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Short Communication

Characterization of a dengue type-specific epitope on dengue 3 virus envelope protein domain III

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Dengue virus (DENV) is a mosquito-borne disease caused by four genetically and serologically related viruses termed DENV-1, -2, -3 and -4. The DENV envelope (E) protein ectodomain can be divided into three structural domains designated ED1, ED2 and ED3. The ED3 domain contains DENV type-specific and DENV complex-reactive antigenic sites. To date, nearly all antigenic studies on the E protein have focused on DENV-2. In this study, the epitope recognized by a DENV-3 type-specific monoclonal antibody (mAb 14A4-8) was mapped to the DENV-3 ED3 domain using a combination of physical and biological techniques. Epitope mapping revealed that amino acid residues V305, L306, K308, E309, V310, K325, A329, G381 and I387 were critical for the binding of mAb 14A4-8 and amino acid residues T303, K307, K386, W389 and R391 were peripheral residues for this epitope. The location of the mAb 14A4-8 epitope overlaps with the DENV complex-reactive antigenic site in the DENV-3 ED3 domain.

The disease dengue is caused by four serologically and genetically related viruses, termed DENV types 1–4, which belong to the genus *Flavivirus*, family *Flaviviridae*. Dengue is the most important mosquito-borne viral disease in terms of the number of cases each year and its geographical distribution (Gubler, 2004). Over three billion people, in more than 100 countries, are at risk from DENV infection, with at least 50 million infections each year. The disease DEN has several clinical manifestations including DEN fever, DEN haemorrhagic fever (DHF) and DEN shock syndrome (DSS) (Kurane, 2007; Leong *et al.*, 2007; Pang *et al.*, 2007). Severe DHF, also known as DSS, can occur when fluid leaks into interstitial tissue spaces, leading to hypovolaemic shock.

The four DENVs are single-stranded, positive-sense RNA viruses with a genome of approximately 11 kb. The genome contains a single open reading frame encoding three structural proteins: capsid (C), pre-membrane/membrane (prM/M) and a major envelope glycoprotein (E) and seven non-structural proteins (Rice *et al.*, 1985). The E protein is associated with viral attachment, fusion, cellular tropism and the induction of a protective immune response (Mukhopadhyay *et al.*, 2005). The E protein forms homodimers on the surface of virions and consists of three domains: a central β-barrel (domain I: ED1), an elongated dimerization region (domain II: ED2) and a C-terminal immunoglobulin-like region (domain III: ED3) (Rey *et al.*, 1995). Among these domains, ED3 contains the critical epitopes recognized by neutralizing antibodies. In previous studies, we have mapped a DENV complex-reactive (i.e. epitopes shared by the four DEN viruses only) antigenic site for DENV-2 (Gromowski *et al.*, 2008) and DENV-3 (Matsui *et al.*, 2009), and DENV type-specific (i.e. recognized by one DENV only) antigenic site for DENV-2 (Gromowski & Barrett, 2007). These antigenic sites are located on the lateral surface of ED3 and are recognized by monoclonal antibodies (mAbs) with strong neutralizing activity (Gromowski & Barrett, 2007; Gromowski *et al.*, 2008; Matsui *et al.*, 2009).

Previous studies have identified DENV-3 type-specific mAb 1H9 that was found to recognize residue 386 in ED3 by the generation of a mAb neutralization resistant variant (Serafin & Aaskov, 2001). Recently, we reported the identification of an antigenic site on the DENV-3 ED3 that is recognized by DENV complex-reactive mAbs. The epitopes were characterized by a combination of physical

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binding of mAbs to recombinant ED3 (rED3), epitope mapping using rED3 mutants and biological activities of neutralization and haemagglutination inhibition (Matsui *et al.*, 2009). This study showed that the critical residues recognized by DENV complex-reactive mAbs varied significantly for DENV-2 and DENV-3 ED3. In this study, we have characterized an epitope on DENV-3 ED3 that is recognized by DENV-3 type-specific mAb 14A4-8 by a combination of physical and biological assays.

DENV-1, -2, -3 and -4 strains OBS7690, New Guinea C, H87 and 703-4, respectively, were used in this study. Mosquito C6/36 cells were used to propagate each of the four DENVs. Cells were maintained at 28 °C in Dulbecco's modified essential medium (DMEM) containing 10 % fetal bovine serum (FBS) and supplemented with tryptose phosphate broth (Sigma-Aldrich). Monkey kidney Vero cells were used in plaque reduction neutralization tests (PRNT) and were maintained in DMEM supplemented with 8 % FBS incubated at 37 °C in a 5 % CO_2 incubator.

mAb 14A4-8 was a kind gift of Dr Robert Putnak (WRAIR, Silver Spring, MD, USA). The DENV complex-reactive mAb MDVP-55A (Immunology Consultants Laboratory, Inc.), which is known to bind to DENV-1, -2, and -3 (Matsui *et al.*, 2009) was used as a control.

