Original article

Comparison of infection-neutralizing and -enhancing antibody balance induced by two distinct genotype strains of dengue virus type 1 or 3 DNA vaccines in mice

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Abstract

Dengue viruses have spread throughout tropical and subtropical countries, and vaccine development is urgently needed. However, one concern is that induction of insufficient levels of neutralizing antibodies in vaccines may increase disease severity because of a hypothetical mechanism termed antibody-dependent enhancement of infection. This study used two distinct genotype strains of dengue virus types 1 and 3 (DENV1 and DENV3, respectively) to compare antibody responses in a mouse-DNA vaccine model. As expected, a conventional neutralization test using Vero cells showed higher antibody titers in homologous rather than heterologous combinations of genotype strains used for mouse immunization and the neutralization test, for each of DENV1 and DENV3. However, our assay system using K562 cells to measure the balance of neutralizing and enhancing antibodies indicated that Vero cell-neutralizing antibody titers did not always correlate with enhancing activities observed at subneutralizing doses. Rather, induction of enhancing activities depended on the genotype strain used for mouse immunization. The genotype/strain difference also affected IgG subclass profiles and potentially the composition of antibody species induced in mice. This study suggests that enhancing activities of dengue virus-induced neutralizing antibodies may vary according to the genotype and has implications for vaccine antigen development.

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1. Introduction

Dengue fever (DF) and dengue hemorrhagic fever (DHF) are mosquito-borne infectious diseases of global importance [1-4]. These diseases are distributed in tropical and subtropical regions with an estimated 50-100 million cases and more than 20,000 deaths reported annually. Without appropriate treatment, the mortality of severe cases is greater than 20%. Furthermore, over 40% of the world’s population lives under threat of dengue. Since there is no specific antiviral treatment available for dengue diseases, the development of an effective dengue vaccine is urgently needed.

The causative agent is any one of four serologically and phylogenetically distinct species of dengue viruses, termed dengue virus types 1-4 (DENV1, DENV2, DENV3 and DENV4) of the genus Flavivirus in the family Flaviviridae [5]. In dengue viruses, the species as a taxonomic rank is termed “serotype”. Based on the nucleotide sequences of the envelope (E) protein coding region, each serotype is composed
of three to five genotypes, excepting sylvatic strains [6]. For DENV1 and DENV3, four genotypes exist. Variations in deduced amino acid sequences of the E protein among the four serotypes are approximately 30% [7,8], whereas those among genotypes of each serotype are 3–4% [9,10].

Several approaches have been used for dengue vaccine development, some of which are in the advanced phase of clinical trials [11–14]. All the vaccine strategies to date have used a tetravalent formulation of the vaccines, i.e., they contain antigens of the four serotypes. The tetravalent vaccine is recommended for dengue vaccine, since disease severity correlates to secondary heterotypic infection. The tetravalent approach to dengue vaccine development uses one selected genotype strain for each serotype. Thus, it is critical for a single vaccine strain of a particular genotype to induce responses against strains of the other genotypes from the same serotype.

Antibody-dependent enhancement (ADE) of infection is a hypothetical mechanism that describes increased dengue disease severity related to secondary heterotypic infection [15]. This is thought to be caused by cross-reactive non-neutralizing antibodies, termed “enhancing antibodies”, which can facilitate virus infection in monocytes/macrophages by an Fc gamma receptor (FcγR)-mediated mechanism. Neutralizing antibodies may have infection-enhancing activity at sub-neutralizing doses in vitro, and all the currently developed dengue vaccines can induce neutralizing antibodies; thus, insufficient induction of neutralizing antibodies by a dengue vaccine may increase disease severity in humans [3,16]. Therefore, it is necessary that the dengue tetravalent vaccine can induce balanced neutralizing antibody responses against all serotypes and that the representative genotype strain selected for vaccine antigen can induce sufficient neutralizing antibodies against other genotype strains.

