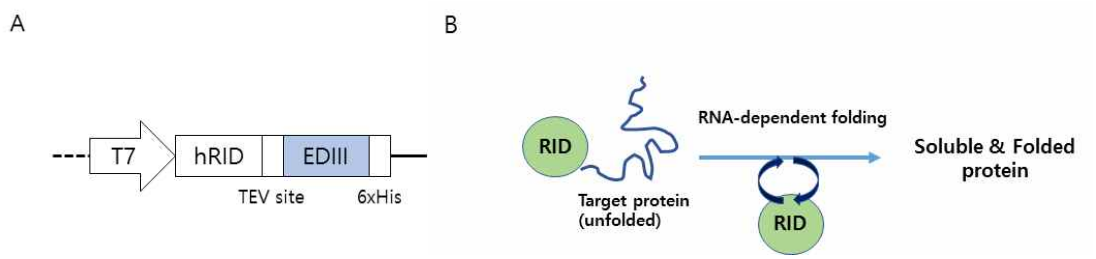

Figure. 7. Expression system of hRID fusion protein in *E. coli*

The hRID fusion protein mediates the protein folding and increase in solubility. (A) Expression system of soluble recombinant proteins fused with hRID (B) RNA-as-chaperone performs proper protein folding and expression of soluble proteins.

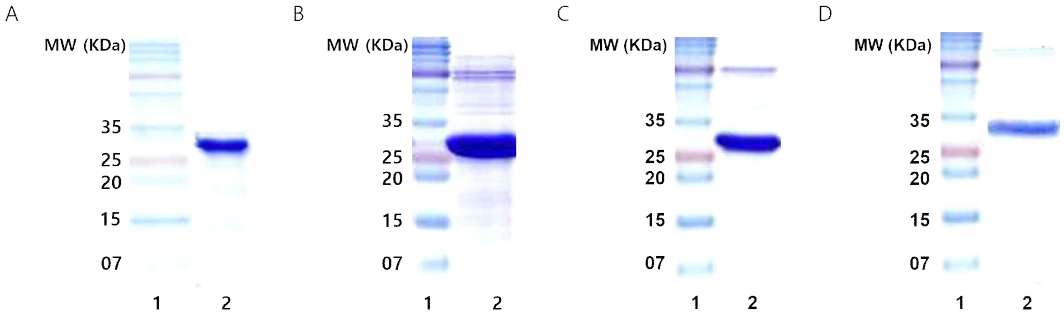

Figure. 8. Expression of soluble hRID-DENV EDIII recombinant fusion proteins

SDS-PAGE analysis of soluble hRID-DENV EDIII recombinant fusion protein expression in *E. coli*. [28]. Molecular weight (MW) markers (lane 1) (A) DENV-1 recombinant protein fraction (lane 2) (B) DENV-2 recombinant protein fraction (lane 2) (C) DENV-3 recombinant protein fraction (lane 2) (D) DENV-4 recombinant protein fraction (lane 2)

Table 4. DENV EDIII monoclonal antibodies produced using soluble hRID-DENV EDIII recombinant fusion proteins

Serotype	Clone	Isotype
1	3A6	IgG2b
	6A7	IgG2b
	9A4	IgG1
	10F7	IgG2b
	16A6	IgG1
	20C9	IgG2b
2	1A10	IgG2b
	3C7	IgG1
	7D7	IgG1
	8G6	IgG1
	11H5	IgG2a
	12G4	IgG1
	13H8	IgG2a
	23A7	IgG2a
	24B11	IgG2a
3	4E8	IgG1
	6A10	IgG2b
	8A5	IgG2b
	14A8	IgG2b
	16A10	IgG1
	17H4	IgG1
	22C8	IgG2b
	23D8	IgG2a
	24H8	IgG1
4	2B11	IgG2b
	3A2	IgG2a
	8C5	IgG2b
	9G3	IgG2a
	12D7	IgG2b

Homologous mapping of antigenic epitopes on DENV EDIII

A total of 92 overlapping peptides corresponding to the sequence of DENV (1-4) EDIII proteins were synthesized and used for epitope mapping by ELISA (Table 3). After the peptides were incubated with DENV EDIII mAbs, they were detected with goat anti-mouse IgG. The absorbance was measured to identify binding epitopes that bound to mAbs. First, we observed that properties of homologous serotypes. The binding properties of DENV-1, all the DENV-1 mAbs were bound to peptide 1-1. Among them, the strongest binding activities were observed between mAbs 6A7, 10F7 and peptides 1-1 and 1-6. The other peptides showed no appreciable binding (Fig. 9). In the case of DENV-2, the highest affinities were observed between mAb 8G6 and peptides 2-12 and 2-13 (Fig. 10). The other peptides showed no binding activity. In the case of DENV-3, all the DENV-3 mAbs were bound with peptides 3-1 and 3-2. The other peptides showed no appreciable binding (Fig. 11). For DENV-4, all the DENV-4 mAbs were bound only against peptide 4-23 most strongly (Fig. 12). Furthermore, we predicted that these dominant epitopes onto the crystal structures of DENV E protein retrieved from PDB (Fig. 13).

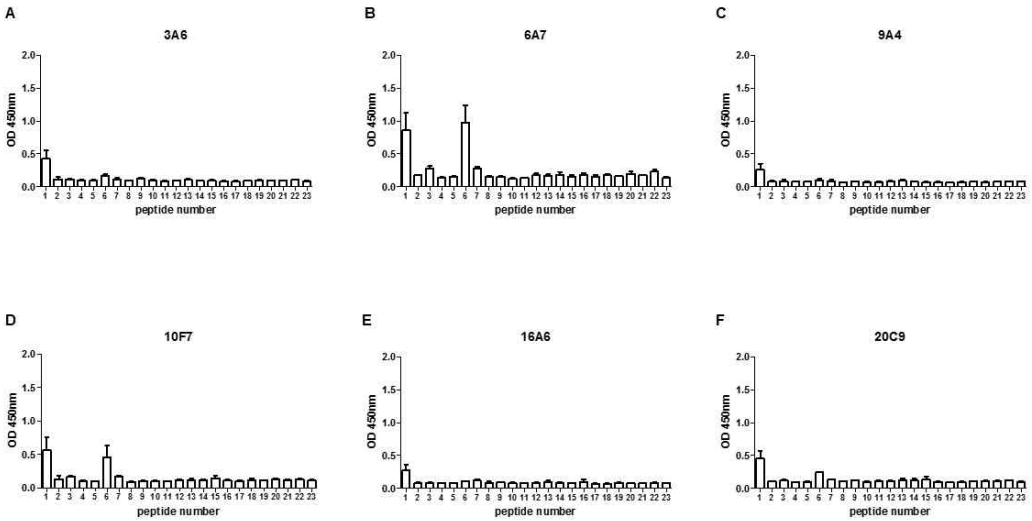

Figure. 9. Epitope mapping of DENV-1 mAbs with DENV-1 EDIII peptides

The overlapping peptides from DENV-1 EDIII protein were synthesized and analyzed by ELISA using corresponding mAbs (A-F). Each 23 peptides were coated on ELISA plates and reacted with DENV-1 EDIII mAbs. All the DENV-1 mAbs showed reactivity with peptide 1-1, and mAbs 6A7 and 10F7 also showed reactivity with peptide 1-6.