The DENV-3 rED3 was constructed and expressed as previously described (Matsui *et al.*, 2009). DENV-1, -2 and -4 rED3 were also used in this study and were produced as previously described (Gromowski & Barrett, 2007).

An indirect ELISA was used to investigate the binding of the mAb 14A4-8 to DENV-3 rED3. A total of 27 single amino acid substitutions were generated for DENV-3 ED3 and evaluated using an indirect ELISA as previously described (Gromowski & Barrett, 2007; Matsui *et al.*, 2009).

For $PRNT_{50}$, mAb 14A4-8 was diluted in PBS starting at 75 mg ml^{-1}. Twofold serial dilutions were prepared and mixed with an equal volume of each DENV strain (~ 50 p.f.u. per well). Virus and mAb mixtures were incubated for 1 h at 37 °C. Subsequently, the virus–mAb mixture was transferred to approximately 80 % confluent Vero cells in six-well plates. After 30 min incubation at room temperature, cell monolayers were overlaid with DMEM containing 2 % FBS and 1 % agar, and incubated at 37 °C. Plaques were observed by staining with neutral red. $PRNT_{50}$ data were converted to per cent neutralization relative to controls; in the absence of mAb and $PRNT_{50}$, concentrations were calculated by undertaking a non-linear regression analysis using Sigmaplot (version 10; Systat Software, Inc.).

The K_d values are presented as mean ± SEM, calculated as previously described (Gromowski & Barrett, 2007) and were analysed using SigmaStat (version 3.1; Systat Software, Inc.). Statistical analysis of the experimental data and controls was performed using the two-tailed Student's *t*-test.

We were interested in mapping the DENV-3 type-specific antigenic site of ED3 for comparison with our previous studies that had mapped the DEN complex-reactive antigenic site of ED3 (Matsui *et al.*, 2009). mAb 14A4-8 was characterized physically using an indirect ELISA based on rED3, and demonstrated specificity for the DENV-3 rED3, with a K_d of 0.58 ± 0.15 μg ml^{-1} (Table 1). mAb 14A4-8 also strongly neutralized DENV-3 ($PRNT_{50}$ of 14.80 ± 4.88 μg ml^{-1}) compared with DENV-1, -2 and -4, which had $PRNT_{50}$ values of 358.05 ± 25.30 μg ml^{-1}, 381.84 ± 27.49 μg ml^{-1}, >1500 μg ml^{-1}, respectively.

To identify the residues involved in the epitope recognized by mAb 14A4-8, a total of 27 single amino acid substitutions were introduced, by site-directed mutagenesis, into the DENV-3 rED3 using a pMal-C_2X plasmid. Twenty-seven different surface accessible residues were selected based on the published structure of the DENV-3 E protein (Modis *et al.*, 2005) and previous studies with DENV-2 (Gromowski *et al.*, 2008). The binding of mAb 14A4-8 was determined by titration in an indirect ELISA with each of the 27 rED3 mutant proteins and compared to the wild-type DENV-3 rED3 (Table 2). The importance of residues for mAb binding was analysed as previously described by Gromowski & Barrett (2007). Briefly, K_d changes of between four- and tenfold were considered 'weak' changes and were regarded as peripheral residues in the epitope (italics in Table 2), while K_d changes greater than tenfold were considered 'strong' changes and were regarded as critical residues in the epitope (bold in Table 2). Amino acids V305, L306, K308, E309, V310, K325, A329, G381 and I387 were identified as critical residues for the epitope recognized by mAb 14A4-8 (Table 2) and are shown in red in Fig. 1(a). Amino acids T303, K307, W389 and R391 were identified as peripheral residues for the epitope recognized by mAb 14A4-8 and are shown in pink in Fig. 1(a). Since only a T303K substitution, and not T303A/S, affected the binding of mAb 14A4-8 to DENV-3 ED3, T303 was considered a 'peripheral' residue to the epitope.