An individual neutralizing antibody species can sometimes show enhancing activity dependent upon its concentration. As both neutralizing and enhancing antibody species are present in the circulation, an assay system that can measure the total of both antibody activities is required for the estimation of in vivo status. However, the conventional neutralization test using Vero or BHK cells only measures neutralizing activity, while the conventional ADE assay system measures enhancing activity but is difficult to use for the measurement of neutralizing activity. One solution may be the use of FcγR-expressing cells [17,18]. In our laboratory, an assay system using semi-adherent K562 cells was established to measure the balance of neutralizing and enhancing activities of antibodies [19].

The purpose of the present study was to analyze the neutralizing and enhancing antibody balance in mice immunized with two distinct genotype strains in a DENV1 or DENV3 DNA vaccine model. For this purpose, strains isolated in Jakarta, Indonesia, D1/JKTA4/88 (DENV1, Genotype IV) and D3/JKTA15/88 (DENV3, Genotype I), were compared with the prototype strains, Mochizuki (DENV1, Genotype I) and H87 (DENV3, Genotype V), respectively. Our study indicated that enhancing antibody levels shown at sub-neutralizing doses in our assay system using K562 cells did not always correlate with neutralizing antibody titers obtained by a conventional test using Vero cells and depended on the viral genotype strain used for mouse immunization.

2. Materials and methods

2.1. Viruses, cells and antibodies

The Mochizuki [20] and D1/JKTA4/88 [21] (A4) strains of DENV1 and the H87 [20] and D3/JKTA15/88 [21] (A15) strains of DENV3 were used throughout the study. There are 18 amino acid differences between the Mochizuki and A4 strains and 10 amino acid differences between the H87 and A15 strains in the E protein coding region (Table 1). C6/36, Vero [22] and K562 [19] cell lines were described previously. Culture fluids harvested from infected C6/36 cells were used as viral antigens for enzyme-linked immunosorbent assay (ELISA), conventional neutralization tests and assays to measure the balance of enhancing and neutralizing activities. Rabbit polyclonal antibody and mouse hyperimmune ascitic fluid against DENV1 or DENV3 were described previously [22]. The monoclonal antibody, flavivirus-crossreactive D1-4G2-4-15 (American Type Culture Collection, Manassas, VA: 4G2) and a panel of monoclonal antibodies previously generated in our laboratory from mice immunized with

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*a The numbering is based on the first amino acid residue in the E protein.
the DENV1 Mochizuki (D1-I-11G12 and D1-V-8E8: 11G12 and 8E8, respectively) or DENV3 H87 (D3-II-7A3, D3-XII-4F6 and D3-XII-4B10: 7A3, 4F6 and 4B10, respectively) (unpublished data) were used in this study.

2.2. DNA vaccine construction

pcDNA3-based plasmids encoding premembrane (prM) and E genes of the A4 (pcD1JKT) or A15 (pcD3JKT) strains were constructed as previously described for the Mochizuki (pcD1ME) or H87 (pcD3ME) strains [20]. Briefly, viral RNA extracted from infected C6/36 cell culture fluids were used for synthesis of cDNA containing prM and E genes of each virus by reverse transcriptase-polymerase chain reaction (RT-PCR). For the RT reaction, gene specific antisense primers containing a sequence corresponding to a portion of the NS1 coding region for DENV1 (5'-CGTCTAGATTTGCAAGACATTGACTT-3') or DENV3 (5'-CCGGAATTCACCATGAATAGAAGAAAAAGATCCGTGA TAGAGACTTITACAT-3') were used. PCR amplification of prM and E genes was performed with sense primers containing a portion of the signal sequence of prM for DENV1 (5'-CCGGAATTCACCATGAATAGAAGAAAAAGATCCGTGA TAGAGACTTITACAT-3') or DENV3 (5'-CCGGAATTCACCATGAATAGAAGAAAAAGATCCGTGA TAGAGACTTITACAT-3') and antisense primers containing the 3' end of the E gene for DENV1 (5'-GCGCAATTCACCAGATAGAAGAAAAAGATCCGTGA TAGAGACTTITACAT-3') or DENV3 (5'-TGCTCTAGATTTGCAAGACATTGACTT-3') and antisense primers containing the 3' end of the E gene for DENV1 (5'-GCGCAATTCACCAGATAGAAGAAAAAGATCCGTGA TAGAGACTTITACAT-3') or DENV3 (5'-TGCTCTAGATTTGCAAGACATTGACTT-3'). The amplified prM-E gene was inserted into a plasmid vector pcDNA3 (Invitrogen, Carlsbad, CA, USA). All plasmid DNAs were purified using a Qiagen plasmid purification kit (Qiagen, Hilden, Germany) and the proper insertion was confirmed by sequencing.