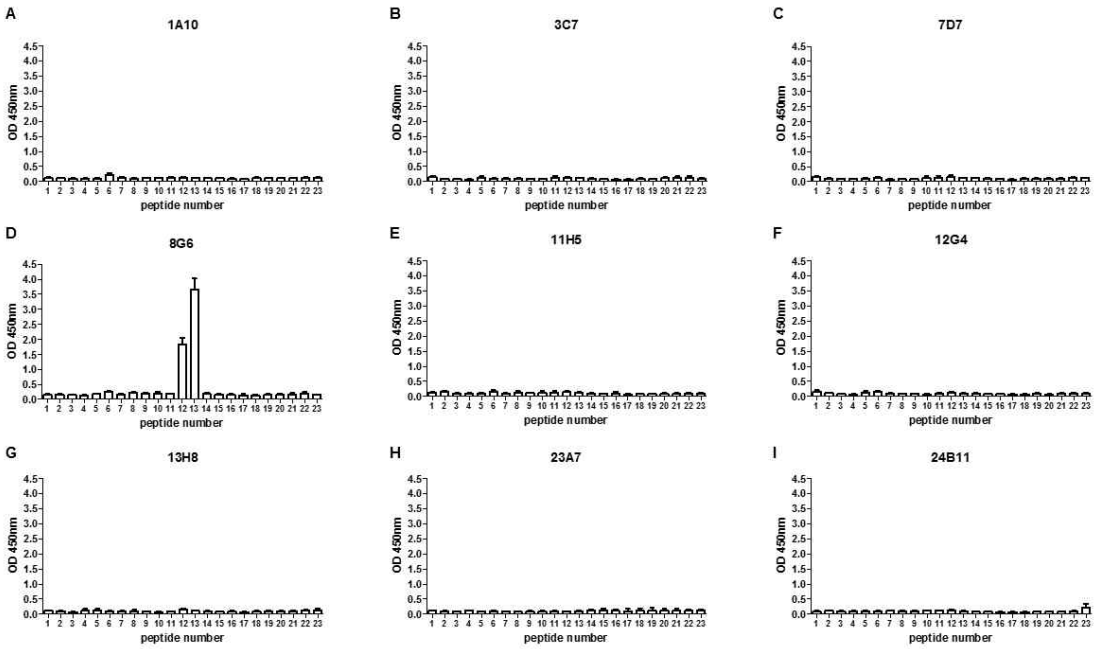

Figure. 10. Epitope mapping of DENV-2 mAbs with DENV-2 EDIII peptides

The overlapping peptides from DENV-2 EDIII protein were synthesized and analyzed by ELISA using corresponding mAbs (A-I). Each 23 peptides were coated on ELISA plates and reacted with DENV-2 EDIII mAbs. Only mAb 8G6 showed reactivity with peptides 2-12 and 2-13.

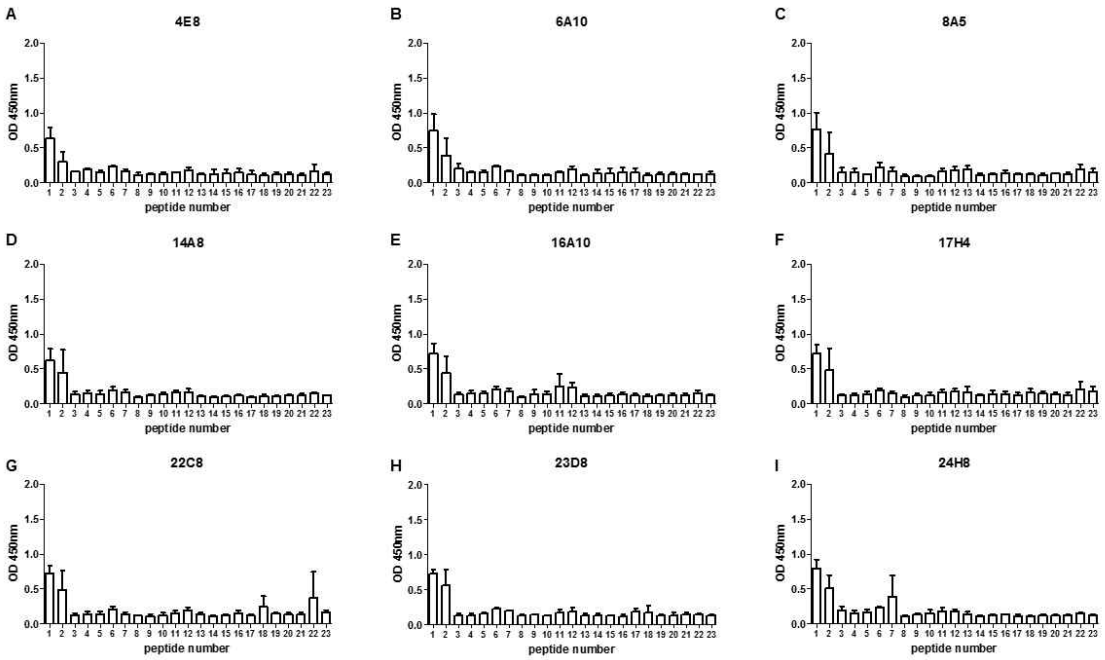

Figure. 11. Epitope mapping of DENV-3 mAbs with DENV-3 EDIII peptides

The overlapping peptides from DENV-3 EDIII protein were synthesized and analyzed by ELISA using corresponding mAbs (A-I). Each 23 peptides were coated on ELISA plates and reacted with DENV-3 EDIII mAbs. All the DENV-3 mAbs showed reactivity with peptides 3-1 and 3-2.

