

# Functional properties of DENV EDIII-reactive antibodies in human DENV-1-infected sera and rabbit antiserum to EDIII

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**Abstract.** The envelope domain III (EDIII) of the dengue virus (DENV) has been confirmed to be involved in receptor binding. It is the target of specific neutralizing antibodies, and is considered to be a promising subunit dengue vaccine candidate. However, several recent studies have shown that anti-EDIII antibodies contribute little to the neutralizing or enhancing ability of human DENV-infected serum. The present study involved an analysis of the neutralization and antibody-dependent enhancement (ADE) activities of EDIII-reactive antibodies in human convalescent sera from patients with primary DENV-1 infection and rabbit antiserum immunized with recombinant DENV-1 EDIII protein. The results indicated that serum neutralization was not associated with titres of EDIII-binding antibodies in the human DENV-1-infected sera. The depletion of anti-EDIII antibodies from these serum samples revealed that the anti-EDIII antibodies of the patients contributed little to neutralization and ADE. However, the EDIII-reactive antibodies from the rabbit antiserum exhibited protective abilities of neutralization at a high dilution (~1:50,000) and ADE at a low dilution (~1:5,000) for the homotypic DENV infection. Notably, the rabbit antiserum displayed ADE activity only at a dilution of 1:40 for the heterotypic virus infection, which suggests that EDIII-reactive antibodies may be safe in secondary infection with heterotypic viruses. These results suggest that DENV EDIII is not the predominant antigen of the DENV infection process; however, purified or recombinant DENV EDIII may be used as a subunit vaccine to provoke an effective and safe antibody response.

## Introduction

Dengue is a mosquito-borne viral disease caused by four serologically and genetically related viruses termed DENV-1, DENV-2, DENV-3 and DENV-4. Infection with each DENV serotype causes an array of clinical diseases, ranging from dengue without warning signs, dengue with warning signs to severe dengue-dengue haemorrhagic fever and dengue shock syndrome (1-4). Individuals infected with one serotype can acquire lifelong homotypic immunity (5); however, those suffering secondary DENV infection with another serotype may have a greater risk of progressing to severe dengue (6). Antibody-dependent enhancement (ADE) by pre-existing cross-reactive, weakly neutralizing antibodies has been suggested to be important in disease severity by promoting virus entry into Fcγ receptor-bearing cells (7). The presence of ADE impedes the development of dengue vaccines to induce protective neutralizing antibodies without enhancing viral replication.

The surface of the mature DENV particle is covered with 90 head-to-tail homodimers of envelope glycoprotein (E) (8), which is the major surface protein of flaviviruses involved in multiple processes, such as viral adsorption, membrane fusion and cell tropism. The E protein ectodomain can be divided into three distinct structural domains designated as I (EDI), II (EDII) and III (EDIII). Structurally, EDIII has been proposed to be a putative receptor-binding domain (9). EDIII-reactive antibodies are found in convalescent sera from patients infected with dengue (10,11).

The EDIII-reactive antibodies can function as effective neutralizers and also may possess varying degrees of enhancing activity (12-19). Recent studies have revealed that these antibodies from virus-infected serum are considered to contribute little to neutralizing and enhancing activities (11,20-22). Thus, it could be presumed that antibodies from patient sera are induced by a number of different epitopes on complex DENV *in vivo*, and not all protective epitopes can serve as predominant epitopes to provoke an effective neutralizing antibody response or ADE; however, protective non-predominant epitopes may serve as purified subunit vaccine candidates to induce a protective response. Additionally, the majority of research on the role of EDIII-reactive

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antibodies of human convalescent serum focused on DENV-2 and DENV-3 infections (11,22), however, >60% of patients with dengue in southern China have DENV-1 infection. In this study, therefore, neutralization and ADE activity as well as the EDIII-binding antibody titre on homotypic and heterotypic DENVs in human DENV-1-infected sera and rabbit antiserum immunized with recombinant EDIII protein were evaluated. Furthermore, the contribution of EDIII-reactive antibodies was verified by depleting EDIII-reactive antibodies from human sera and rabbit antiserum using Dynabead-EDIII conjugates.

## Materials and methods

**Ethics statement.** The current study was approved by the ethics committee of the Zhujiang Hospital of Southern Medical University (Guangzhou, China).

**Patients.** The DENV-1-infected convalescent patients were recruited by follow-up phone calls and achieving consensus. A total of 30 primary DENV-1-infected convalescent human serum samples were collected from the Guangzhou Centre for Disease Control and Prevention (Guangzhou, China). The convalescent human serum samples were obtained by venous blood.

**Animals.** New Zealand white rabbits (n=3) were purchased from the Laboratory Animal Centre of Southern Medical University. The animals were housed at 25°C, with 60% relative humidity and free access to food and water. The serum was collected from the jugular vein of the rabbit after anaesthesia. The rabbits were sacrificed with pentobarbital (30 mg/kg weight) at 9 weeks.

**Cells and viruses.** LLC-MK2 (#CCL-7) and K562 (#CCL-243) (American Type Culture Collection, Manassas, VA, USA) cells were cultured in a 5% CO<sub>2</sub> incubator at 37°C supplemented with 10% fetal bovine serum (FBS, Gibco; Thermo Fisher Scientific, Inc., Carlsbad, CA, USA) in minimal essential medium (MEM; Gibco, Thermo Fisher Scientific, Inc.) and RPMI-1640 medium (Gibco), respectively. The four dengue serotype strains (DENV-1, Hawaii; DENV-2, New Guinea-C; DENV-3, Guanxi-80-2; and DENV-4, H241, provided by the Center for Disease Control and Prevention of Guangzhou, China) used in this study were propagated in C6/36 *Aedes albopictus* cells (American Type Culture Collection; #CRL-1660) in MEM containing 10% FBS at 33°C for three to five days in the presence of 5% CO<sub>2</sub> as described previously (23).

