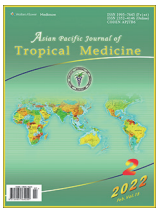

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Systematic mutational analysis of epitope-grafted ED3 immunogenicity reveals a DENV3-DENV4 bi-serospecific ED3 mutant

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ABSTRACT

Objective: To identify the residue determinants of the serospecificity and sero-cross-reactivity of dengue virus (DENV) envelope protein domain 3 (ED3), which contains two major putative epitopes of DENV.

Methods: We constructed ED3 from DENV3 (3ED3) and DENV4 (4ED3), and six epitope-grafted variants, where we transferred epitope 1 ($L^{304}I$, $K^{305}D$, $V^{309}M$, and $S^{310}A$) and/or epitope 2 ($D^{383}N$, $K^{384}S$, $K^{387}T$, and $N^{389}H$) of 4ED3 onto 3ED3 and *vice-versa*. Swiss albino mice aged 3-4 weeks were immunized against wildtype and epitope-grafted ED3 variants and anti-ED3 IgG antibody responses were determined using ELISA.

Results: Mouse immunization using 3ED3 and 4ED3 generated serotype-specific antisera, as expected. Similarly, most epitope-grafted ED3s produced antisera serospecific to the template ED3 with little or no cross-recognition of ED3 of the serotype from which the epitopes were taken. These indicated that a mere grafting of the epitope was not sufficient to transfer serospecificity, contrary to our expectations. However, one epitope-grafted ED3 mutant, where epitope 1 of 3ED3 was grafted onto 4ED3 ($4ED3^{epi1}$), generated antisera that was serospecific to both 4ED3 and 3ED3.

Conclusions: The $4ED3^{epi1}$ is a chimeric ED3 that produces antisera possessing serospecificity to both 3ED3 and 4ED3 onto a common 4ED3 scaffold. The $4ED3^{epi1}$, therefore, provides a unique tool for analyzing serospecificity and sero-cross-reactivity in dengue. We believe that chimeric ED3 may provide a template for future recombinant ED3 possessing serospecificity of multiple DENVs onto a single scaffold and may pave a way developing tri-and/or tetravalent anti-DENV antisera.

KEYWORDS: Dengue virus serospecificity; Sero-cross-recognition; Anti-ED3 antibodies; Epitope-grafting; Chimeric ED3

1. Introduction

Dengue fever, a mosquito-borne viral disease, is caused by the dengue virus (DENV), which is a flavivirus classified into four serologically distinct serotypes (DENV 1-4). It is a major public health issue in tropical and subtropical regions[1,2], with 390 million cases reported every year, and 40% of the world's population at risk[3,4]. Primary infection by a DENV may provoke a high fever for a few days, but the patient usually recovers and may gain a long-lasting immunity against the infecting serotype[5]. However, a secondary heterotypic infection can lead to severe syndromes such as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS)[6,7]. The severity is thought to be caused by antibodies produced during the primary infection, which would be cross-reacting but sub-neutralizing against the secondary infecting

Significance

The serospecificity determinant of dengue viruses (DENVs) is still a bottle neck of dengue research. Putative epitope grafting from the ED3 of DENV3 (3ED3) onto the ED3 of DENV4 (4ED3) generated a bivalent chimeric ED3 possessing serospecificity of both 3ED3 and 4ED3. The chimeric bivalent ED3 could be recognized by both anti-3ED3 and anti-4ED3 sera and anti-chimeric ED3 sera recognized both 3ED3 and 4ED3. Therefore, the bivalent chimeric ED3 may provide a template for future recombinant ED3 possessing serospecificity of multiple DENVs onto a single scaffold and may pave a way developing tri-and/or tetravalent anti-DENV antisera.

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DENV serotype. This phenomenon is coined as the Antibody Dependent Enhancement (ADE)[7,8]; ADE can be caused by natural dengue infection and also demonstrated in artificial immunization studies[9,10], which is a factor making the development of the dengue vaccine cumbersome[11,12].

The single-stranded RNA genome of DENV encodes ten gene products: The capsid (C), pre-membrane (prM), envelope (E), and seven nonstructural (NS) proteins[13]. The E-protein mediates virus-host attachment, and it is composed of three domains (ED1-ED3)[14]. ED2, and accessorily ED3, are involved in the attachments of DENV to the host cell, and ED3 is the major target of neutralizing antibodies[15]. ED3s from all four DENV serotypes have a high sequence similarity (70%-88%) and are structurally identical within an root mean square deviation (RMSD) of 0.5-1.0 Å[16]. Monoclonal antibodies against ED3 are neutralizing DENV in cell culture studies and is fully corroborated by *in vivo* experiments in model mice[17–19]. Furthermore, immunization using ED3 can be long-lasting[20–22], suggesting that DENV's ED3 is an essential factor in determining DENV's serotype[23–26].