2.3. Mouse experiments

Groups of six 4-week-old male ddY mice (Japan SLC, Shizuoka, Japan) were immunized twice at a 3-week interval by injection with 100 μg of each DNA plasmid using a spring-powered needle-free jet injector (ShimaJET; Shimadzu, Kyoto, Japan). ddY mice were selected for this study as they produce high levels of neutralizing antibodies after immunization with dengue DNA vaccines [22]. Mice were retro-orbitally bled immediately before the second immunization (week 3) and every week after the second immunization until week 7. Sera pooled per group and time point were examined for neutralizing antibody titers, IgG1 and IgG2a antibody levels and the balance of neutralizing and enhancing antibodies.

2.4. Neutralization test

Neutralizing antibodies elicited in immunized mice were titrated using conventional plaque reduction assays performed on Vero cells with each strain of DENV1 and DENV3 in the presence of complement, as previously described [20]. The plaques were visualized by immunostaining using a 4G2 monoclonal antibody (see below). The neutralizing antibody titer was expressed as the maximum serum dilution yielding a 90% reduction in plaque numbers.

2.5. Assay for the balance of neutralizing and enhancing antibody activity

This assay was performed essentially following the previously described method [19]. Briefly, serial 10-fold dilutions of heat-inactivated antibody specimens prepared in a 96-well poly-L-lysine coated plate were mixed with the virus preparation containing 1 × 10^5 focus forming units (FFUs) per well. After incubation at 37 °C for 2 h, K562 cell suspension containing 1 × 10^5 per well was added and further incubated at 37 °C for 48 h. To determine infected cell numbers, cell layers were fixed and immunostained. The assay was performed in duplicate and the mean number of infected cells calculated. To minimize inter-plate variation, infected cell counts obtained with the test specimens were adjusted with the mean of infected cell counts obtained from eight negative controls without antibody specimens set in the same plate at 1 × 10^5. The cut-off value for differentiating enhancing/neutralizing from non-enhancing/non-neutralizing activity was calculated from the mean ± three times the standard deviation [SD] of the numbers of infected cells obtained with negative controls. In the assay system, which included complement, virus preparations containing Low-Tox-M Rabbit Complement (Cedarlane, Hornby, Canada) at a final concentration of 5% were used before being mixed with antibody dilutions.

2.6. Immunohistochemistry

Infected cells were immunostained as previously described [22]. Briefly, following rinsing and fixation, cells were incubated with a monoclonal antibody 4G2, biotinylated anti-mouse IgG, avidin-biotinylated peroxidase complex and VIP substrate (Vector Laboratories, Burlingame, CA, USA).

2.7. ELISA for measuring antibody levels

An ELISA to measure IgG1 and IgG2a antibody levels was performed as previously described [20]. Briefly, 96-well microplates coated with rabbit hyperimmune sera against DENV1 or DENV3 were incubated with Mochizuki, A4, H87 or A15 antigen, serial dilutions of sera starting at 1:100 dilution, followed by alkaline phosphatase-conjugated anti-mouse IgG1 or IgG2a and p-nitrophenyl phosphate. Tests were performed in duplicate. The ELISA antibody titer was the maximum serum dilution that showed an absorbance value greater than the mean + three times the SD of absorbance values obtained with normal mouse sera.

3. Results

3.1. Neutralizing antibodies induced in immunized mice

To compare neutralizing antibody titers against different genotype strains, mice were immunized with pcD1JKT or
pcD1ME in a DENV1 model and pcD3JKT or pcD3ME in a DENV3 model. Sera from immunized mice were examined using a conventional Vero cell neutralization test using the A4 or Mochizuki strain as a DENV1 antigen and the A15 or H87 strain as a DENV3 antigen. Thus, neutralizing antibody titers were compared in homologous and heterologous combinations between genotype strains used for mouse immunization and those used for the neutralization test (Fig. 1).