**Expression, purification and labelling of DENV recombinant EDIII proteins.** DENV rEDIII proteins of the four dengue serotypes were produced as described previously (24). In brief, the genes encoding DENV EDIII of the four DENV serotypes were amplified and cloned separately into a pPIC9K vector (Invitrogen, Thermo Fisher Scientific, Inc.), and the 6X His tag coding sequence (synthesized by Sangon Biotech Co., Ltd., Shanghai, China) was added downstream. Recombinant plasmids were then transferred into *Pichia pastoris* GS115 (Invitrogen, Thermo Fisher Scientific, Inc.). The expression of rEDIII protein for each DENV serotype was initiated using methanol induction, and the protein was subsequently purified

using Ni-nitrilotriacetic acid affinity chromatography (Qiagen, Hilden, Germany). The purified rEDIII protein was used to prepare the Dynabead-histidine-tagged rEDIII conjugates (Thermo Fisher Scientific, Inc.) and horseradish peroxidase (HRP)-rEDIII conjugates by a periodate method (25).

**Serum samples.** A total of 30 primary DENV-1-infected convalescent human serum samples confirmed by RT-PCR and a dengue IgG and IgM capture enzyme-linked immunosorbent assay (ELISA; Panbio, Windsor, Australia) were collected from the Guangzhou Centre for Disease Control and Prevention (Guangzhou, China). The 30 serum samples were obtained from patients aged 10-75 years, 15 males and 15 females. The control sera were obtained from healthy individuals without dengue. The collection and use of human sera were in compliance with the Ethics Committee of the Zhujiang Hospital of Southern Medical University (Guangzhou, China). Rabbit antisera against DENV-1 rEDIII were prepared in New Zealand white rabbits using the following procedure: The rabbits were subcutaneously immunized with 500 µg purified DENV-1 rEDIII emulsified with Freund's adjuvant (Sigma-Aldrich, St. Louis, MO, USA) at ten-day intervals for 8 weeks followed by intravenous boosters at three days prior to serum collection. purified DENV-1 rEDIII (50 µg) was injected through the ear marginal intravenously. Three days later, the serum was collected from jugular vein of the rabbit after pentobarbital anaesthesia.

RT-PCR was performed according to a previously described protocol (26). Briefly, RT-PCR was performed using an ABI 7300 PCR thermal cycler (Applied Biosystems; Thermo Fisher Scientific, Inc.). With the DEN-1 assay as an example, the PCR mixture included a final volume of 20 µl with 10 µl one-step RT-PCR Master kit (Qiagen China Co., Ltd., Shanghai, China), 0.5 µl (10 pmol ml<sup>-1</sup>) each primer, 1.5 µl (2 pmol ml<sup>-1</sup>) probe, 5.5 µl distilled water and 2 µl extracted RNA. The amplification conditions were as follows: An initial step at 50°C for 30 min and 95°C for 15 min, followed by 40 cycles of 95°C for 15 sec, annealing and elongation at 60°C for 60 sec, with fluorescence acquisition in single mode. Negative controls included human RNA, C6/36 RNA, distilled sterile water and PCR mixture. All experiments were repeated four times. The PCR mixture for the remaining three DEN assays were the same as for DEN-1. Amplified products were detected by agarose gel electrophoresis and sequence identification. The sequencing was undertaken on an ABI PRISM 3730 DNA Sequencer. Primers and probes used in group-specific and serotype-specific real-time assays for DENV are as previously described (27).

**Detection of EDIII-binding antibodies by double-antigen sandwich ELISA.** A double-antigen sandwich ELISA was performed to evaluate the titres of the EDIII-binding antibodies in the serum samples described above. Microwell plates (Costar Corning, Inc., Corning, NY, USA) were coated with 100 µl/well DENV rEDIII at a concentration of 0.1 µg/ml (for DENV-1 and DENV-2) or 0.05 µg/ml (for DENV-3 and DENV-4) overnight at 4°C followed by blocking with 2.5 g casein sodium salt, 1.21 g Tris-base, 2 g gelatin, 20 g sucrose, 0.2 g Merthiolate, and 5 ml Tween 20 in 1000 ml dH<sub>2</sub>O (Sigma-Aldrich). The diluted serum samples were added (100 µl/well) and incubated for 1 h at 37°C. After

Table I. Neutralization titres and anti-DENV-1 EDIII serum titres of DENV-1-infected sera.