Despite numerous studies, the precise residue determinants of DENV serospecificity and sero-cross-reactivity are still to be fully identified[27–29]. In this study, we report the immunogenicity, serospecificity, and sero-cross-reactivity of ED3 from DENV3 (3ED3) and DENV4 (4ED3) and their epitope-grafted variants in Swiss albino mice. As expected, both 3ED3 and 4ED3 were serospecific: antisera recognized only the respective antigens. Further, the grafting of the putative epitope 1 and 2 individually or conjointly onto 3ED3 from 4ED3 and *vice-versa* caused minor changes in the serospecificity of the antisera, which was against our initial expectation that serospecificity could be transferred by grafting the epitopes. However, we observed that antisera raised against 4ED3 onto which epitope 1 from 3ED3 was grafted ($4ED^{epi1}$), was serospecific to both 4ED3 and 3ED3. Thus, $4ED3^{epi1}$ generated a DENV3-DENV4 bivalent antisera, and we hope that it may provide a template for producing a tetravalent antiserum in the future.

2. Materials and methods

2.1. Mutant design, expression and purification

The sequences of DENV3 ED3 (3ED3) and DENV4 ED3 (4ED3) were retrieved from UniProt (ID P27915:1 and P09866, respectively)[30,31] and synthetic genes encoding ED3 sequences were cloned into a pET15b vector along with six His (His6-tag) and a thrombin cleavage site at the N-terminus, as reported previously[16]. The putative epitope residues (putative epitope 1: $L^{304}I$, $K^{305}D$, $V^{309}M$, $S^{310}A$; epitope 2: $D^{383}N$, $K^{384}S$, $K^{387}T$, and $N^{389}H$) were grafted from 3ED3 onto 4ED3 and *vice-versa* through site-directed mutagenesis[23]. All ED3 variants were expressed in *Escherichia coli* JM109 (DE3) pLysS as inclusion bodies and purified as previously reported[32]. Protein identities and purity were confirmed by analytical HPLC, and MALDI-TOF mass spectroscopy. The purified proteins were lyophilized and stored at -30 ℃ until use.

2.2. Circular dichroism (CD) measurement

Samples for CD spectra measurements were prepared at 5-10 μM concentration in Phosphate Buffered Saline (PBS, pH 7.4) and acetate buffer, pH4.5, and centrifuged just before CD measurement to remove aggregates that might have accumulated during sample preparation[33]. CD spectra were measured using a 1 mm cuvette with a JASCO J-820 spectropolarimeter in the wavelength range 200-260 nm at different temperatures.

2.3. Modeling of the epitope–grafted variants

The surface structure models for the epitope-grafted 3ED3s and 4ED3s were generated using the crystal structures of 3ED3 (3vtt.pdb)[16] and 4ED3 (3we1.pdb)[34], respectively, as templates. In short, we added the target epitope residues (Figure 1A) to the template structures using Crystallographic Object-Oriented Toolkit (COOT)[35], assuming that the backbone structures remained unchanged. The side-chains were manually configured based on Richardson's penultimate rotamer library[36] with no and/or minimal side-chain steric clashes as judged by Molprobity[37]. The surface structure models were generated using Pymol graphics[38].

2.4. Artificial immunization studies

The artificial immunization studies were carried out in Swiss albino mice of 3-4 weeks of age (age at the start of immunization) under the ethical clearance number CUBIO210005 issued by the Ethical Review Board of the University of Chittagong, Bangladesh. All mice were randomly assigned into eight experimental groups and one control group, each group having five mice. Group 1 mice received wildtype 3ED3 as immunogen while group 2, 3, and 4 had $3ED3^{epi1}$, $3ED3^{epi2}$ and $3ED3^{epi1,2}$ variants, respectively. Similarly, groups 5, 6, 7 and 8 were injected with wildtype 4ED3, $4ED3^{epi1}$, $4ED3^{epi2}$ and $4ED3^{epi1,2}$ variants, respectively. In general, experimental groups were repeatedly immunized with wildtype ED3s and their epitope-grafted variants at weekly intervals for five times at 20 μg/dose in 100 μL of PBS (pH7.4) plus 100 μL of Freund's Adjuvants, as previously reported[23]. The control group received only 100 μL PBS plus 100 μL Adjuvants at weekly intervals for five times. The first dose was given subcutaneously in a complete Freund's Adjuvant, and doses 2-5 were injected intraperitoneally in incomplete Freund's Adjuvant. The dose-specific antibody response was monitored through tail bleeding after each round of immunization at day 7 using ELISA (*per se* next section). After the 5th dose, mice were sacrificed by cervical dislocation at day 7 and sera were prepared from 500-1 000 μL of heart-blood and were preserved at 30 ℃ until use.