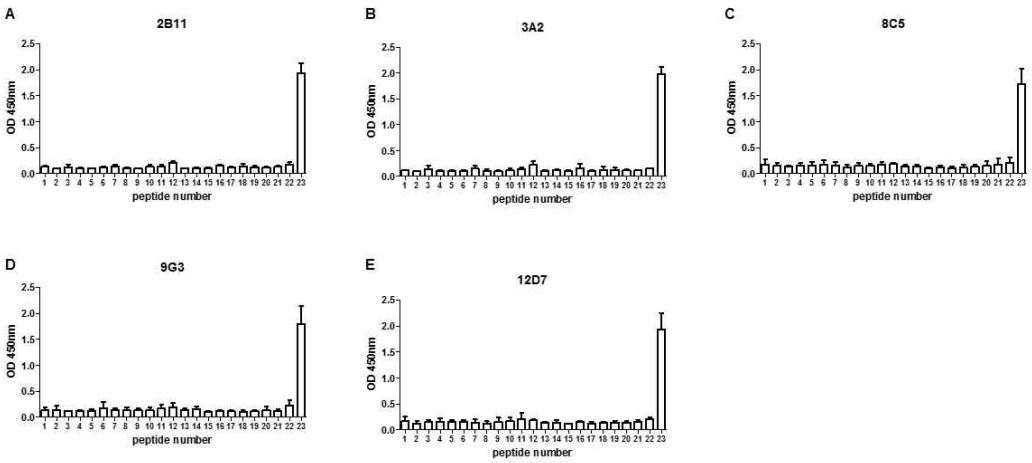

Figure. 12. Epitope mapping of DENV-4 mAbs with DENV-4 EDIII peptides

The overlapping peptides from DENV-4 EDIII protein were synthesized and analyzed by ELISA using corresponding mAbs (A-E). Each 23 peptides were coated on ELISA plates and reacted with DENV-4 EDIII mAbs. All the DENV-4 mAbs showed reactivity only with peptide 4-23.

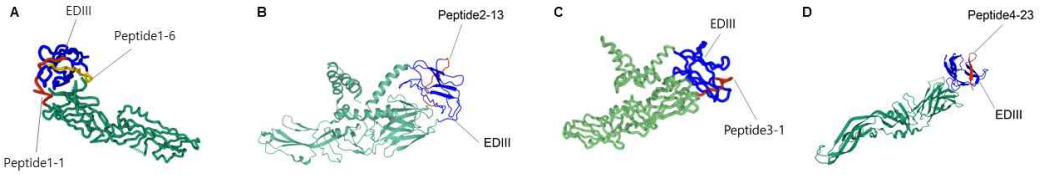

Figure. 13. Epitope mapping onto crystal structures

These crystal structures indicate four serotypes of DENV E proteins. The EDIII were colored in blue, and each binding epitopes were colored red and yellow. (A) DENV-1 (PDB code: 3J05). (B) DENV-2 (PDB code: 3J27). (C) DENV-3 (PDB code: 3J6U). (D) DENV-4 (PDB code: 3UAJ).

Heterologous mapping of antigenic epitopes on DENV EDIII

We also tested that heterologous binding properties of the DENV mAbs (1-4) with EDIII overlapping peptides. Properties of DENV-1 mAbs, all the DENV-1 mAbs were bound against peptides 3-1. The other peptides showed no appreciable binding (Fig. 14). In the case of DENV-2 mAbs, all the DENV-2 mAbs were bound against peptides 1-1 and 3-1. The other peptides showed no binding activity (Fig. 15). In the case of DENV-3 mAbs, all the DENV-3 mAbs showed reactivity only with peptide 1-1 (Fig. 16). In the case of DENV-4 mAbs, all the DENV-4 mAbs were bound against peptides 1-1 and 3-1. The other peptides showed no appreciable binding (Fig. 17). Overall, the peptides 1-1 and 3-1 showed the common binding activity against four DENV serotypes.

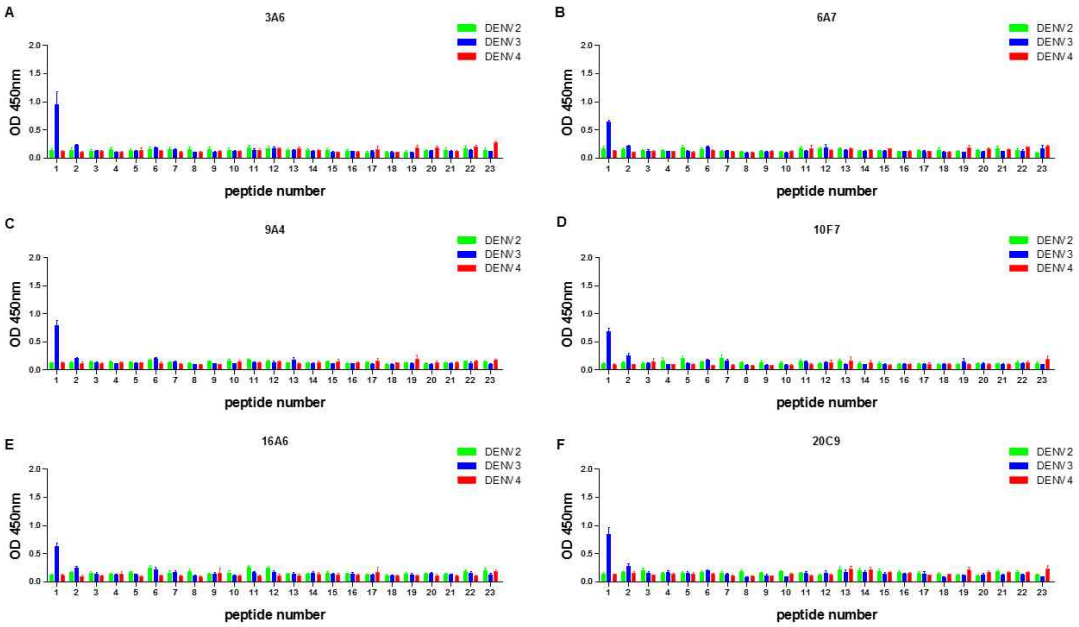

Figure. 14. Epitope mapping of DENV-1 mAbs with heterologous DENV EDIII peptides

The epitope mapping of DENV-1 mAbs with heterologous DENV EDIII peptides was analyzed by ELISA (A-F). Each 23 peptides (DENV-2, DENV-3, DENV-4) were coated and reacted with the DENV-1 EDIII mAbs. All the DENV-1 mAbs showed binding activity with peptide 3-1.