Patient number	NT50 titres <sup>a</sup>				Anti-DENV-1 EDIII serum titres <sup>b</sup>
	DENV-1	DENV-2	DENV-3	DENV-4	
1	950.2	227.3	194.7	63.2	40
2	1190.0	262.0	237.9	237.7	80
3	1630.0	273.0	89.7	86.0	40
4	1827.0	228.4	167.3	150.1	80
5	1020.0	1023.0	918.5	271.6	40
6	2896.0	2153.0	918.0	302.7	80
7	2290.0	597.8	1155.0	276.2	40
8	1316.0	2357.0	681.7	70.6	20
9	1043.0	318.8	586.2	141.6	20
10	587.1	108.1	164.6	<20	5
11	4114.0	1399.0	1061.0	368.1	80
12	2666.0	302.2	159.4	543.7	<5
13	3708.0	133.1	36.2	<20	10
14	794.4	50.05	24.7	<20	5
15	529.7	103.4	331.2	<20	10
16	3666.0	141.8	204.2	48.9	20
17	1419.0	164.9	142.0	102.5	20
18	568.2	47.8	32.8	<20	20
19	1763.0	337.8	291.3	<20	10
20	2582.0	325.5	38.9	<20	40
21	1219.0	34.2	<20	<20	<5
22	1836.0	440.7	34.6	<20	20
23	153.6	28.35	28.5	<20	20
24	4707.0	630.6	1302.0	75.0	80
25	962.5	88.4	129.3	<20	10
26	10684.0	742.7	2156.0	385.2	80
27	240.4	68.8	79.0	<20	40
28	2013.0	395.2	75.7	<20	<5
29	4960.0	634.5	503.0	<20	<5
30	4178.0	57.6	44.3	<20	20

<sup>a</sup>NT50 neutralization titres of the sera are shown as reciprocal serum dilutions at which the viral infection was inhibited by 50%. <sup>b</sup>Anti-DENV-1 EDIII serum titres are displayed the highest reciprocal dilution that gave an indicator of >3 standard deviations of the indicator produced for the control human sera. DENV, dengue virus; EDIII, envelope domain III; NT50, 50% neutralizing titers.

washing, diluted horseradish peroxidase (HRP)-conjugated DENV rEDIII with 100  $\mu$ l/well was added to the plates, and an additional incubation was performed for 40 min at 37°C. Finally, the colour reactions were visualized by adding tetramethylbenzidine (TMB, KPL, Gaithersburg, VA, USA) substrate (100  $\mu$ l/well) and quenched with an equal volume of 0.3 mol/l sulphuric acid. The absorbance was recorded at 450 nm on an ELISA plate reader (Bio-Tek, Winooski, VT, USA).

*Depletion of DENV EDIII-binding antibodies from sera.* Dynabeads (1- $\mu$ m diameter) were conjugated with histidine-tagged rEDIII according to the manufacturer's protocol (Thermo Fisher Scientific, Inc.). Briefly, the Dynabeads were washed thoroughly 4 times with binding/wash buffer

(0.05 mol/l sodium-phosphate, 0.3 mol/l NaCl and 0.01% Tween-20) by standing the tube on a magnet for 2 min and discarding the supernatant. An excess of rEDIII protein was then added to the Dynabeads and mixed. The mixture was incubated on a roller (Grant Instruments, Cambridge, UK) for 30 min at 4°C. In general, Dynabeads have the capacity to isolate 15  $\mu$ g EDIII/mg beads. Naked Dynabeads served as a negative control and were incubated with binding buffer only under the same conditions. After washing thoroughly as mentioned above, the serum sample was incubated with the Dynabead-EDIII complex on a roller for 2 h at 4°C. This depletion procedure for each serum sample was repeated at least three times with a new Dynabead-EDIII complex, and the depletion efficiency was confirmed by a double-antigen sandwich ELISA with rEDIII.

**In vitro neutralization assay using enzyme-linked immunospot microneutralization test (ELISPOT-MNT).** The ELISPOT-MNT was performed as previously described with several modifications (28). Briefly, serially diluted serum samples were blended with equal volumes of virus containing ~200 PFUs per well, then serum and virus mixtures were incubated for 1 h at 37°C. The serum and virus mixtures were then added to the LLC-MK2 cell monolayers cultured in 96-well plates (Greiner Bio-One, Frickenhausen, Germany) and incubated for 1.5 h. Next, these cells were covered with semi-solid MEM medium containing 1% methylcellulose (Sigma-Aldrich) and 5% heat-inactivated FBS. The cell monolayers were incubated at 37°C for two to three days. Then the cells were fixed and stained with the anti-DENV non-structural protein 1 (NS1) monoclonal antibody 5F10A7. Following the addition of HRP-conjugated polyclonal goat anti-mouse IgG (1:300; cat no. A5278; Sigma-Aldrich), plaques were visualized with AEC solution (SK4200; Vector Laboratories, Burlingame, CA, USA) and counted with an automated ELISPOT instrument (Cellular Technology Ltd., Shaker Heights, OH, USA). A 50% reduction in the number of plaques compared with the virus dose control absenting a target serum was calculated as follows: % reduction =  $100 \times [1 - (\text{average number of plaques for each dilution} / \text{average number of plaques for virus dose control})]$ .

**In vitro enhancement assay by NS1 capture ELISA-based ADE assay (ELISA-ADE).** The assessment of ADE by measuring DENV NS1 production in Fcγ receptor-expressing K562 cell culture supernatants was performed by modified ELISA-ADE as previously described (29). Briefly, serially diluted serum samples were incubated with an equal volume of DENV at a multiplicity of infection of 0.5 or 0.125 in 96-well plates for 1 h at 37°C. The K562 cells were adjusted to a concentration of  $5 \times 10^4 / 100 \mu\text{l}$  and added to the virus-antibody mixture. Following incubation for 2 h at 37°C, the K562 cells were washed twice and cultured in RPMI-1640 medium containing 5% FBS for 4 days at 37°C. The 96-well plates were placed at -80°C overnight, and the NS1 levels in the freeze-thawed 96-well plate supernatants were used to evaluate the severity of viral infection using an NS1 antigen-capture ELISA previously developed in our laboratory (23). To distinguish between enhancing and non-enhancing activities, a cut-off value was set as a mean absorbance at 450 nm plus 3 standard deviations using eight wells containing the virus control in the absence of antibodies. The K562 cells incubated with DENV alone were set as the infection baseline from which fold enhancement was determined.

