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Rapid Communication

Characterization of dengue complex-reactive epitopes on dengue 3 virus envelope protein domain III

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ABSTRACT

The disease dengue (DEN) is 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 contains the DENV type-specific and DENV complex-reactive (epitopes shared by DENV 1–4) antigenic sites. In this study the epitopes recognized by four DENV complex-reactive monoclonal antibodies (MAbs) with neutralizing activity were mapped on the DENV-3 ED3 using a combination of physical and biological techniques. Amino acid residues L306, K308, G381, I387, and W389 were critical for all four MAbs, with residues V305, E309, V310, K325, D382, A384, K386, and R391 being critical for various subsets of the MAbs. A previous study by our group (Gromowski, G.D., Barrett, N.D., Barrett, A.D., 2008. Characterization of dengue complex-specific neutralizing epitopes on the envelope protein domain III of dengue 2 virus. J. Virol 82, 8828–8837) characterized the same panel of MAbs with DENV-2. The location of the DENV complex-reactive antigenic site on the DENV-2 and DENV-3 ED3s is similar; however, the critical residues for binding are not identical. Overall, this indicates that the DENV complex-reactive antigenic site on ED3 may be similar in location, but the surprising result is that DENV 2 and 3 exhibit unique sets of residues defining the energetics of interaction to the same panel of MAbs. These results imply that the amino acid sequences of DENV define a unique interaction network among these residues in spite of the fact that all flavivirus ED3s to date assume the same structural fold.

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Introduction

The disease dengue (DEN) 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 geographic distribution (Gubler, 2004). Over 3 billion people, in more than 100 countries, are at risk from DENV infection, with at least 50 million infections each year. Clinical symptoms range from a self-limited, acute, febrile disease called DEN fever (DF) to the more severe DEN hemorrhagic fever (DHF), and DEN shock syndrome (DSS) (Halstead, 2007). The 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 nonstructural proteins (Rice et al., 1985). The DENV E protein has three domains: a central β-barrel (domain I: ED1), an elongated dimerization region (domain II: ED2), and a C-terminal immunoglobulin (Ig)-like module (domain III: ED3) (Rey et al., 1995). The DENV ED3 contains type-specific and complex-reactive (i.e., epitopes shared by DENV 1–4 only) epitopes that are the dominant neutralization determinants (Roehrig, 2003). In this study, we analyzed epitopes on the DENV-3 ED3 that are recognized by DENV complex-reactive monoclonal antibodies (MAbs). These were characterized by a combination of physical binding of MAbs to a recombinant ED3 (rED3), epitope mapping using rED3 mutants, and biological activities of neutralization and hemagglutination inhibition. Interestingly, the antigenic site recognized by this panel of DENV complex-reactive MAbs on the DENV-2 ED3 (Gromowski et al., 2008) was in a similar location on the DENV-3 ED3. However, in this study, we show that the critical residues for binding varied significantly for the DENV-3 ED3. This indicates that the DENV

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complex-reactive antigenic site on ED3 may be similar in location, but unique in amino acid composition in defining the energetics of interaction between DENV ED3s and MAbs for each of the four DENVs.

Results

Characterization of MAbs

The binding of four affinity purified IgG2a MAbs (MDVP-55A, GTX29202, MA1-27093, and 20-783 -74014) to DENV-3 and DENV-2 were compared by indirect ELISA, HI, and $PRNT_{50}$. All four MAbs demonstrated tighter binding to DENV-2 rED3 than to DENV-3 rED3 with Kds ranging from 0.5 ± 0.1 nM to 0.7 ± 0.2 nM for the DENV-2 rED3 and 2.5 ± 0.2 nM to 7 ± 1 nM for the DENV-3 rED3 (Table 1). The $PRNT_{50}$ concentration was determined for each of the four DENV complex-reactive MAbs with DENV-2 and DENV-3. The four MAbs had $PRNT_{50}$ values with DENV-3 that ranged from 150 ± 10 nM to 279 ± 7 nM, which was significantly higher than the physical binding Kd that ranged from 2.50 ± 0.2 nM to 7 ± 1 nM. Similar results were obtained for DENV-2. The ratio of $PRNT_{50}$ values to Kd values (i.e., $PRNT_{50}$/Kd) was calculated as it reflects the magnitude of the shift of the PRNT curve relative to the physical binding curve (Table 1). For DENV-3 the $PRNT_{50}$ to Kd ratio was large (greater than 20-fold) for all four MAbs indicating that there was a large shift of the PRNT curve to the right and away from the physical binding curve. The $PRNT_{50}$ to Kd ratio for DENV-3, compared to DENV-2, was higher with MAbs MDVP-55A and 20-783-74014, but similar for MAbs GTX29202 and MA1-27093 (Table 1) indicating that the MAbs neutralized DENV-3 weaker than DENV-2 even when the physical binding was normalized. In HI assays, the MAbs had titers for DENV-3 ranging from 25 nM to 67 nM and these values were much higher than the HI titers previously reported for this set of MAbs with DENV-2 (Gromowski et al., 2008) (Table 1).