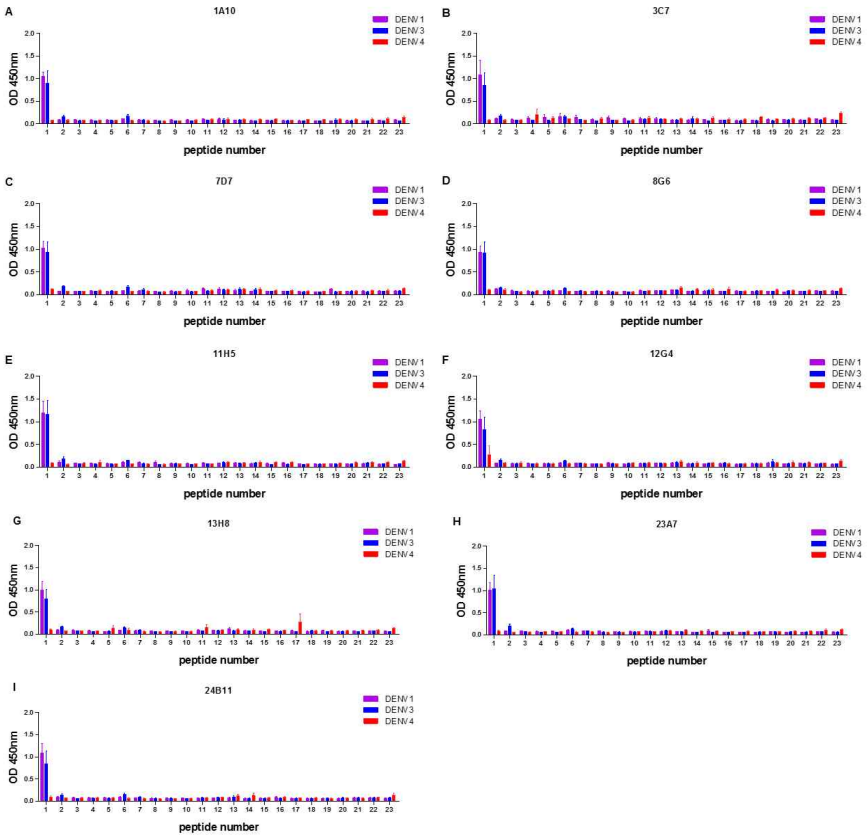

Figure. 15. Epitope mapping of DENV-2 mAbs with heterologous DENV EDIII peptides

The epitope mapping of DENV-2 mAbs with heterologous DENV EDIII peptides was analyzed by ELISA (A-I). Each of 23 peptides (DENV-1, DENV-3, DENV-4) were coated and reacted with the DENV-2 EDIII mAbs. All the DENV-2 mAbs showed binding activity with peptides 1-1 and 3-1.

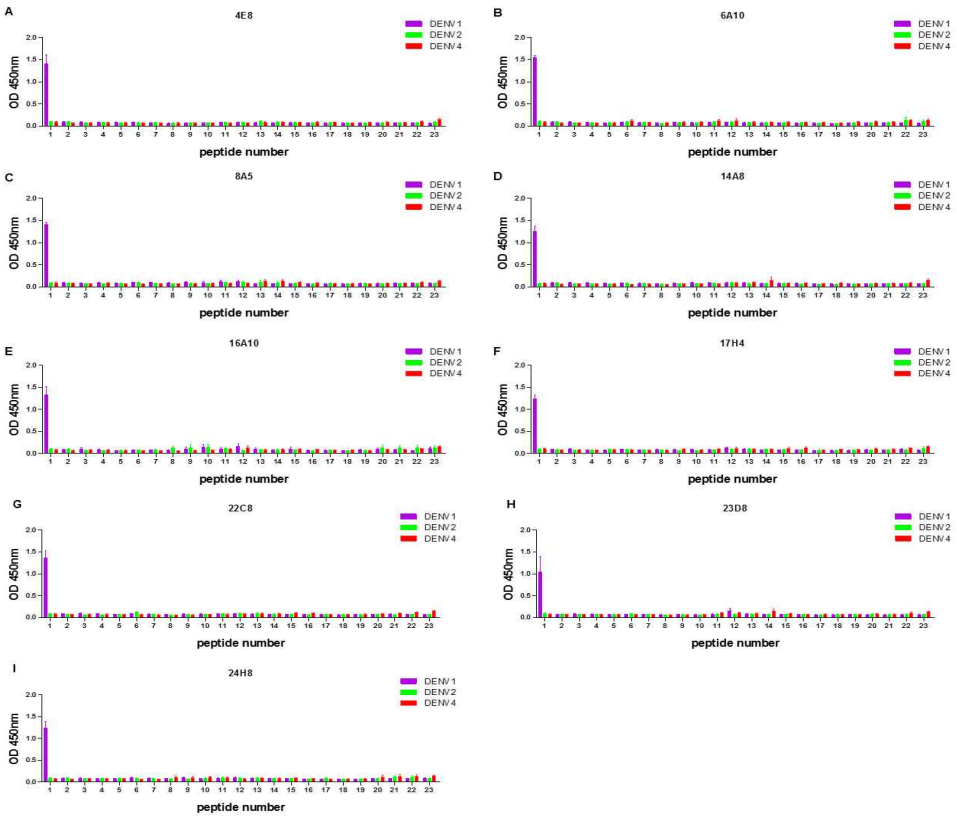

Figure. 16. Epitope mapping of DENV-3 mAbs with heterologous DENV EDIII peptides

The epitope mapping of DENV-3 mAbs with heterologous DENV EDIII peptides was analyzed by ELISA (A-I). Each 23 peptides (DENV-1, DENV-2, DENV-4) were coated and reacted with the DENV-3 EDIII mAbs. All the DENV-3 mAbs showed binding activity with peptide 1-1.

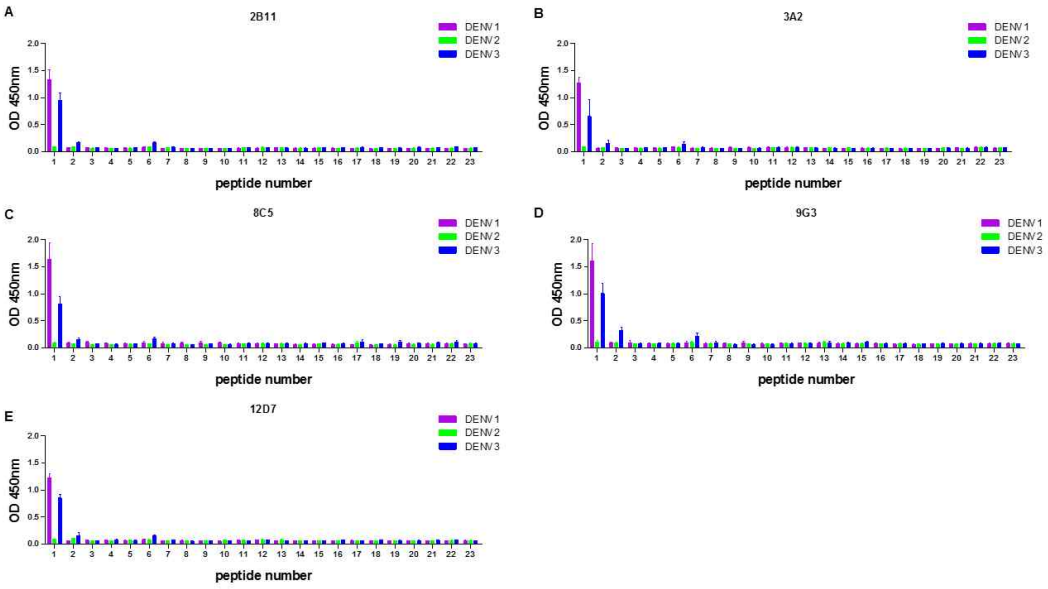

Figure. 17. Epitope mapping of DENV-4 mAbs with heterologous DENV EDIII peptides

The epitope mapping of DENV-4 mAbs with heterologous DENV EDIII peptides was analyzed by ELISA (A-E). Each 23 peptides (DENV-1, DENV-2, DENV-3) were coated and reacted with the DENV-4 EDIII mAbs. All the DENV-4 mAbs showed binding activity with peptides 1-1 and 3-1.