**Statistical analysis.** Statistical analyses were performed using GraphPad Prism version 5.04 (GraphPad Software Inc., La Jolla, CA, USA) and Predictive Analytics Suite Workstation (PASW; version 20.0; IBM SPSS, Armonk, NY, USA). The 50% neutralizing titers (NT50) of the sera corresponding to a 50% plaque reduction, were computed using a nonlinear regression (curve fit) analysis. The peak enhancement titre (PET) of the serum corresponded to the serum dilution in the highest fold enhancement. The differences in neutralization among the four DENV serotypes in the human convalescent serum samples were compared by the analysis of variance

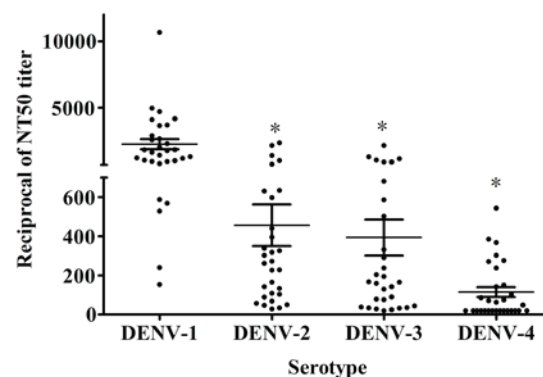


Figure 1. Neutralization titres against all four DENV serotypes in convalescent sera from DENV-1-infected patients. The neutralization titres of thirty serum samples were measured using enzyme-linked immunospot microneutralization test. The NT50 neutralization titres of the serum samples were expressed as reciprocal serum dilutions at which the viral infection was inhibited by 50%. \* $P < 0.05$  vs. DENV-1. DENV, dengue virus; NT50, 50% neutralizing titers.

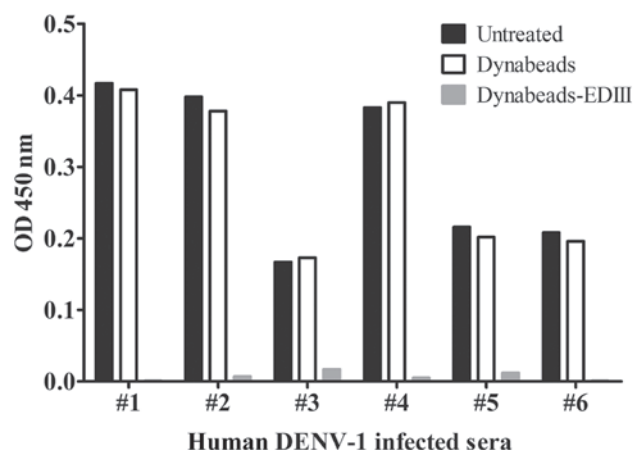


Figure 2. Binding properties of EDIII-reactive antibodies in DENV-1-infected human sera. Six primary DENV-1-infected convalescent human serum samples #1-6 were depleted of homologous EDIII-reactive antibodies using Dynabead-EDIII conjugates. Total homologous EDIII-reactive antibodies were assessed for DENV-1 EDIII binding by double-antigen sandwich enzyme-linked immunosorbent assay, and the absolute OD value at 450 nm is shown on the y-axis. The data display one of two representative experiments. EDIII, envelope domain III; DENV, dengue virus; OD, optical density.

and least significant difference multiple comparison tests. The correlation between neutralization activities and EDIII-specific antibody titres to DENV-1 in the human convalescent serum samples was calculated by Spearman's rank correlation test. The neutralization and enhancement differences of the six DENV-1-infected sera prior to and following EDIII antibody-depletion were compared with analysis of variance.

## Results

**Correlation between neutralization and EDIII-reactive antibody titres in convalescent sera from DENV-1-infected patients.** To evaluate the contribution of EDIII-reactive antibodies to the total neutralization activity of convalescent sera from patients infected with DENV-1, serum titres