2.5. Evaluation of serospecificity and sero–cross–reactivity of anti–ED3 sera

Antisera developed in Swiss albino mice were first tested for serospecificity through enzyme-linked immunosorbent assay (ELISA) against the respective ED3s used as antigens and then tested for sero-cross-recognition using the other ED3 variants as coating antigens. Namely, 96-well microtiter plates (NuncTM) were coated with 100 μL/well of ED3s (wild-type ED3s and their epitope-grafted variants) in PBS at 1.5 μg/mL concentration for overnight at room temperature. Unbound ED3s were washed out with PBS, and plates were blocked with 1% BSA in PBS for 45 minutes at 37 ℃. After washing with PBS, mouse anti-ED3 sera were applied at an initial dilution of 1:500 fold, followed by a 3-fold serial dilution in 0.1% BSA-PBS-Tween20 and incubated at 37 ℃ for 2 hours[24]. After an extensive wash with PBS-0.05% Tween20, 100 μL/well of anti-mouse IgG-peroxidase conjugate was added at 1:3 000 dilution in 0.1% BSA-PBS-Tween20 and incubated at 37 ℃ for 90 minutes. Finally, after thorough washing with PBS-Tween, coloring was performed with 100 μL/well of OPD substrate (0.4 mg/mL in 50 mM citrate buffer, pH4.5 supplemented with 4 mM H_2O_2) for 20 minutes followed by absorbance measurement at 450 nm using a Multiskan Ascent (Thermofisher) microplate reader. Antibody titers were calculated from power fitting of absorbance *versus* reciprocal of serum dilution using a cutoff of 0.1 ($OD_{450\ nm}$) above the background values using Microsoft excel spreadsheet[32].

3. Results

3.1. Immunogenicity and serospecificity of 3ED3 and 4ED3

Although the 3ED3 and 4ED3 have very similar sequences and structures (Figure 1), 3ED3 and 4ED3 generated 3ED3 and 4ED3-specific IgG responses, respectively without and/or minimal cross-recognition of 4ED3 and 3ED3, respectively (Figure 2). However, antibody titers of anti-3ED3 sera against 3ED3 was slightly higher than those of anti-4ED3 sera against 4ED3, indicating that the 3ED3 was more immunogenic over 4ED3 (Figure 2). These observations clearly suggested that, first, even though the ED3 was a fragmented domain of the whole dengue envelop protein it could

A

3ED3 : GSGMSYAMCLNTFVLKKEVSETQHGTILIKVEYKGEDAPCKIPFSTEDGQGKAHNGRLITANPVVTKKEEPVNIEAEPPFGESNIVIGIGDKALKINWYRKGSSIGK
3ED3^{epi1} : GSGMSYAMCLNTFVIDKEMAETQHGTILIKVEYKGEDAPCKIPFSTEDGQGKAHNGRLITANPVVTKKEEPVNIEAEPPFGESNIVIGIGDKALKINWYRKGSSIGK
3ED3^{epi2} : GSGMSYAMCLNTFVLKKEVSETQHGTILIKVEYKGEDAPCKIPFSTEDGQGKAHNGRLITANPVVTKKEEPVNIEAEPPFGESNIVIGIGNSALTIHWYRKGSSIGK
3ED3^{epi1,2}: GSGMSYAMCLNTFVIDKEMAETQHGTILIKVEYKGEDAPCKIPFSTEDGQGKAHNGRLITANPVVTKKEEPVNIEAEPPFGESNIVIGIGNSALTIHWYRKGSSIGK
4ED3 : GSGMSYTMCSGKFSIDKEMAETQHGTTVVKVKYEGAGAPCKVPIEIRDVNKEKVVGRIISSTPLAENTNSVTNIELEPPFGDSYIVIGVGNSALTLHWFRKGSSIGK
4ED3^{epi1} : GSGMSYTMCSGKFSLKKEVSETQHGTTVVKVKYEGAGAPCKVPIEIRDVNKEKVVGRIISSTPLAENTNSVTNIELEPPFGDSYIVIGVGNSALTLHWFRKGSSIGK
4ED3^{epi2} : GSGMSYTMCSGKFSIDKEMAETQHGTTVVKVKYEGAGAPCKVPIEIRDVNKEKVVGRIISSTPLAENTNSVTNIELEPPFGDSYIVIGVGDKALKLNWFRKGSSIGK
4ED3^{epi1,2}: GSGMSYTMCSGKFSILKEVSETQHGTTVVKVKYEGAGAPCKVPIEIRDVNKEKVVGRIISSTPLAENTNSVTNIELEPPFGDSYIVIGVGDKALKLNWFRKGSSIGK