Neutralizing activity of homologous DENV EDIII mAbs

We generated a total of 29 DENV mAbs using hRID-DENV EDIII recombinant fusion proteins (Table 4). Homologous neutralizing activity was tested by microneutralization test. Virus solutions and antibody mixtures were treated on Vero cell and incubated for 10 days. Crystal violet solution was treated and absorbance was measured at 560 nm. The homologous test showed little or no neutralization activity against the DENV-1, DENV-3, and DENV-4 mAbs, except for the DENV-2 mAbs. In the case of DENV-1, the mAbs 3A6, 6A7, 9A4, and 10F7 showed neutralization activity. In the case of DENV-2, most of the mAbs showed considerable neutralizing activity. In the case of DENV-3, most mAbs showed neutralization activity, except 4E8 and 16A10. All of the DENV-4 mAbs showed neutralization activity. None means negative control treated with PBS instead of mAbs (Fig. 18).

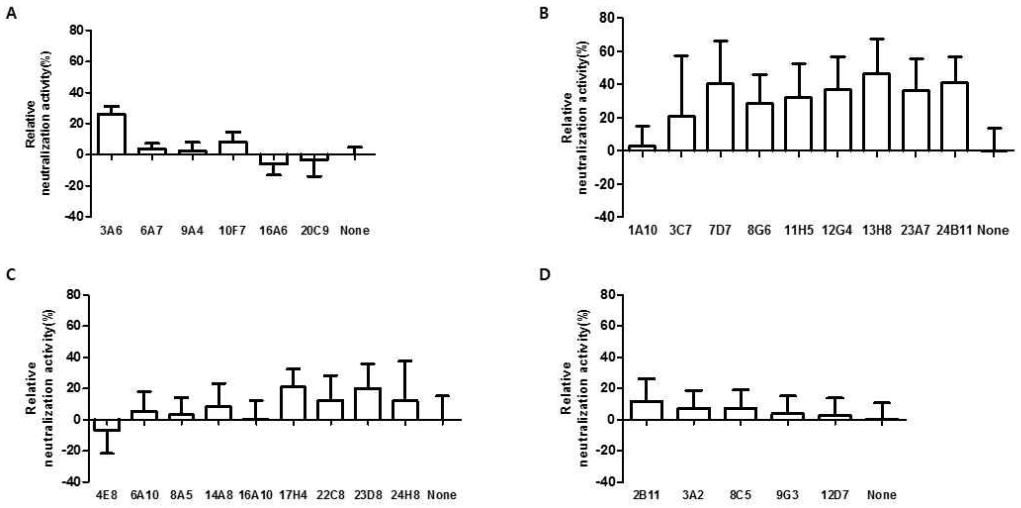

Figure. 18. Neutralizing activity of homologous DENV EDIII mAbs

To identify the neutralizing activity with homologous serotype of DENV, microneutralization test was tested. None means negative control. (A) DENV-1 (B) DENV-2 (C) DENV-3 (D) DENV-4

Neutralizing activity of heterologous DENV EDIII mAbs

We generated a total of 29 DENV mAbs from hRID-DENV EDIII recombinant fusion proteins (Table 4). Heterologous neutralizing activity was confirmed by microneutralization test. Virus solutions and antibody mixtures were treated and incubated for 10 days. Crystal violet solution was treated and absorbance was measured at 560 nm. In the case of DENV-1 and DENV-4, some mAbs showed neutralizing activity, but most of the mAbs represented enhancing viral infection (Fig. 19), (Fig. 22). The strongest level of neutralization was seen against DENV-2 and DENV-3 mAbs (Fig. 20B). For DENV-3, most of the DENV-2 mAbs showed neutralizing activity against DENV-3 (Fig. 21B). Furthermore, DENV-1 mAbs 16A6 and 20C9 showed neutralizing activity only against heterologous DENVs (Fig. 20, 21 and 22). DENV-4 mAb 12D7 has neutralizing activity against all four DENV serotypes (Fig. 18, 19, 20 and 21). The other mAbs showed no appreciable activity. None means negative control treated with PBS instead of mAbs.

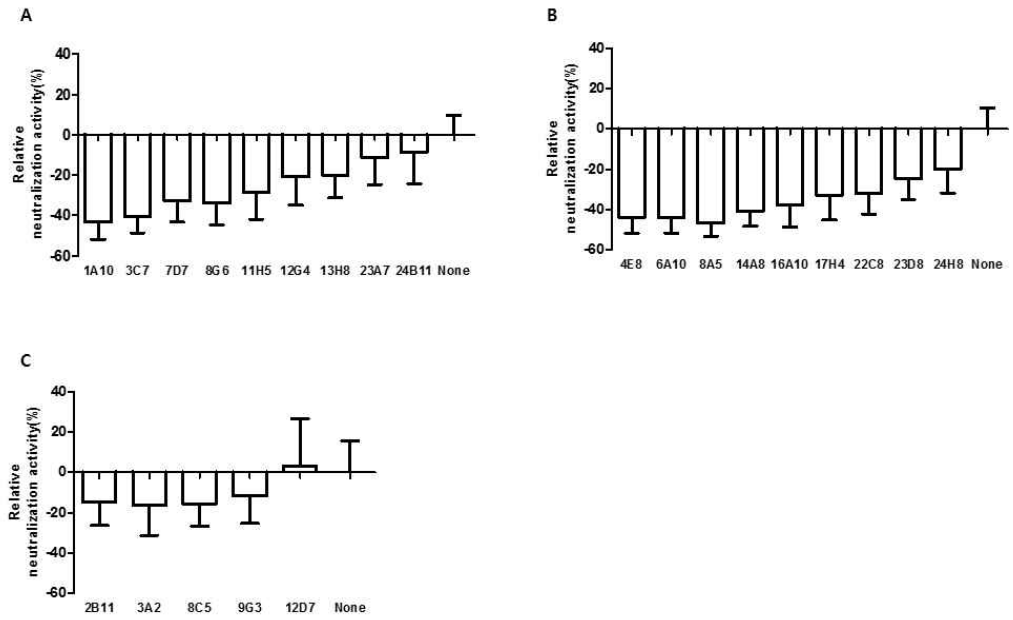

Figure. 19. Neutralizing activity of heterologous DENV EDIII mAbs against DENV-1

To identify neutralizing activity between DENV-1 and heterologous mAbs, microneutralization test was tested. None means negative control. (A) DENV-2 EDIII mAbs (B) DENV-3 EDIII mAbs (C) DENV-4 EDIII mAbs