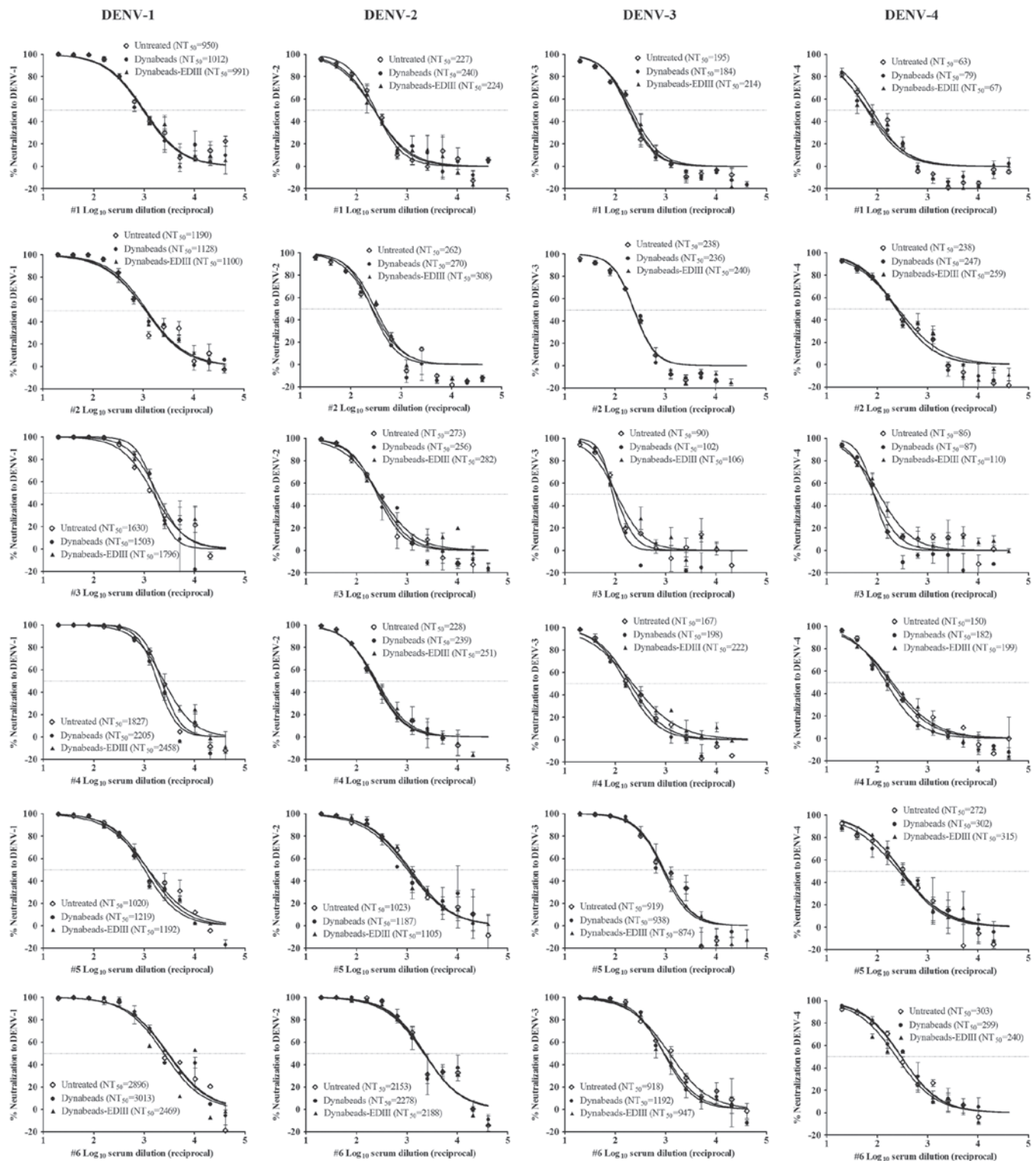


Figure 3. Neutralization properties of EDIII-reactive antibodies in DENV-1-infected human sera. The neutralization activities of the above six DENV-1-infected human serum samples for all four DENV serotypes were measured in parallel in untreated, Dynabeads and Dynabead-DENV-1 EDIII absorption serum samples. The percentage of plaque reduction is shown on the y-axis, the log reciprocal dilution of the serum is shown on the x-axis, and the dashed line indicates 50% plaque reduction. For each serum sample, the neutralization curve fits generated by nonlinear regression analysis are presented. The data show one of two independent experiments in duplicate. The error bars represent the standard error of the mean of duplicate wells. EDIII, envelope domain III; DENV, dengue virus; NT50, 50% neutralizing titers.

specific to DENV-1 recombinant EDIII (rEDIII) protein in 30 human serum samples were detected by a double-antigen sandwich ELISA. In addition, the NT50 of these sera were detected by ELISPOT-MNT. The results indicated that the

EDIII-binding antibody titres of the patient serum samples were very low that was not correlated with the serum NT50 titres ( $R=0.215$ ,  $P=0.253$ ) (Table I), implying that EDIII-reactive antibodies may not exhibit a major role in