B

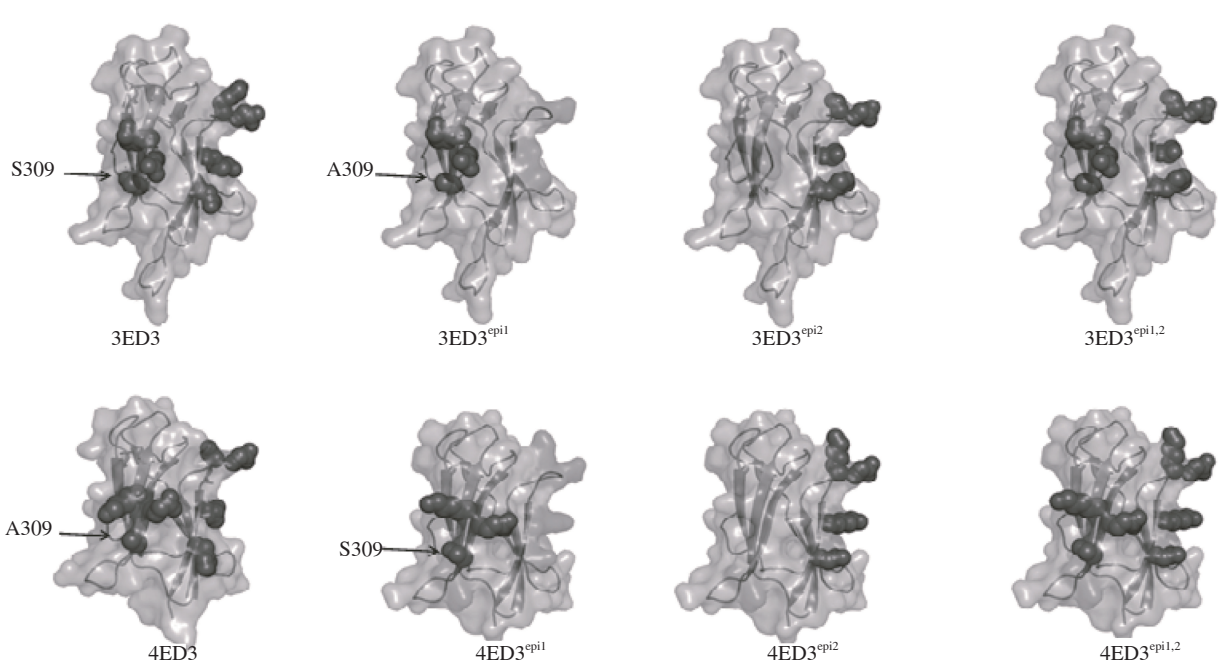

Figure 1. Sequences and structures of ED3s and their epitope-grafted variants. (A) Sequences of ED3 variants. Putative epitope residues grafted from 4ED3 onto 3ED3 and *vice-versa* are in blue. (B) Surface model structures of 3ED3, epitope-grafted 3ED3s (3ED3^{epi1}, 3ED3^{epi2}, 3ED3^{epi1,2}), 4ED3 and epitope-grafted 4ED3 (4ED3^{epi1}, 4ED3^{epi2}, 4ED3^{epi1,2}). Structures of 3ED3 and 4ED3 were generated from 3vtt.pdb[16] and 3we1.pdb[34], respectively, while the surface structures of epitope-grafted ED3s were generated from their modeled structures (*per se* Materials and methods). Grafted epitope residues on the protein surface are in dark grey/black spheres.

Table 1. Solvent accessibility of the putative epitope residues on 3ED3 and 4ED3.

Epitopes	Residue position	Residue identities and ASA				Side-chain position
		3ED3 (3vtt.pdb)		4ED3 (3we1.pdb)		
		Identity	ASA (%)	Identity	ASA (%)	
Epitope 1	304	Leu	23.5±4.2	Ile	17.6±1.8	Buried
	305	Lys	40.5±2.6	Asp	36.8±1.8	Partially buried
	308	Val	9.6±4.2	Met	5.9±2.6	Buried
	309	Ser	67.4±1.3	Ala	80.2±8.0	Surface
Epitope 2	383	Asp	83.4±13.0	Asn	85.3±0.5	Surface
	384	Lys	76.0±5.7	Ser	42.3±9.6	Surface/Partially Buried
	387	Lys	60.4±1.8	Thr	56.2±3.2	Surface
	389	Asn	60.3±4.6	His	50.3±0.5	Surface

Residues substituted on ED3s (residues grafted onto 3ED3 from 4ED3 and *vice-versa*); ASA: solvent Accessible Surface Area expressed as percentage of the total side-chain exposed to the surface; ASA was calculated using 3vtt.pdb and 3we1.pdb; side-chain. ASA <30%, 30%-50%, and >50% were considered as buried, partially buried, and surface-exposed, respectively.

maintain the serospecificity of respective DENVs, and secondly, although the 3ED3 and 4ED3 have very similar sequences and structures their immunogenicity are different. Altogether, these observations indicated that the envelop protein domain 3 of dengue viruses is worth considering as a simplified model to explore the determinants of DENVs serospecificity at atomic level.