Binding affinity of MAbs with rED3 mutants

A total of 27 single amino acid substitutions were introduced into the DENV-3 rED3 by site-directed mutagenesis at 19 different surface accessible residues that 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 Kd for each of the four DENV complex-reactive MAbs 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 analyzed as previously described by Gromowski and Barrett (2007) where mutant proteins resulting in a change in Kd of between 4- and 10-fold were considered "weak" changes in affinity (italicized in Table 2) and those having a change in affinity that was greater than 10-fold were considered "strong" changes (bolded in Table 2). Mutations that resulted in weak and strong changes in affinity are summarized in Fig. 1.

Epitope analysis

The epitope analysis was carried out in a similar manner to that described by Gromowski and Barrett (2007). Those mutations that had a "strong" effect (i.e., greater than 10-fold change in Kd) on MAb binding affinity were considered as being critical epitope residues. Overall, the four MAbs were found to involve a total of 13 residues in ED3 but only L306, K308, G381, I387 and W389 (Fig. 1a, colored red) were shared by all four MAbs. The other nine residues were mutations that had a "strong" or "weak" effect depending on the particular MAb. These residues were as follows: V305, E309, V310, K325, D382, A384Q, K386, and R391 (Fig. 1a, colored pink). Overall, the four DENV complex-reactive MAbs recognize a set of overlapping epitopes that form a single antigenic site on the lateral surface of ED3.

Table 2
Relative Kd for each MAb/rED3 mutant combination

Number of amino acid	Different of anti DENV MAbs			
	MDVP-55A	GTX29202	MA1-27093	20-783-74014
DENV-3 wt	1.00	1.00	1.00	1.00
L301G	*5.74*	2.43	2.34	2.48
T303A	1.40	1.89	2.00	3.34
T303K	2.69	*6.69*	1.86	1.40
T303S	0.70	1.19	>**48**	2.74
V305G	>**78**	**19.49**	4.70	*9.39*
L306G	>**78**	>**64**	>**48**	>**32**
K307G	0.21	0.18	0.17	0.42
K308A	>**78**	**54.89**	>**48**	>**32**
K308G	>**78**	>**64**	>**48**	>**32**
K308Q	>**78**	>**64**	>**48**	>**32**
E309G	>**78**	**28.67**	>**48**	*6.91*
V310G	*5.92*	>**64**	>**48**	>**32**
K321G	1.64	1.30	1.04	2.25
K325G	>**78**	**32.70**	*5.92*	>**32**
A329G	*7.99*	>**64**	1.34	>**32**
G381E	**24.43**	**14.13**	>**48**	>**32**
D382E	3.55	1.96	2.97	**17.32**
D382G	*6.36*	*7.82*	2.04	*6.73*
D382N	3.18	1.62	0.71	0.90
D382P	*4.44*	2.70	*6.60*	**16.35**
K383G	*8.86*	3.05	*6.54*	*9.06*
A384Q	**12.86**	*9.89*	>**48**	>**32**
K386G	0.45	**37.42**	>**48**	>**32**
K386N	>**78**	3.41	0.60	>**32**
I387G	>**78**	>**64**	**34.06**	>**32**
W389G	>**78**	>**64**	>**48**	>**32**
R391G	>**78**	*8.46*	**34.18**	>**32**

The effects of mutations in ED3 are classified as either weak changes (in italics) and strong changes (in bold).

Comparison of DENV complex-reactive antigenic site on DENV-2 and DENV-3 ED3s

The DENV-3 ED3 is 100 amino acids and consists of residues 293 to 393 of the E protein. The DENV-2 ED3 is also 100 amino acids; but the entire E protein is two amino acids longer and so the numbering of DENV-2 ED3 is from 295 to 395 of the E protein. The DENV complex-reactive antigenic site was localized to amino acids on analogous surfaces of ED3 for DENV-3 and DENV-2 (Figs. 1c and d, respectively). Amino acid residues L306, K308, G381, I387, and W389 were critical for all four MAbs on the DENV-3 ED3 and are equivalent to V308, K310, E383, L389 and W391 on the DENV-2 ED3. Residue K310 for DENV-2 (K308 for DENV-3) was the only common critical amino acid for

Table 1
A comparison of the Kd, PRNT50, and HI titer for the DENV complex-reactive MAbs with DENV-2 and -3

DENV MAbs	Kd (nM)		$PRNT_{50}$ (nM)		Ratio $PRNT_{50}$/Kd		HI titer (nM)	
	DEN3V	DEN2V	DEN3V	DEN2V[a]	DEN3V	DEN2V	DEN3V	DEN2V[a]
MDVP-55A	2.50 ± 0.24	0.52 ± 0.11	176.8 ± 12.8	3.0 ± 0.2	70.7	5.8	25.0	0.63
GTX29202	7.30 ± 1.04	0.44 ± 0.02	148.3 ± 14.3	5.9 ± 0.3	20.3	13.4	25.0	0.94
MA1-27093	6.49 ± 1.04	0.43 ± 0.03	159.9 ± 12.7	7.5 ± 0.5	24.6	17.4	50.0	1.25
20-783-74014	5.78 ± 1.20	0.73 ± 0.15	278.5 ± 6.5	11.8 ± 1.3	48.2	16.2	66.7	1.25

[a] The Kd values with DENV-2 virus $PRNT_{50}$ and HI concentrations were previously reported (Gromowski et al., 2008).