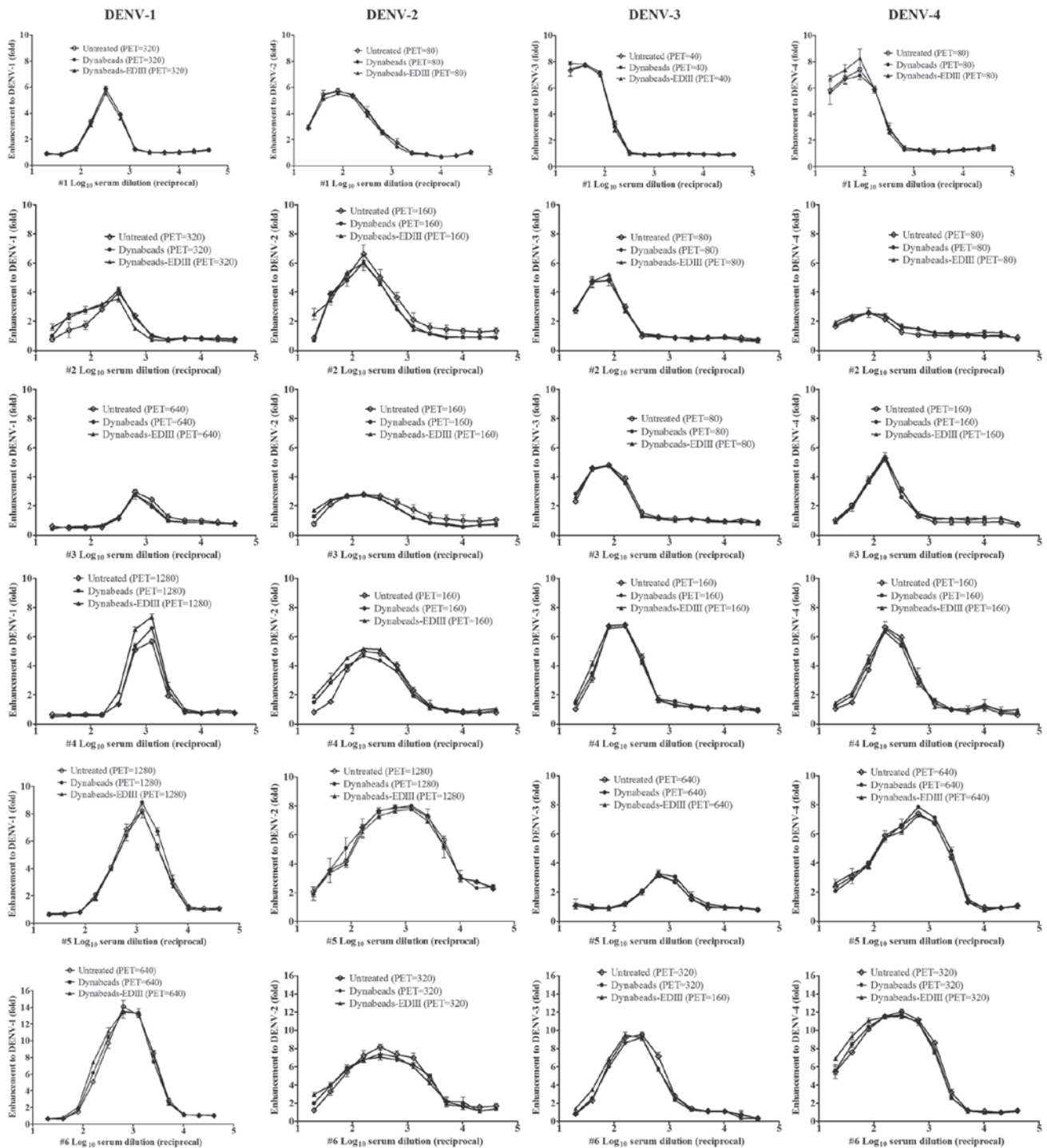


Figure 4. Enhancement properties of EDIII-reactive antibodies in DENV-1-infected human sera. The peak enhancement titres of thirty serum samples were assessed using ELISA-ADE. The ADE activities of the six DENV-1-infected human serum samples for all four dengue serotypes were measured in untreated, Dynabead and Dynabead-DENV-1 recombinant EDIII absorption serum samples. Fold enhancement is shown on the y-axis, and the log reciprocal dilution of the serum is shown on the x-axis. The data show one of two independent experiments in duplicate. The error bars represent the standard error of the mean of duplicate wells. EDIII, envelope domain III; DENV, dengue virus; NT50, 50% neutralizing titers; PET, peak enhancement titre; ADE, antibody-dependent enhancement.

neutralization in DENV-infected human sera. Furthermore, the mean reciprocal NT50 values for DENV-1, DENV-2, DENV-3 and DENV-4 in these 30 sera were 2250, 455.8, 393.6 and 114.1, respectively. A significant difference was identified among four serotypes of NT50 in these 30 sera ( $F=15.412$ ,  $P<0.001$ ) and the sera had a significantly higher NT50 to DENV-1 than the heterogeneous serotypes ( $P<0.05$ )

(Fig. 1 and Table I), which are consistent with the DENV-1 infection profile.

*Role of EDIII-reactive antibodies in human DENV-1-infected sera.* To further identify the functional properties of EDIII-reactive antibodies in DENV-1 convalescent serum, the neutralization and ADE activities of EDIII antibody-depleted