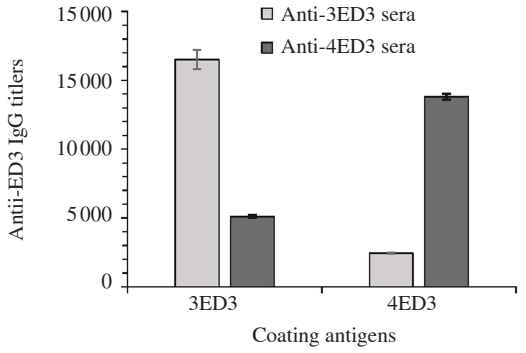

Figure 2. Effects of epitope-grafting on the serospecificity of 3ED3 and 4ED3. Averaged antibody titers (bars) with standard deviations are shown. Legends are shown within the panel. Anti-3ED3 and anti-4ED3 sera mostly recognized 3ED3 and 4ED3, respectively with minimal cross-recognition. Control group mice did not shown any recognition of either ED3s (data not shown).

3.2. Effects of epitope-grafting on ED3s' serospecificity

We grafted the putative epitope 1 ($L^{304}I$, $K^{305}D$, $V^{309}M$, and $S^{310}A$) and epitope 2 ($D^{383}N$, $K^{384}S$, $K^{387}T$, and $N^{389}H$) individually and conjointly from 4ED3 onto 3ED3, and *vice-versa* (Figure 1). We developed antisera against the two wildtype ED3s and their six epitope-grafted variants. Now let us first consider the effects of epitope grafting on the serospecificity of 3ED3. The antisera raised against wildtype 3ED3 (hereafter named as anti-3ED3 sera) showed reduced recognition of epitope-grafted 3ED3 variants (reduced antibody titers against 3ED3 variants), where epitope 1 and 2 from 4ED3 were grafted onto the 3ED3 template (Figure 3A; Supplemental Figure 1). Similarly, antisera raised against epitope-grafted 3ED3s exhibited the strongest recognition for the respective epitope-grafted 3ED3 variant against which they were raised, and a reduced recognition for the wildtype 3ED3 (Figure 3A; Supplemental Figure 1A). In summary, these reduced recognition of epitope-grafted 3ED3s by anti-3ED3 sera and reduced recognition of 3ED3 by anti-epitope-grafted 3ED3 sera clearly suggested that both epitopes play a major role in defining 3ED3's serospecificity.

Corresponding experiments with wildtype 4ED3 and its epitope-grafted variants showed quite similar but not identical trend (Figure 3B). Namely, the antibody titers of anti-4ED3 sera against epitope-1-grafted 4ED3 ($4ED3^{epi1}$) was slightly reduced while titers against epitope-2-grafted variants of 4ED3 remained almost unchanged (Figure 3B; Supplemental Figure 2). However, anti-$4ED3^{epi1}$ sera showed similar recognition of both 4ED3 and $4ED3^{epi1}$, suggesting that epitope 1 might not be a determinant for 4ED3 serospecificity. Interestingly, both anti-$4ED3^{epi2}$ and anti-$4ED3^{epi1,2}$ sera had very reduced recognition of wildtype 4ED3, indicating that epitope 2 might play a major role in determining 4ED3 serospecificity (Figure 3B).

Finally, let us consider sero-cross-recognition of 3ED3 and 4ED3 by antisera raised against the epitope-grafted ED3s. First, anti-3ED3 sera could not cross-recognize any of the epitope-grafted 4ED3s where the epitopes were taken from 3ED3, except a moderate recognition of $4ED3^{epi1}$ (Figure 4A). These observations indicated that grafting of epitope 1 from 3ED3 onto 4ED3 could at least partially transfer the serospecificity of 3ED3 onto $4ED3^{epi1}$. However, none of the antisera raised against epitope-grafted 3ED3s showed any cross-recognition of 4ED3 (Figure 4A), suggesting that serospecificity of 4ED3 could not be transferred onto 3ED3 by epitope-grafting. Similarly, none of the antisera raised against 4ED3 and its epitope-grafted variants cross-recognized neither wildtype 3ED3 nor its epitope-grafted variants, except the anti-$4ED3^{epi1}$ sera, which showed a significant recognition of 3ED3 (Figure 4B), in addition to recognizing 4ED3 (Figure 4B; Supplemental Figure 3). Therefore, sero-cross-recognition of $4ED3^{epi1}$ by anti-3ED3 sera and cross-recognition of 3ED3 by anti-$4ED3^{epi1}$ sera clearly indicated that the serospecificity of DENV3 (3ED3) was almost entirely transferred onto DENV4 (4ED3)