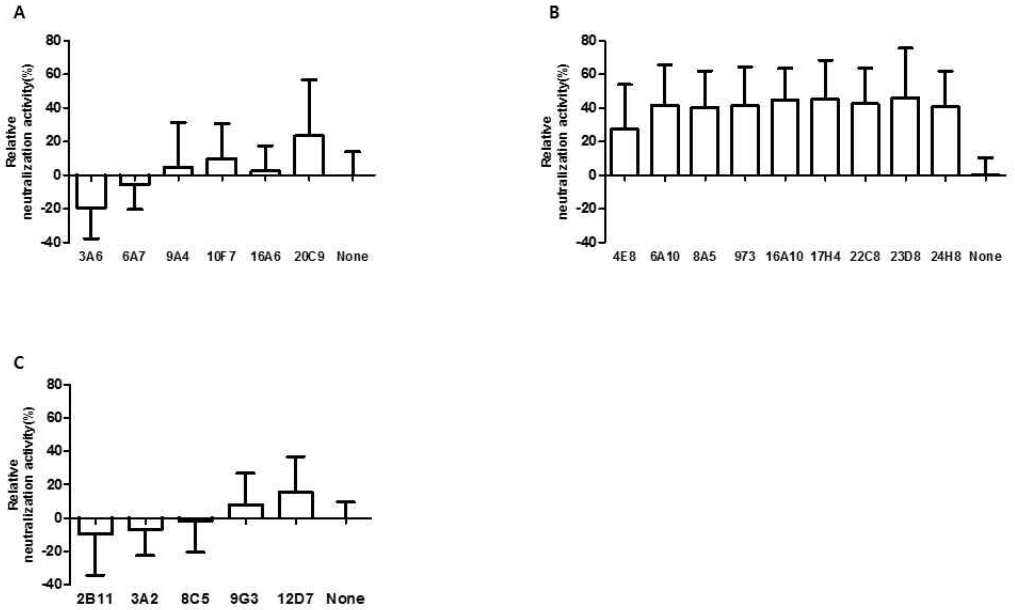

Figure. 20. Neutralizing activity of heterologous DENV EDIII mAbs against DENV-2

To identify neutralizing activity between DENV-2 and heterologous mAbs, microneutralization test was tested. None means negative control. (A) DENV-1 EDIII mAbs (B) DENV-3 EDIII mAbs (C) DENV-4 EDIII mAbs

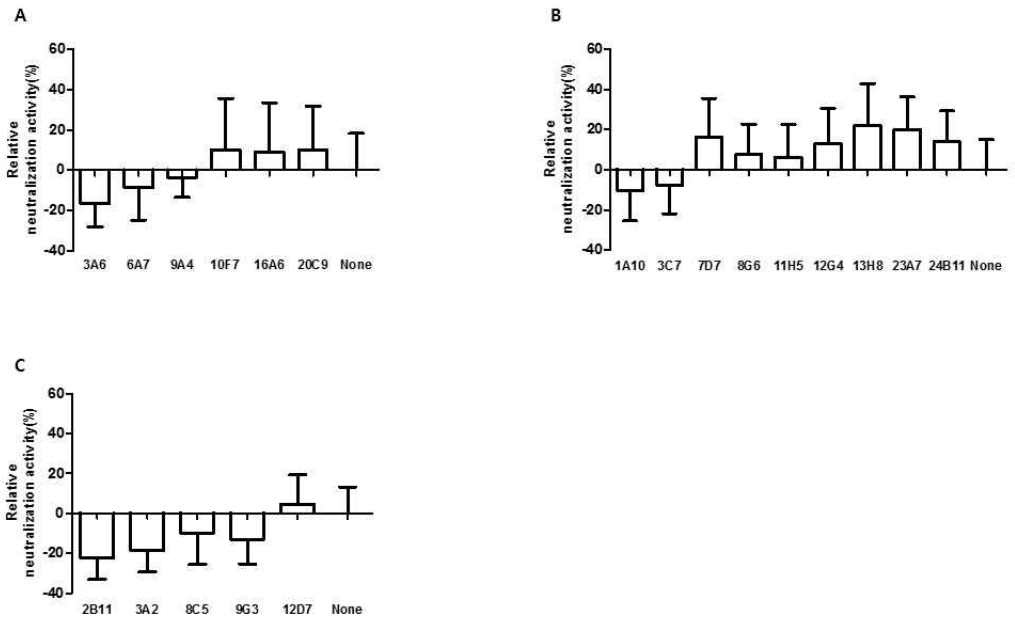

Figure. 21. Neutralizing activity of heterologous DENV EDIII mAbs against DENV-3

To identify neutralizing activity between DENV-3 and heterologous mAbs, microneutralization test was tested. None means negative control. (A) DENV-1 EDIII mAbs (B) DENV-2 EDIII mAbs (C) DENV-4 EDIII mAbs