Neutralizing antibody titers were detected in all combinations 3 weeks after the first immunization (week 3), increased after the second immunization (week 4 or 5) and maintained until the end of the experiment (week 7). For DENV1 (Fig. 1A), the neutralizing antibody titer against the A4 strain at week 7 was 1:2560 in pcD1JKT-immunized mice and 1:80 in pcD1ME-immunized mice, a 32-fold higher titer in the homologous combination compared with the heterologous combination. Titors against the Mochizuki strain were almost the same between pcD1JKT- and pcD1ME-immunized mice. For DENV3 (Fig. 1B), homologous combinations induced 4-fold higher titers against A15 and 2-fold higher titers against H87 than heterologous combinations at week 7, demonstrating a total 8-fold difference between the homologous and heterologous combinations. The increased homologous/heterologous differences shown by DENV1 (32-fold) compared with DENV3 (8-fold) strains were consistent with the greater amino acid difference shown by DENV1 (3.7%) compared with DENV3 (2.0%), respectively. These results indicated that, as expected, homologous combinations provided higher neutralizing antibody titers than heterologous combinations.

### 3.2. Balance of neutralizing and enhancing antibodies induced in sera from immunized mice

Since the Vero cell neutralization test can only measure neutralizing but not enhancing activities, the balance of neutralizing and enhancing activities was measured by our assay system using semi-adherent K562 cells (Fig. 2). The assay was performed in the presence and absence of complement. The neutralizing and enhancing antibody balance against A4 and Mochizuki (Fig. 2A) and A15 and H87 (Fig. 2B) was determined from sera pooled from immunized mice at weeks 3 and 7 that were obtained from the experiment shown in Fig. 1.

In the absence of complement, similar to conventional ADE assays, most serum samples exhibited dose (dilution)-dependent antibody activities against the A15 or H87 strain obtained with sera from pcD3JKT- or pcD3ME-immunized mice. The assays were performed in the presence (closed circle) or absence (open circle) of complement. Dotted lines indicate cut-off values to differentiate enhancing/neutralizing activity from non-enhancing/non-neutralizing activity (mean ± 3 × SD obtained with eight negative controls lacking antibodies). The assays were performed in duplicate and each datum represents a mean value obtained in two separate experiments with SDs (indicated by bars).

Fig. 1. Neutralizing antibody titers induced in DNA vaccine-immunized mice. Mice were immunized twice and bled just before the second immunization (week 3) and every week for 4 weeks after the second immunization. Pooled sera were examined for conventional Vero cell neutralization testing. (A) Neutralizing antibody titers against the A4 (left panel) or Mochizuki (right panel) strains induced by pcD1JKT (closed circle) or pcD1ME (open circle). (B) Neutralizing antibody titers against the A15 (left panel) and the H87 (right panel) strains induced by pcD3JKT (closed circle) or pcD3ME (open circle).

Fig. 2. Balance of neutralizing and enhancing activities in sera from DNA vaccine-immunized mice. Sera obtained at weeks 3 and 7 in Fig. 1 were used to measure neutralizing and enhancing antibody balance. (A) Dose (serum dilution)-dependent antibody activities against the A4 or Mochizuki strain obtained with sera from pcD1JKT- or pcD1ME-immunized mice. (B) Dose-dependent antibody activities against the A15 or H87 strain obtained with sera from pcD3JKT- or pcD3ME-immunized mice. The assays were performed in the presence (closed circle) or absence (open circle) of complement.
dependent antibody activity patterns showing neutralizing activities at low (10^1 and/or 10^2) dilutions, enhancing activities at middle (10^2–10^3) dilutions and no activity at high (10^5 and 10^6) dilutions, both for DENV1 and DENV3. Since the abscissa indicates serum dilutions in Fig. 2, increases in antibody levels cause the dose-dependent antibody activity curve to shift to the right. Thus, comparison of the antibody activity curves against the Mochizuki strain obtained with pcD1JKT-immunized mice at weeks 3 and 7 (Fig. 2c, d) indicated an increase in antibody levels. However, most others showed a difference in the antibody activity curves between weeks 3 and 7. For instance, pcD3JKT-immunized mice showed different antibody activity patterns against the H87 strain: enhancing activities appeared at week 7, but not at week 3 (Fig. 2k, l). This indicated a qualitative difference in antibody responses induced in mice between weeks 3 and 7, as well as a quantitative difference.