B

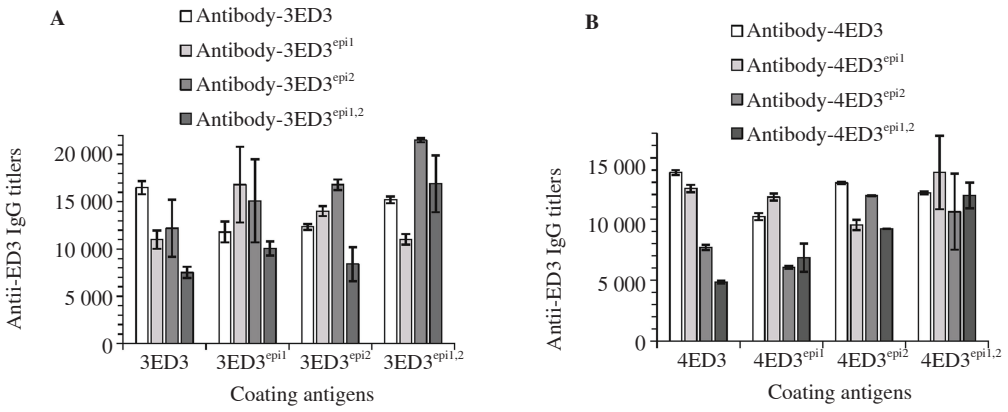

Figure 3. Effects of epitope-grafting on the serospecificity of 3ED3 and 4ED3. A. Antibody titers of anti-3ED3, anti-3ED3^{epi1}, anti-3ED3^{epi2} and anti-3ED3^{epi1,2} sera against 3ED3 variants. B. anti-4ED3, anti-4ED3^{epi1}, anti-4ED3^{epi2} and anti-4ED3^{epi1,2} sera against 4ED3 variants. Averaged antibody titers (bars) with standard deviations are shown. Antibody titers of anti-3ED3 sera reduced noticeably following epitope-grafting. Similarly, antisera raised against epitope-grafted 3ED3 showed reduced recognition of wildtype 3ED3. Anti-4ED3 also showed the highest antibody titers against widltype 4ED3. Interestingly, grafting of the putative epitope 1 onto 4ED3 did not much affect serospecific recognition of anti-4ED3 sera and *vice-versa*, however, anti-sera raised against 4ED3^{epi2} and 4ED3^{epi1,2} showed reduced recognition of wildtype 4ED3.

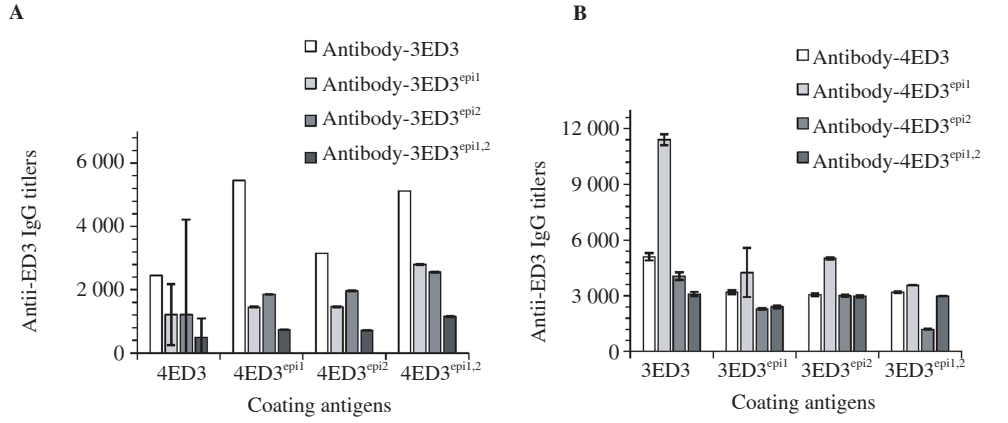

Figure 4. Effects of epitope-grafting on sero-cross-recognition of 3ED3 and 4ED3. A. Antibody titers of anti-3ED3, anti-3ED3^{epi1}, anti-3ED^{3epi2} and anti-3ED3^{epi1,2} sera against 4ED3 variants. B. Anti-4ED3, anti-4ED3^{epi1}, anti-4ED3^{epi2} and anti-4ED3^{epi1,2} sera against 3ED3 variants. Averaged antibody titers (bars) with standard deviations are shown.

through grafting of epitope 1 (Figure 5). Furthermore, sero-cross-recognition of both 3ED3 and 4ED3 by anti-4ED3^{epi1} sera and *vice-versa* suggested that 4ED3^{epi1} is a chimeric ED3 possessing serospecificity of both DENV3 (3ED3) and DENV4 (4ED3) onto a common scaffold.

3.3. Structural origin of serospecific recognition of ED3s by anti–ED3s sera

Grafting of the putative epitope 1 onto 3ED3 reduced serospecific recognition of anti-3ED3 sera (Figure 3). Among the four residues substituted, three were buried in 3ED3's interior and were thus not expected to affect serospecific recognition of 3ED3 by anti-3ED3 sera (Table 1)[2]. The remaining S^{309}A substitution was the only residue. Thus, it is tempting to consider that the S^{309}A mutation was the main factor behind the reduced detection of anti-3ED3 sera by 3ED3^{epi1} and low antibody titers of anti-3ED3^{epi1} sera against 3ED3 (Figure 1, Table 1). However, this interpretation would need to be confirmed by single mutation analysis in the future.