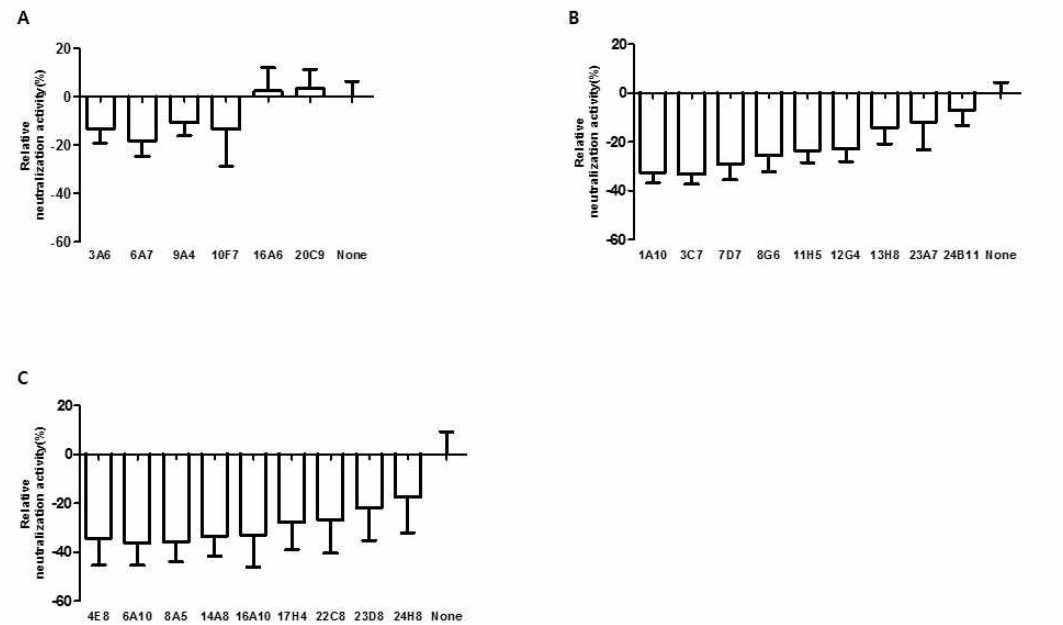

Figure. 22. Neutralizing activity of heterologous DENV EDIII mAbs against DENV-4

To identify neutralizing activity between DENV-4 and heterologous mAbs, microneutralization test was tested. None means negative control. (A) DENV-1 EDIII mAbs (B) DENV-2 EDIII mAbs (C) DENV-3 EDIII mAbs

Table 5. Summary table for results of binding and neutralizing test

DENV-1		3A6	6A7	9A4	10F7	16A6	20C9			
DENV-1	Binding	1	2	1	2	1	1			
	Neutralizing	++	+	+	+	-	-			
DENV-2	Binding	0	0	0	0	0	0			
	Neutralizing	-	-	+	+	+	++			
DENV-3	Binding	1	1	1	1	1	1			
	Neutralizing	-	-	-	+	+	+			
DENV-4	Binding	0	0	0	0	0	0			
	Neutralizing	-	-	-	-	+	+			
DENV-2		**1A10**	**3C7**	**7D7**	**8G6**	**11H5**	**12G4**	**13H8**	**23A7**	**24B11**
DENV-1	Binding	1	1	1	1	1	1	1	1	1
	Neutralizing	-	-	-	-	-	-	-	-	-
DENV-2	Binding	0	0	0	2	0	0	0	0	0
	Neutralizing	+	++	4+	+++	+++	+++	4+	+++	+++
DENV-3	Binding	1	1	1	1	1	1	1	1	1
	Neutralizing	-	-	+	+	+	+	++	+	+
DENV-4	Binding	0	0	0	0	0	0	0	0	0
	Neutralizing	-	-	-	-	-	-	-	-	-
DENV-3		**4E8**	**6A10**	**8A5**	**14A8**	**16A10**	**17H4**	**22C8**	**23D8**	**24H8**
DENV-1	Binding	1	1	1	1	1	1	1	1	1
	Neutralizing	-	-	-	-	-	-	-	-	-
DENV-2	Binding	0	0	0	0	0	0	0	0	0
	Neutralizing	++	4+	4+	4+	4+	4+	4+	4+	4+
DENV-3	Binding	2	2	2	2	2	2	2	2	2
	Neutralizing	-	+	+	+	+	++	+	++	+
DENV-4	Binding	0	0	0	0	0	0	0	0	0
	Neutralizing	-	-	-	-	-	-	-	-	-
DENV-4		**2B11**	**3A2**	**8C5**	**9G3**	**12D7**				
DENV-1	Binding	1	1	1	1	1				
	Neutralizing	-	-	-	-	+				
DENV-2	Binding	0	0	0	0	0				
	Neutralizing	-	-	-	+	+				
DENV-3	Binding	1	1	1	1	1				
	Neutralizing	-	-	-	-	+				
DENV-4	Binding	1	1	1	1	1				
	Neutralizing	+	+	+	+	+				

Discussion

In this study, we aimed to identify functional properties of the DENV EDIII mAbs elicited by soluble recombinant proteins. The soluble hRID-DENV EDIII recombinant fusion proteins were expressed in *E. coli*. After mice were immunized with the recombinant proteins, B-lymphocytes were collected, and DENV EDIII mAbs were produced using hybridoma technology. DENV EDIII peptide overlapping libraries were also synthesized, and epitope mapping and neutralization tests were performed.

According to previous studies, EDIII plays an important role in binding host receptors in a serotype-specific manner [30-32]. However, EDIII-specific antibodies form a small part of the neutralizing antibody response; they do not fully represent the considerable range of type specific neutralization activity [33-37]. Furthermore, subneutralizing concentrations of antibodies cause ADE in cells expressing FcγR, and sufficient receptor occupancy and antibody concentration are critical for effective neutralization [38]. Additionally, DENV viral breathing, in which the structure of E protein and glycan is rearranged, occurs primarily at temperatures above 37°C; therefore, some neutralizing mAbs bind epitopes that are exposed temporarily during viral breathing [39-41].

In this study, although the mAbs of all four serotypes bound to peptide 1-1, most of the DENV-1 mAbs did not exhibit neutralizing activity. The reason for this observation may be that antibodies targeting only EDIII proteins tend not to show neutralization. Thus, peptide 1-1 may be a

common binding epitope among the four DENV serotypes. Moreover, DENV-1 mAbs 6A7 and 10F7 exhibited binding activity in response to peptides 1-1 and 1-6. When the epitopes were mapped onto the crystal structure, the two peptides were close to each other, indicating that mAbs 6A7 and 10F7 target peptides 1-1 and 1-6 simultaneously.

In the case of DENV-2, only mAb 8G6 bound strongly to peptides 2-12 and 2-13. In a previous study, peptide 2-13 also showed binding activity in response to goose-derived anti-DENV-2 IgY, so we predicted that mAb 8G6 would be a potential antibody [42,43]. In terms of neutralization, most of the DENV-2 mAbs showed considerable neutralizing activity in DENV-2 and DENV-3, although mAb 8G6 showed homologous neutralizing activity similar to that of other DENV-2 mAbs. Therefore, DENV-2 mAb 8G6 and peptides 2-12 and 2-13 may be a potential neutralizing antibody and binding epitopes, respectively. The lack of binding activity may be due to factors such as the avidity and morphology of the peptides during peptide coating. Moreover, it is assumed that some neutralizing mAbs bind with hidden epitopes that are transiently exposed during incubation at 37°C.

In the case of DENV-3, all DENV-3 mAbs bound to peptides 3-1 and 3-2. Peptide 3-1 bound to mAbs of all four DENV serotypes. Therefore, peptide 3-1 may be a common binding epitope among the four DENV serotypes. In terms of neutralization, most of the DENV-3 mAbs showed neutralizing activity in response to DENV-2. This result indicates that DENV-3 mAbs target structurally similar sites in DENV-2 and DENV-3.