Homologous combinations between genotype strains used for mouse immunization and the assay antigen in the absence of complement provided higher enhancing activities at subneutralizing doses than heterologous combinations in pcD1ME- and pcD1JKT-immunized mice against the A4 strain (Fig. 2a, b, e, f). However, this was not the case for pcD1JKT- and pcD1ME-immunized mice against the Mochizuki strain, especially at week 3 (Fig. 2c, g). The heterologous combination provided higher enhancing activities than homologous combinations. Similarly, in DENV3, homologous/heterologous combinations did not correlate with levels of enhancing activities obtained at subneutralizing doses.

The inclusion of complement in the assay system is considered closer to the situation in vivo, since complement exists in the circulation. The neutralization test in the present study (Fig. 1) also included complement. Overall, enhancing activities were reduced by the addition of complement, and in some sera (Fig. 2h, i, l), enhancing activities shown at subneutralizing doses in the absence of complement were abolished by its addition. Comparison between neutralizing antibody titers determined using Vero cells (Vero cell neutralizing antibody titers) and the maximum antibody dilution that showed neutralizing activities in the assay using K562 cells (K562 cell neutralizing antibody titers) usually indicated similar values in sera whose enhancing activities were not shown at subneutralizing doses. For instance, pcD1ME-immunized mice had similar Vero and K562 cell neutralizing antibody titers (1:10) against the A4 strain at week 3 (Fig. 2e), and pcD3JKT-immunized mice had a Vero cell neutralizing antibody titer of 1:80 and a K562 cell neutralizing antibody titer of 1:100 against the H87 strain at week 7 (Fig. 2l). In contrast, when enhancing activities were observed at subneutralizing doses, K562 cell neutralizing antibody titers were usually lower than Vero cell neutralizing antibody titers. For instance, pcD1JKT-immunized mice had a Vero cell neutralizing antibody titer of 1:2560 but a K562 cell neutralizing antibody titer of 1:100 against A4 at week 7 (Fig. 2b). Thus, neutralizing activities obtained at a serum dilution of 1:10 in this assay system were affected by enhancing activities shown at subneutralizing doses, resulting from the balance between two opposite activities.

Homologous combinations between viral antigens used for mouse immunization and our assay system did not always provide higher enhancing activities than heterologous combinations in the presence of complement. Similar to results obtained in the absence of complement, homologous combination between pcD1ME-immunized mice and the Mochizuki strain (Fig. 2g, h) did not show higher enhancing activities than the heterologous combination with pcD1JKT-immunized mice (Fig. 2c, d). In addition, homologous combination of pcD3JKT-immunized mice and the A15 strain (Fig. 2i, j) did not show higher enhancing activities than heterologous combination with pcD3ME-immunized mice (Fig. 2m, n). These results indicated that high neutralizing antibody titers obtained by the Vero cell neutralization test did not correlate with enhancing activities at subneutralizing doses in the assay system using K562 cells.

Comparison between genotype strains used for mouse immunization in the presence of complement indicated that pcD1ME and pcD3JKT induced less enhancing activities at subneutralizing doses than pcD1JKT and pcD3ME, respectively. In addition, a comparison between genotype strains used for the assay using K562 cells suggested that Mochizuki and H87 strains tended to induce less enhancing activities than the A4 and A15 strains, respectively. The highest enhancing activity for DENV1 was observed in the combination of pcD1JKT-mmunized mice and A4, and for DENV3 it was observed in the combination of pcD3ME-mmunized mice and A15. These results suggested that enhancing activities at subneutralizing doses might depend on the combination of genotype strains used for mouse immunization and those used for the assay to measure the balance of neutralizing and enhancing antibodies.