On the other hand, all the four residues in epitope 2 are surface exposed and cause a significant modification in the size and properties of the side-chains (K^{384}S, K^{387}T, and N^{389}H). Grafting of epitope 2 from 4ED3 onto 3ED3 template (3ED3^{epi2} and 3ED3^{epi1,2}) induced noticeable local structural changes as assessed by circular dichroism (Figure 6; Supplemental Figure 4). Such local significant structural changes may provide a rationale for the origin of the serospecificity.

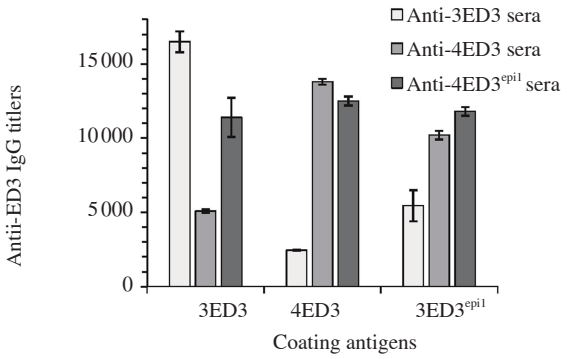

Figure 5. Sero-cross-recognition of both 3ED3 and 4ED3 by anti-4ED3^{epi1} sera. Averaged antibody titers with standard deviations are shown. Both anti-3ED3 and anti-4ED3 sera recognized 4ED3^{epi1} and anti-4ED3^{epi1} sera cross-recognized both 3ED3 and 4ED3 suggesting that 4ED3^{epi1} is a chimeric ED3 possessing sero-specificity of both 3ED3 and 4ED3 onto a common ED3 scaffold.

In contrast, grafting of the putative epitope 1 from 3ED3 onto 4ED3 significantly improved anti-3ED3 antisera's recognition of 4ED3^{epi1} (Figure 3) without compromising the serospecific recognition of anti-4ED3 sera. The sero-cross-recognition of 4ED3^{epi1} by anti-3ED3 sera is attributed to a single substitution: Ser309 on 4ED3 (from 3ED3) (Supplemental Figure 3). Furthermore, this interpretation is fully corroborated with the observation that antisera raised against 4ED3^{epi1} could almost equally recognize 3ED3, 4ED3, and 4ED3^{epi1}, suggesting that Ser309 is responsible for the 4ED3^{epi1} chimeric ED3 detecting both anti-ED3s (Figure 5).

Furthermore, epitope-grafting from 3ED3 onto 4ED3 did not affect or barely affected the overall structure as assessed by the secondary structure content estimated from circular dichroism (CD) spectra measured at 0.20 mg/mL concentration in acetate buffer, pH4.5 at 30 ℃. (Figure 6) nor the serospecificity of 4ED3 (Figure 3 and 4). Besides, the CD spectra of 4ED3 and 4ED3^{epi1} remained almost unchanged under the immunization condition (at 0.2 mg/mL in PBS; Supplemental Figure 4). This may explain why anti-4ED3^{epi1} sera had strong antibody titers against not only itself (and 4ED3^{epi1}) but also against 3ED3, and 4ED3. The grafting of the putative epitope 2 barely or moderately affected the CD spectrum of 4ED3[25], but it is yet unclear whether such small structural change is responsible for the loss of recognition.