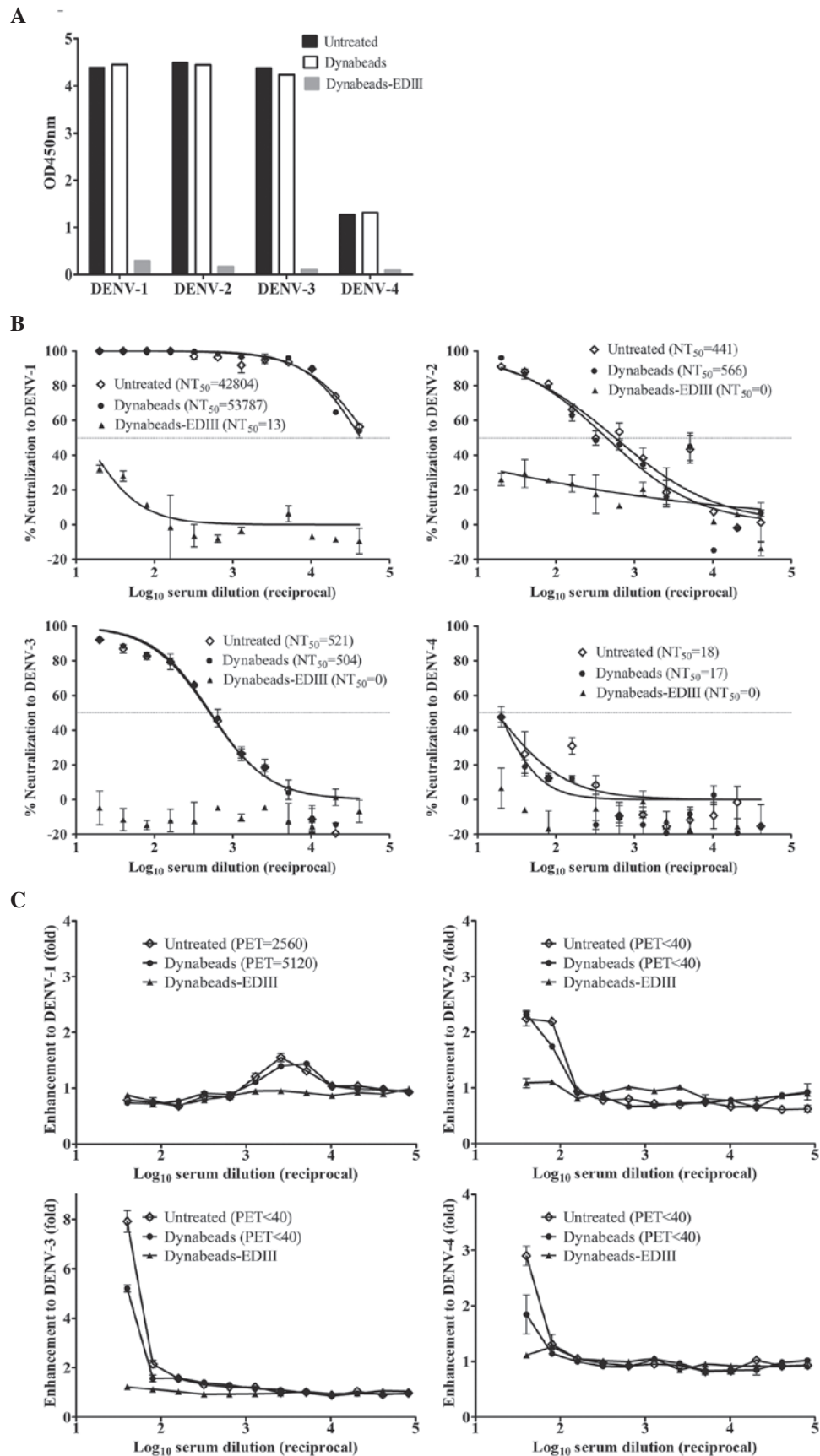


Figure 5. Functional properties of EDIII-reactive antibodies in rabbit antiserum. (A) EDIII-reactive antibodies were depleted from rabbit antiserum using Dynabead-DENV-1 reEDIII, and the antiserum was assessed for DENV-1, DENV-2, DENV-3 and DENV-4 EDIII-binding properties by ELISA. The neutralization (B) and enhancement (C) properties for the four dengue serotypes in the rabbit antiserum before and after the depletion of EDIII-reactive antibodies were measured with enzyme-linked immunospot microneutralization test and ELISA-ADE. The data display one of two representative experiments. The error bars represented the standard error of the mean of duplicate wells. EDIII, envelope domain III; DENV, dengue virus; NT50, 50% neutralizing titers; PET, peak enhancement titre; ADE, antibody-dependent enhancement; ELISA, enzyme-linked immunosorbent assay.

sera were measured. First, six human convalescent serum samples from patients with primary DENV-1 infection were incubated with Dynabeads-DENV-1 rEDIII conjugates to deplete the EDIII-binding antibodies. As shown in Fig. 2, the depletion successfully removed the EDIII-reactive antibodies in the sera compared with the naked Dynabead absorption control. Then, ELISPOT-MNT and ELISA-ADE were performed to measure the neutralization and ADE activities of the EDIII-specific antibody-depleted sera. Untreated sera and Dynabead absorption sera were used as controls. No significant difference in the neutralization titers in the group treated with Dynabeads-EDIII compared with the group treated with Dynabeads control in the six sera samples for DENV-1 ( $F=0.030$ ,  $P=0.971$ ), DENV-2 ( $F=0.006$ ,  $P=0.994$ ), DENV-3 ( $F=0.028$ ,  $P=0.972$ ) and DENV-4 ( $F=0.039$ ,  $P=0.962$ ) (Fig. 3). In addition, there was no observed significant difference in the enhancement in the group treated with Dynabeads-EDIII compared with the group treated with Dynabeads control in the 6 sera samples for DENV-1 ( $F=0.000$ ;  $P>0.05$ ), DENV-2 ( $F=0.000$ ;  $P>0.05$ ), DENV-3 ( $F=0.028$ ;  $P=0.973$ ) and DENV-4 ( $F=0.000$ ;  $P>0.05$ ) (Fig. 4). The results indicate no significant loss of neutralization or ADE activity for the four dengue serotypes after the removal of EDIII-specific antibodies from the human sera. Therefore, this suggested that EDIII-specific antibodies in primary DENV-1-infected convalescent sera exhibit minor roles in the neutralization and enhancement of viral replication.