3.3. Balance of neutralizing and enhancing activities in monoclonal antibodies

As amino acid differences between two distinct genotype strains may relate to the difference in dose-dependent neutralizing and enhancing activity patterns observed in mouse sera (Fig. 2), a panel of monoclonal antibodies against the E protein of DENV1 or DENV3, as well as flavivirus-crossreactive 4G2, was used to compare the balance of neutralizing/enhancing activities against two genotype strains of each of DENV1 (Fig. 3A) and DENV3 (Fig. 3B). These monoclonal antibodies recognized different epitopes on three domains of E (EDI, EDII, EDIII). 4G2 recognizes E169 (EDI) and E275 (the hinge region between EDI and EDII) of DENV2 [41]. The epitope sites recognized by DENV1 monoclonal antibodies were E309 and E325 (EDIII for 11G12) and E325 (EDIII for 8E8), while those for DENV3 monoclonal antibodies were E180 (EDI for 7A3), E327 (EDIII for 4F6) and E382 (EDIII for 4B10) (unpublished data). This comparison was performed in the absence of complement.

All monoclonal antibodies against DENV1 (Fig. 3A) showed differences between dose-dependent antibody activity patterns against the A4 and Mochizuki strains. The greatest difference was obtained with 11G12 that had high enhancing activities...
against the Mochizuki strain at IgG concentrations ranging from approximately $10^3$–$10^6$ ng/ml, but almost no activity against the A4 strain. Enhancing activities shown by 4G2 and 8E8 were higher against A4 than for Mochizuki over a wide range of IgG concentrations. For DENV3 monoclonal antibodies (Fig. 3B), differences between antibody activity patterns against A15 and H87 were also observed, although they were not as great as those for DENV1 monoclonal antibodies. These results indicated that the difference between genotype strains in antibody activity patterns was due to the difference in epitopes on the E protein between these genotype strains.

3.4. IgG antibody subclass profiles induced in immunized mice

The difference in enhancing activity levels obtained under assay conditions including complement (Fig. 2) could be explained by the difference in IgG subclass levels contained in sera, since each subclass has different affinities to complement [23]. Thus, antibodies induced by different genotype strains were analyzed to determine their IgG1 and IgG2a profiles (Fig. 4). The same sera used for experiments in Fig. 2 were used for this purpose.

Overall, levels of IgG2a antibodies were higher than those of IgG1 antibodies with an IgG1:IgG2a antibody ratio mostly ranging from 1:1 to 1:8 (Fig. 4). The ratios were similar between weeks 3 and 7 with a difference of 2-fold or less, except for ratios induced in pcD1ME-immunized mice where 4- to 8-fold differences were observed (Fig. 4c, d). Comparison with the dose-dependent antibody activity pattern in the presence of complement (Fig. 2) suggested a correlation between the enhancing activities at subneutralizing doses and the IgG1:IgG2a antibody ratio (Fig. 4). For DENV1, when the

![Fig. 3](image-url) Balance of neutralizing and enhancing activities using a panel of monoclonal antibodies. (A) Dose (IgG concentration)-dependent antibody activities against the Mochizuki (closed circle) or A4 (open circle) strain using DENV1 monoclonal antibodies as indicated (the antibody code and subclass are shown in each panel). (B) Dose-dependent antibody activities against the H87 (closed circle) or A15 (open circle) strain obtained with DENV3 monoclonal antibodies as indicated (the antibody code and subclass are shown in each panel). The assays were performed in the absence of complement. Dotted lines indicate cut-off values to differentiate enhancing/neutralizing activities from non-enhancing/non-neutralizing activities (mean ± 3 SD obtained with eight negative controls lacking antibodies). The assays were performed in duplicate and each datum represents a mean value obtained in two separate experiments with SDs (indicated by bars).