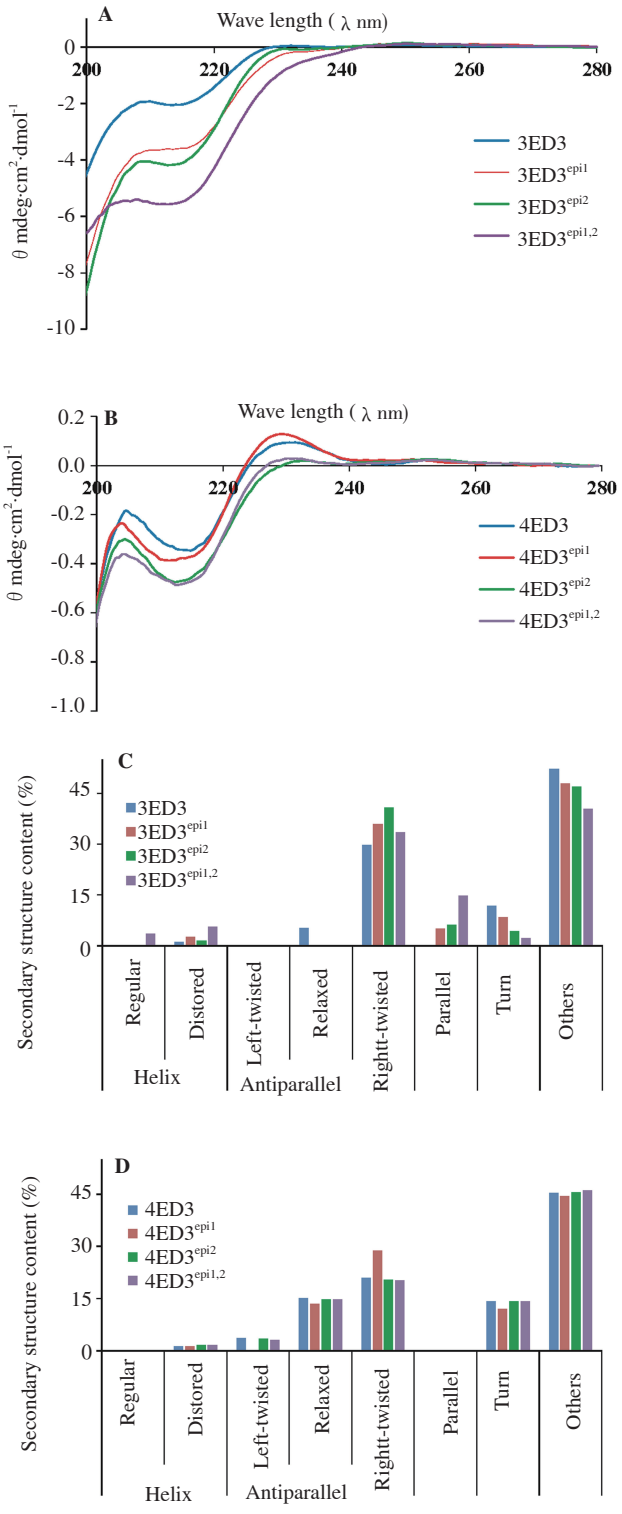

Figure 6. Effects of epitope-grafting on the structures of 3ED3 and 4ED3. A. The CD-spectra of 3ED3 and its epitope-grafted variants. B. The CD-spectra of 4ED3 and its epitope-grafted variants. CD-spectra were measured at 0.20 mg/mL concentration in acetate buffer, pH 4.5 at 30 ℃. The secondary structure contents of 3ED3 and 4ED3 variants estimated using bestsel[39] are shown in panel C, and D, respectively. Epitope-grafting on 3ED3 caused a noticeable change in the CD spectrum, but not on 4ED3.

4. Discussion

There are controversies over the residue identities determining DENV serospecificity and sero-cross-reactivity among the four DENV serotypes[18,38]. Moreover, these putative epitope residues have been reported to reside at different locations including membrane, non-structural and different domains envelop proteins[21,27,28]. In this report the effects of epitope-grafting on the serospecific recognition of 3ED3 clearly suggested that both the epitopes 1 and 2 are essential for the serospecificity of 3ED3, fully corroborated with previous report on DENV2[17]. In contrary, antisera raised against 4ED3 exhibited a titer almost identical against all three epitope-grafted 4ED3s. Moreover, antisera raised against the epitope-grafted ED3s did not improve sero-cross-recognition of 3ED3 and 4ED3, except the anti-4ED3^{epi1} sera, which showed a significant recognition of 3ED3, in addition to recognizing 4ED3. This observation indicated that the serospecificity of DENV3 (3ED3) was almost entirely transferred onto DENV4 (4ED3) through grafting of epitope 1. Though we do not have very good explanation for the rationale of structural changes following mutation and their consequences on DENV serospecificity at atomic level, an Ala-scanning mutagenesis at the putative epitope residues that we report here may reveal the exact residue identities of DENV serospecificity. Furthermore, the identification of 4ED3^{epi1} as a chimeric ED3 possessing bi-serospecificity to both 3ED3 and 4ED3 is an important finding from an application viewpoint. This is because 4ED3^{epi1} may serve as a simplified template for further exploring serospecificity and sero-cross reaction among DENV serotypes, and combining serospecificity of all four DENVs onto a single scaffold[21]. Furthermore, it might be used as a scaffold for producing a trivalent and, perhaps, a tetravalent antiserum against DENV.

In summary, this is most likely the first systematic examination of the effect of epitope grafting on the immune response and serospecificity of ED3 from DENV, which has four distinct serotypes. The results are not easily interpreted in terms of the serospecificity transfer, but we identified one mutant that holds a bivalent serospecificity against DENV3 and DENV4. The identification of 4ED3^{epi1} as a chimeric ED3 might act as a powerful tool for analyzing serospecificity and cross-reactivity in dengue; and we hope that it can provide a common scaffold that recognizes all four serotypes and possibly open the way for the design of a tetravalent dengue vaccine.

Conflict of interest statement

We declare that we have no conflict of interest.

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Authors' contributions

MMI designed the project and wrote the manuscript; MS and NH performed the experiment; MRM optimized the immunization dose and protocol. All authors read and approved the manuscript.

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