**Role of EDIII-reactive antibodies in rabbit antiserum.** Previously, a patient was shown to develop serum antibodies against various DENV proteins after DENV infection (30-33). Based on the consideration that EDIII may be a non-predominant antigen in complex antigenic components of DENV, and EDIII could not induce effective antibody response in natural infection because of the antigenic competition, the present study aimed to determine whether purified DENV-1 rEDIII protein would result in effective protective neutralizing antibody, in addition to the roles of EDIII reactive antibodies in rabbit antiserum. The rabbit antiserum exhibited a strong binding capacity to recombinant EDIII protein with a titre of 1:10,000, which was verified by double antigen sandwich ELISA as that used for human convalescent sera (data not shown), and further confirmed by removing EDIII-specific antibodies using Dynabead-DENV-1 rEDIII (Fig. 5A). Notably, the antiserum displayed concentration-dependent neutralization, in which the rabbit antiserum exhibited an NT50 of ~50,000 for DENV-1, and an NT50 of ~500 for DENV-2 and DENV-3, but without neutralization of DENV-4 (Fig. 5B). In contrast to its neutralization activity, the rabbit antiserum showed markedly lower dilution-dependent ADE activity, and the PET against DENV-1 (~1:5,000) was higher than that of the other serotypes (<1:40) (Fig. 5C). All of the above neutralization and ADE activities were mostly depleted by absorption with Dynabead-EDIII conjugates (Fig. 5B and C). These results indicated that antibodies induced by DENV-1 rEDIII protein in rabbit serum possess stronger neutralization activity and lower ADE on homotypic virus replication, and the lower neutralization and ADE on heterotypic infection. The results also suggest that only a small fraction of

rabbit anti-EDIII antibodies could enhance an infection with heterotypic DENV.

## Discussion

In this study, the correlation between serum neutralization titres and EDIII-reactive antibody titres binding to EDIII protein was determined in 30 convalescent serum samples from patients with primary DENV-1 infection as shown in Table I. Notably, no correlation was identified between high levels of serum NT50 titres and low levels of EDIII-binding titres in patient serum samples implies that EDIII-reactive antibodies may not be involved in neutralization. In total, 6 of 30 convalescent serum samples were treated with Dynabead-DENV-1 rEDIII to remove specific antibodies and it was demonstrated that the serum neutralization and enhancement activities on homotypic and heterotypic DENV replication were unchanged prior to and following the depletion. The depletion with Dynabead-EDIII was demonstrated to be sufficient and specific, since the recombinant EDIII proteins were expressed with the methylotrophic yeast, *Pichia pastoris*, which is closer to the conformation of natural EDIII than the prokaryotic expression protein conjugates (34-36). This depletion further confirmed that EDIII-specific antibodies may be insignificant for the antibody protective response in human convalescent sera, which is in accordance with the reports on DENV by several groups (11,20-22).

To identify the role of EDIII-reactive antibodies by effective immunization, recombinant EDIII protein was purified for immunization to prepare rabbit antiserum. In addition to a high titre of binding to DENV1 rEDIII protein, the EDIII-reactive antibodies of rabbit antiserum showed strong neutralization activity and corresponding ADE activity on homologous serotype virus DENV-1, compared with the weak neutralization and ADE on heterogeneous virus infection. All the above-mentioned activities of rabbit antiserum were completely removed by treatment with Dynabead-EDIII. These results suggest that EDIII-reactive antibodies are critical in the neutralization and ADE activities in rabbit antiserum. Notably, the results also suggest that the ADE of heterotypic DENV only occurs at high concentrations of anti-EDIII antibody, which means that only a small fraction of the anti-EDIII antibody in rabbit antiserum possess ADE activity.

These results supported our hypothesis that DENV EDIII may be not a predominant antigen in complex components of DENV under antigen competition *in vivo*, consequently, DENV EDIII could not induce an effective antibody neutralization or ADE response in the natural infection process. However, immunization using purified recombinant EDIII protein without antigen competition by other virus components could provoke the antibody response with strong neutralization and weak ADE effects, which suggests that this protein could be considered as a subunit vaccine candidate. There were also reports that EDIII-specific human antibody can inhibit DENV infection in a dose-dependent manner (37), a tetravalent vaccine containing two bivalent DENV EDIIIs could induce protective antibody responses against all four DENV serotypes (38), and >50% of the total IgG was targeted against EDIII when mice were vaccinated with DENV virus-like particles (39). In our previous study, two monoclonal antibodies were generated,



2D73 and 3E31, and were shown to possess strong neutralization abilities, but only 2D73 caused ADE (data not shown). This finding may be attributed to the structure of the different epitopes of EDIII protein, which may affect the properties of antibody response, particularly the production of neutralizing or enhancing antibodies. It has been demonstrated that an improvement in the anti-DENV immune response with increasing neutralizing may be achieved by modifying the DENV EDIII protein (40,41). Thus, we hypothesise that the ideal EDIII epitope candidate or epitope combinations would be identified or restructured to induce the antibody response with strong neutralization and weak or no ADE.

The ideal dengue vaccine should induce a life-long protective response against all four DENV serotypes, thereby preventing the risk of ADE. Thus far, only a chimeric recombinant DNA dengue vaccine developed by Sanofi Pasteur completed clinical trial phase 3, this vaccine can provide 60% protection to DENV-1, and 80-90% protection against DENV-3 and DENV-4, but no protection against DENV-2 (42). Based on the epidemiological and aetiological characteristics of dengue infection in southern China, DENV-1 is the most predominant serotype (accounting for >60% of all patients), DENV-2 is occasionally detected, and DENV-3 and DENV-4 are seldom observed (43-46). Therefore, our research has been focused primarily on DENV-1 EDIII in human convalescent serum samples and DENV-1 EDIII immunized rabbit serum, in which the neutralization and ADE on heterotypic DENVs were also observed. In conclusion, the present study demonstrated that purified DENV-1 EDIII protein as a vaccine candidate would be safe to protect from heterotypic DENV infections.

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