![Fig. 4](image-url) ELISA of IgG1 and IgG2a antibody titers in sera from DNA vaccine-immunized mice. Sera obtained at weeks 3 and 7 in Fig. 1 were used for ELISA. (A) Titers against the A4 or Mochizuki strain obtained with sera from pcD1JKT- or pcD1ME-immunized mice. (B) Titers against the A15 or H87 strain obtained with sera from pcD3JKT- or pcD3ME-immunized mice. Open bars indicate the IgG1 subclass and closed bars indicate IgG2a.
ratio was \( \leq 1:2 \) as shown in pcD1JKT-immunized mice assayed against A4 (Fig. 4a), high levels of enhancing activities were observed (Fig. 2a, b). In contrast, when the ratio was 1:4 as shown in pcD1JKT-immunized mice assayed against Mochizuki (Fig. 4b), low levels of enhancing activities close to the cut-off value were observed (Fig. 4c, d). Furthermore, when the ratios were 1:32 or higher as shown in pcD1ME-immunized mice (Fig. 4c, d), no enhancing activities were observed (Fig. 2e–h). The relationship between the IgG1:IgG2 ratio and the maximum number of infected cells obtained from each panel of Fig. 2 is shown in a scatter graph (Fig. 5A). A significant correlation coefficient of \(-0.807 \) (\( P < 0.02 \)) was observed. For DENV3, IgG1:IgG2a ratios of \( \leq 1:2 \) (Fig. 4g) tended to provide higher levels of enhancing activities (Fig. 2m, n) than those of 1:2 to 1:8 (Fig. 4e, f, h; Fig. 2i–l, o, p), with a correlation coefficient between the IgG1:IgG2a ratio and enhancing activities (\(-0.614 \)) that was not significant (\( P > 0.05 \); Fig. 5B). A potential reason why statistical significance was not obtained for DENV3 is the absence of serum samples showing low IgG1:IgG2a ratios similar to those obtained from pcD1ME-immunized mice. Results from DENV1- and DENV3-immunized mice showed a significant correlation with a correlation coefficient of \(-0.621 \) (\( P < 0.02 \); Fig. 5C). These results suggested that differences in enhancing activities between genotype strains in the presence of complement in our assay system depended upon the difference in the IgG1:IgG2a antibody ratio observed between these genotype strains.

4. Discussion

Since multiple DENV genotypes are present in each of DENV1-4 and frequent shifts of the predominantly circulating genotype occur in many different geographic regions [24–26], the ability of a vaccine to successfully cover this diversity is crucial. Detailed studies on antigenic determinants using monoclonal antibodies against DENV1 [27], DENV2 [28] and DENV3 [29] demonstrated variations from one genotype to another, so that several monoclonal antibodies inefficiently neutralized at least one strain of a distinct genotype. The genotype difference may also affect the disease protective capacity of a vaccine, since a recent report of the first trial to evaluate the protective efficacy of a dengue tetravalent vaccine described low protection against DENV2 infection [30]. This may have potentially been due to a difference between genotype strains used for the vaccine antigen and circulating at the study site. An important point in the development of dengue vaccines is that the induction of insufficient levels of neutralizing antibodies may cause vaccine-derived enhancement of viral infection [3,16].

Infection-enhancing antibody activity was observed in vitro at subneutralizing doses of sera from patients or immunized animals [15]. Usually, levels of enhancing activity shown at subneutralizing doses do not correlate with levels of neutralizing antibody titers as determined by the conventional neutralization test [31–35]. In the present study, homologous combinations of genotype strains used for mouse immunization and assay antigen provided higher antibody titers than heterologous combinations in the Vero cell neutralization test (Fig. 2). In contrast, use of our assay to measure the balance of neutralizing and enhancing antibodies did not show a correlation of enhanced levels in homologous combinations compared with heterologous combinations (Fig. 4). Since our assay measured the balance between neutralizing and enhancing antibodies in serum, neutralizing activity expressed as K562 cell neutralizing antibody titers were affected by the presence of enhancing antibodies, consistent with the results obtained using Fc\( \gamma \) receptor-expressing cells [17,36]. Furthermore, the titers were generally lower than Vero cell neutralizing antibody titers in relation to the level of enhancing activity at subneutralizing doses, which varied according to the genotype strain used for mouse immunization.