Of the nine critical residues in the mAb 14A4-8 epitope, four (L306, K308, G381 and I387) are identical to critical epitope residues of four DEN complex-reactive epitopes identified previously (Matsui *et al.*, 2009). The other five

Table 1. Reactions of mAb 14A4-8 with different DEN viruses

Comparison of mAb 14A4-8 with each of the four DENV in rED3 ELISA and neutralization test ($PRNT_{50}$). Titres are shown in concentrations (μg ml^{-1}).

Viruses	Indirect ELISA (K_d)	$PRNT_{50}$
DENV-1	>1500	358.05 ± 25.30
DENV-2	>1500	381.84 ± 27.49
DENV-3	0.58 ± 0.15*	14.80 ± 4.88
DENV-4	>1500	>1500

*The *P*-value was compared with DENV-1, -2 and -4 (<0.05).

Table 2. Relative K_d for mAb/rED3 mutants using wild-type DENV-3 strain H87 as a value of 1.0

The larger the number the weaker the binding of mAb 14A4-8 to the rED3. 'Critical' residues are shown in bold and 'weak' residues are shown in italic.

No. amino acid	mAb 14A4-8
DENV3 wt	1.00
L301G	0.92
T303A	1.88
T303K	**28.17**
T303S	2.55
V305G	**70.54**
L306G	**>356.63**
K307G	*4.37*
K308A	**>356.63**
K308G	**>356.63**
K308Q	**>356.63**
E309G	**>356.63**
V310G	**>356.63**
K321G	1.49
K325G	**>356.63**
A329G	**>356.63**
G381E	**>356.63**
D382E	0.83
D382G	1.38
D382N	1.44
D382P	1.57
K383G	1.25
A384Q	0.31
K386G	*6.67*
K386N	2.18
I387G	**19.90**
W389G	*5.71*
R391G	*4.07*

critical residues are unique to subsets of the five epitopes identified (one type-specific in this study and four DEN complex-reactive identified previously). mAb 14A4-8 recognizes an epitope that has a unique set of critical epitope residues compared with any of the DENV complex-specific epitopes identified previously. For example, the critical residues V305, L306, K308, E309, K325, G381 and I387 for mAb 14A4-8 were the same as those for the DENV complex-reactive epitope recognized by mAb MDVP-55A (Fig. 1b) (Matsui *et al.*, 2009). In contrast, the peripheral residues for each epitope depended on the particular mAb, with residue K307 being specific for the epitope recognized by mAb 14A4-8.

Although the structure of the ectodomain of DENV-3 E protein has been resolved by X-ray crystallography, there have been few studies on epitopes of the DENV-3 E protein (Modis *et al.*, 2005). We have previously reported the mapping of DENV-3 complex-reactive epitopes on the surface of DENV-3 rED3 (Matsui *et al.*, 2009) and in this report, we undertook physical and biological characterization of the DENV-3 type-specific epitope recognized by mAb 14A4-8 that bound to ED3 of the DENV-3 E protein. To date only one study has mapped a type-specific epitope on the E protein of DENV-3 (Serafin & Aaskov, 2001), which used a mAb neutralization resistant variant to map the epitope to residue 386 (coloured cyan in Fig. 1a). In this study, we found that residue 386 was not part of the epitope recognized by mAb 14A4-8, rather the critical residues for binding of this DENV-3 type-specific mAb to the DENV-3 rED3 were V305, L306, K308, E309, V310, K325, A329, G381 and I387. Residues T303, K307, W389 and R391 were considered to be peripheral to the mAb 14A4-8 epitope (Fig. 1a). Since many of these residues overlap with the previous mapping of the DENV complex-reactive epitope recognized by mAb MDVP-55A (compare Fig. 1a and Fig. 1b), it appears that like DENV-2 ED3 (Gromowski *et al.*, 2008) the DENV-3 type-specific antigenic site overlaps with the DENV complex-reactive antigenic site on DENV-3 ED3. Furthermore, the DENV-3 type-specific and DEN complex-reactive antigenic sites appear to be essentially on top of each other, with much more overlap than is seen with the DENV-2 type-specific and DEN complex-reactive antigenic sites (Gromowski *et al.*, 2008). This may be unique for the epitope recognized by mAb 14A4-8 and not necessarily true for all DENV-3 ED3 type-specific epitopes, but more mAbs will be required to test this hypothesis.