In the case of DENV-4, all DENV-4 mAbs exhibited binding activity in response to peptide 4-23 and this suggests that peptide 4-23 is a binding epitope. DENV-4 mAb 12D7 exhibited broad-spectrum neutralizing activity in response to all four DENV serotypes; therefore, mAb 12D7 may be a broad-spectrum neutralizing antibody that targets a structurally similar antigenic site in the four DENV serotypes. The lack of neutralization may have occurred because the EDIII-specific antibodies tend not to exhibit neutralizing activity.

Additionally, some results showed that the neutralizing activity represented viral infection enhancement by mAbs. In previous studies, ADE was caused by various complement receptors and occurred in cells that do not express FcγR, such as fibroblasts and epithelial cells [44-46]. Therefore, the enhancement of the viral infection is attributed to ADE caused by other mechanisms. Overall, peptides 1-1, 1-6, 2-12, 2-13, 3-1, and 4-23 showed binding activity in response to DENV EDIII mAbs. Among them, peptides 2-12, 2-13 and 4-23 may be effective neutralizing epitopes, and mAb 8G6 may be effective antibodies. MAb 12D7, which showed broad-spectrum antiviral activity, may be helpful for developing therapeutic agents against all four serotypes. Further studies are required to increase the neutralizing activity of the mAbs. Moreover, this study had limitations; the binding activity of only linear peptides was tested, and only antibodies specific to EDIII were examined. Therefore, further studies are necessary to investigate the binding activity of other peptide types and to evaluate antibodies targeting additional antigenic sites, at various concentrations.

논 문 개 요

매년, 전 세계적으로 약 4억 명의 사람들이 뎅기바이러스에 감염된다. 뎅기바이러스는 1형부터 4형까지 네 가지 혈청형을 가지며 뎅기바이러스의 감염은 대부분 무증상 또는 미약한 증상을 보이지만, 다른 혈청형에 의한 두번째 감염은 항체의존면역증강 (antibody-dependent enhancement)을 발생시키는 특성을 가지고 있다. 이로 인해 중증 뎅기열 (dengue fever), 뎅기 출혈열 (dengue hemorrhagic fever), 뎅기 쇼크 증후군 (dengue shock syndrome)등의 심각한 증상을 일으키며 사망에 이를 수 있다. 뎅기바이러스는 주로 열대지방과 아열대 지방에서 많이 발생한다. 그러나 최근에는 지구온난화의 가속화로 인하여 모기의 서식지가 증가함에 따라 뎅기열 발병 또한 증가하고 있는 추세이다. 현재 뎅기바이러스에 특이적으로 작용하는 치료제는 없으며, 2019년 FDA의 승인을 받은 뎅기바이러스 백신, Dengvaxia, 역시 부작용으로 인한 사용의 한계가 있다. 그러므로 여전히 뎅기바이러스의 네가지 혈청형을 모두 예방할 수 있는 백신 또는 치료제의 개발이 필요한 상황이다.

이 연구에서는, *E. coli* 발현 시스템을 이용하여 뎅기바이러스 재조합단백질 항원을 수용성의 형태로 발현하였고, 이를 이용하여 뎅기바이러스의 단클론항체를 생산한 후 이들의 특성을 확인하였다. 수용성 재조합 단백질을 생산하는 과정에서, *E. coli* 발현 시스템 내의 적절한 단백질의 번역 후 변형 (post translational modification) 과정을 수행하기 위하여 타겟 단백질에 human RNA-interacting domain (hRID)을 융합한 hRID-DENV EDIII 재조합 융합 단백질을 이용하였으며, 하이브리도마 기술을 이용하여 각 혈청형 별 단클론항체를 생산하였다. 또한 에피토프 매핑을 위한

뎅기바이러스 E protein domain III (EDIII)의 펩타이드 라이브러리를 합성하였다. 효소면역분석법 (enzyme-linked immunosorbent assay)을 이용하여 에피토프 매핑을 진행하였으며, microneutralization test를 이용하여 각 혈청형의 뎅기바이러스에 대한 단클론항체의 중화능을 확인하였다. 결과적으로, 단클론항체와 EDIII 펩타이드가 상당한 결합력을 나타냄에도, 뎅기바이러스의 EDIII만을 타겟하는 단클론항체는 대체적으로 중화능이 부족함을 확인하였으며, 반대로 단클론항체와 EDIII 펩타이드가 결합력을 보이지 않음에도 불구하고 몇몇 단클론항체는 뎅기바이러스에 대한 중화능을 나타내는 것을 확인하였다. 특히 2형 뎅기바이러스의 단클론항체 8G6 과 펩타이드 2-12 와 2-13 간의 결합력과 중화능은 모두 상당한 효과를 나타냈으며, 펩타이드 2-12 와 2-13은 단클론항체 8G6의 핵심 중화 에피토프 일 것 이라고 추측한다. 뎅기바이러스 4형의 단클론항체 12D7은 뎅기바이러스 네 가지 혈청형 모두에 중화능을 보이는 광범위 항바이러스 효과 (broad-spectrum antiviral activity)를 확인하였다.

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감사의 글

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많은 것들을 배웠고 후회 없는 연구실 생활을 할 수 있었습니다. 무엇보다도 저의 일들을 선배의 일처럼 진심으로 들어주고 응원해준 점 너무 감사하고 선배 덕분에 소소한 기념일들을 축하하며 살아가는 낭만도 배울 수 있어 즐거웠습니다. 저의 일이지만 항상 같이하자며 도와주는 예은선배 감사합니다. 처음 들어왔을 때 선배에게 세포실험을 배우면서 저도 선배처럼 따뜻한 선배가 되겠다고 다짐했습니다. 수다스러운 저에게 호응해주고 즐겁게 웃어주는 선배가 있어서 마음 편한 연구실 생활을 할 수 있었습니다. 진심으로 저를 도와주고 응원해 주셔서 너무 감사하고 선배 덕분에 2021년 마지막날을 즐거운 추억으로 마무리할 수 있었습니다. 어른처럼 저를 응원해주시고 먼저 챙겨주었던 다빈선배 감사합니다. 저녁의 연구실을 보면 선배와 같이 실험하던 기억이 떠올라 즐겁습니다. 선배의 지혜 덕분에 많은 것 들을 배울 수 있었습니다. 언제나 선배의 행복한 미래를 응원하겠습니다. 바쁜 와중에 많은 도움을 주신 나은선배 감사합니다. 아무것도 몰랐던 제가 선배 덕분에 무사히 졸업과정을 마무리하고 있습니다. 항상 선배의 일처럼 먼저 챙겨 주시고 무엇이든 친절히 도와주셔서 감사합니다. 선배가 있어서 졸업준비를 하는 동안 많은 힘이 되었습니다. 그리고 도움요청에 자신의 일처럼 도와준 제 친구 수진이에게도 감사의 인사를 전합니다.

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