Antigenic epitopes located on viral surface proteins are involved in neutralizing and enhancing activity [8,37]. For flaviviruses, neutralization is a “multiple-hit” phenomenon where viruses are neutralized when the number of antibody molecules bound to the virion exceeds a certain threshold [38]. However, recent studies have reported the presence of cryptic epitopes that are accessible only through viral “breathing” [39,40]. In the present study, we used a panel of monoclonal antibodies that showed various dose-dependent antibody activity patterns in the assay using K562 cells. For example,
against DENV1. 4G2 showed both neutralizing and enhancing activities depending on its concentration, while 11G12 and 8E8 only showed enhancing activity. These monoclonal antibodies recognized different epitopes: 11G12 (E309 and E325, EDIII) and 8E8 (E325, EDIII) for DENV1 monoclonal antibodies and 7A3 (E180, EDI), 4F6 (E327, EDIII) and 4B10 (E382, EDIII) for DENV3 monoclonal antibodies as mapped by neutralization escape (unpublished data). In addition, 4G2 was reported to recognize an epitope containing E169 (EDI) and E275 (EDI/II hinge region) of DENV2 [41]. The greatest difference in activity patterns shown by 11G12 against different DENV1 strains was consistent with one of the epitope sites recognized by the monoclonal antibody (E309) being identical to one of the amino acid differences between the two strains (Table 1). The difference observed with 4G2 and 8E8 may also be explained by the amino acid differences between the two strains (Table 1). Although not identical, the sites recognized by the monoclonal antibodies were close to the positions where amino acid differences were observed between the two strains. In contrast, DENV3 monoclonal antibodies showed less difference in activity patterns between the two DENV3 strains. This may be explained as the epitope sites were relatively distant from the positions of amino acid differences between the two strains. Although the amino acid at E169 was different between the two DENV3 strains, the flavivirus-crossreactive antibody, 4G2 recognizing E169 of DENV2, showed similar activity patterns against H87 and A15. This suggests that the amino acid substitution from Ala to Val at this position did not cause significant effects on 4G2 binding in these DENV3 strains. As the monoclonal antibodies showed various antibody activity patterns, differences between genotype strains in serum antibody activity patterns may be due to the difference in antibody species induced in mice by these distinct genotype strains used as immunogens, as well as the difference in epitopes on the E protein between the genotype strains used for assay antigens.

Levels of enhancing activity of mouse monoclonal antibodies were reduced by the inclusion of complement in the assay system, and were dependent upon the IgG subclass [42–44]. When using immunized mouse sera, a significant effect of complement was observed on the enhancing activity at subneutralizing doses, dependent on the IgG antibody subclass profile. In particular, when using pcD1ME-immunized mouse sera where IgG2a antibodies were induced at much higher levels (>32-fold) than IgG1 antibodies, enhancing activity in the absence of complement was abolished by the addition of complement. Since immunization conditions (DNA vaccine, route and dose) were consistent in the present mice experiments, these results suggest that the Mochizuki strain tended to induce a stronger Th1 immune response in mice than the A4, A15 and H87 strains. Thus, the type of immune response induced may be controlled by the specific selection of vaccine strains, in addition to the type of vaccine, immunization route and adjuvant that are generally considered for immunomodulation [45].

Several reports have described increased dengue incidence or more severe cases related to the genotype or clade shift of the predominant virus circulating in a particular area [46–50]. Although the preimmune status in these populations was not known, it is possible that immunity against the previous genotype/clade did not protect people from infection with the new strain, or rather it may have had a deteriorative role. Further studies are required to examine whether secondary infection with a strain of a different genotype may lead to more severe disease through the ADE phenomenon.

In conclusion, the present study used a mouse vaccination model to show that enhancing activity, demonstrated by observing neutralizing antibodies induced against DENV1 or DENV3 at subneutralizing doses did not correlate with Vero cell neutralizing antibody titers, and rather depended on the genotype strain used for immunization. Selection of the genotype strain for vaccine antigens may be a potential strategy to reduce the enhancing activity of dengue vaccine-induced neutralizing antibodies. However, the findings obtained in the present study are limited to the mouse DNA vaccine model and further studies are required to characterize the breadth of genotype-specific neutralizing or enhancing antibodies.

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