Previous studies by Sukupolvi-Petty *et al.* (2007) and ourselves (Gromowski & Barrett, 2007) demonstrated that there were two critical residues, K305 and P384, for the DENV-2 type-specific antigenic site, which are equivalent to residues T303 and D382 on DENV-3 ED3. Interestingly, even though we mutated residue T303 to three different residues and residue D382 to four different residues (Table 2), only a T303K substitution affected the binding of mAb 14A4-8 to DENV-3 ED3, suggesting that although the DENV-3 type-specific antigenic site is on the exposed lateral ridge of ED3, the critical residues of the DENV-3 type-specific antigenic site are not exactly analogous to those of the DENV-2 type-specific antigenic site. This is consistent with our previous study, demonstrating that the residues involved in the DENV complex-reactive antigenic site are not identical on DENV-2 and DENV-3 ED3s (Matsui *et al.*, 2009). Furthermore, we have shown previously that the electrostatic charges on the surface of each of the four DENV ED3s differ (Volk *et al.*, 2007) and found that more critical residues were required for binding of both the type-specific and DEN complex-reactive epitopes on DENV-3 (this study and Matsui *et al.*, 2009) compared with DENV-2 ED3 (Gromowski & Barrett, 2007; Gromowski *et al.*, 2008). Taking these results together, we believe that the weaker binding of antibodies to DENV-3 ED3 compared with DENV-2 ED3 is due to differences in surface charge (for DEN complex-reactive antibodies in particular) and in the network of critical epitope residues required to bind the DENV-3 and DENV-2 ED3s. If correct, it suggests that the combination of different residues and surface charges on the four dengue viruses

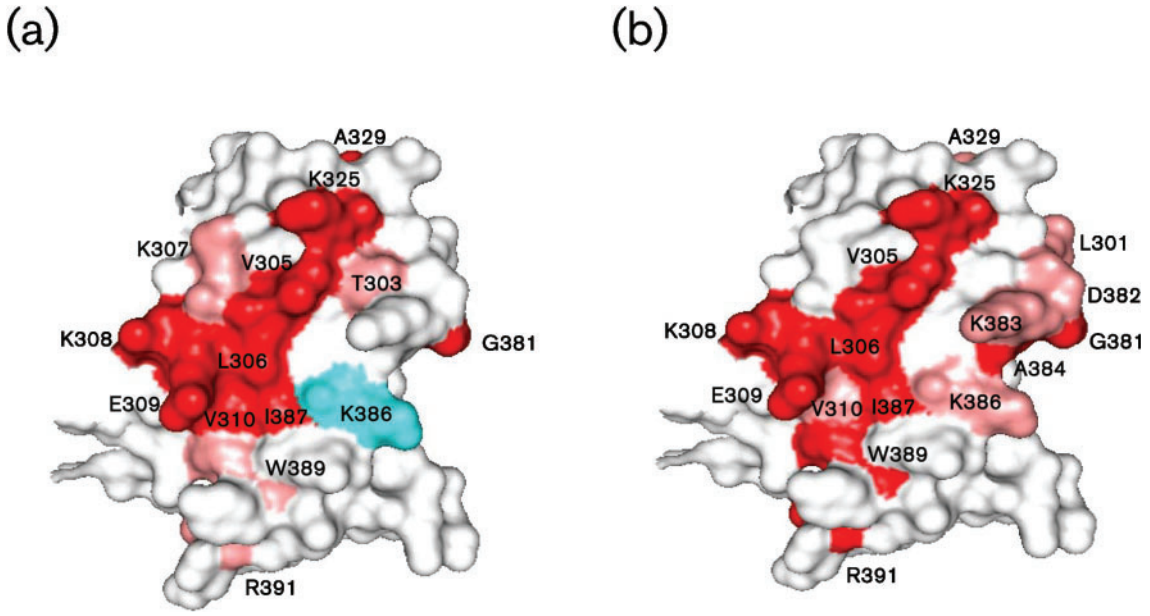

Fig. 1. Predicted epitope for the DENV-3 type-specific mAb 14A4-8 (a) and complex-reactive mAb MDVP-55A (b). Amino acid substitutions that resulted in a change in binding affinity between four- and tenfold were defined as having weak effects on the binding affinity (coloured pink). A greater than tenfold change in binding affinity was considered a strong effect (coloured red). Residue K386, which is recognized by mAb 1H9 (Serafin & Aaskov, 2001) is coloured in cyan for comparison.

results in antibodies distinguishing ED3 of the four DENVs.

The paucity of DENV-3 type-specific mAbs makes analysis of the DENV-3 type-specific antigenic site difficult. There is a need to have more mAbs generated against DENV-3 in order to map additional DENV-3 type-specific epitopes on ED3. However, it is clear that DENV type-specific and DENV complex-reactive antigenic sites are different on DENV-2 and DENV-3 ED3s. It would be interesting to investigate if the same is true for all four dengue viruses.

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