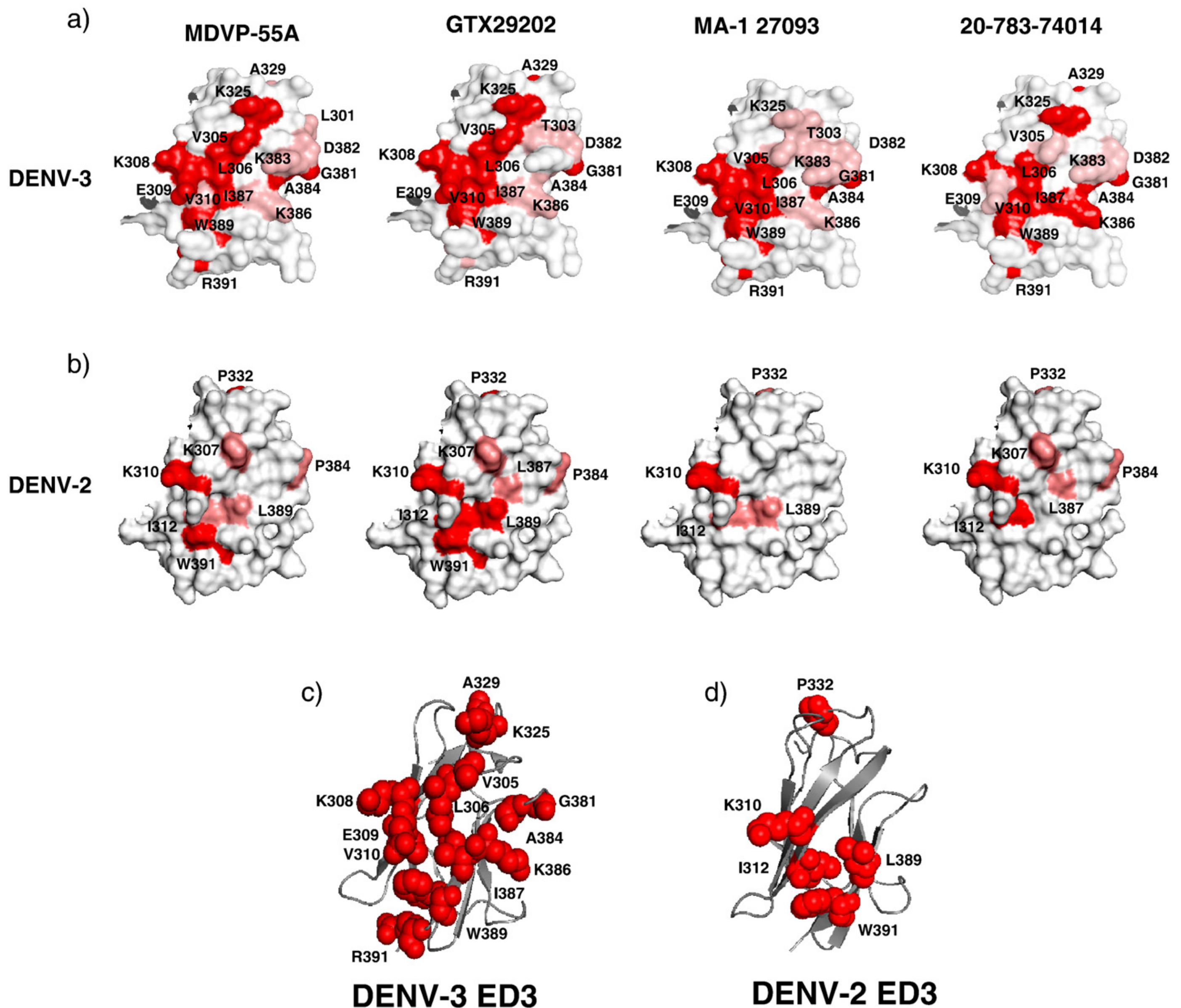

Fig. 1. Comparison of DENV complex-reactive MAb epitopes on DENV-2 and DENV-3 ED3s. The Kd of the 4 DENV complex-reactive MAbs was determined by titration in an indirect ELISA with the DENV-3 rED3 protein. The substitutions that resulted in a change in binding affinity between 4- and 10-fold were defined as having weak effects on the binding affinity (colored pink). Substitutions that resulted in a greater than 10-fold change in binding affinity were considered a strong effect (colored red). (a) Critical residues for all 4 MAb with the DENV-3 ED3 were L306, K308, G381, I387, W389, and R391. (b) For DENV-2, the only shared critical residue for the four MAbs was residue K310 (K308 is equivalent for DENV-3) as reported by Gromowski et al. (2008) All of the critical residues within the DENV complex-reactive antigenic site for DENV-3 are in panel (c) and DENV-2 is in panel (d) for comparison.

binding of the DENV complex-reactive MAbs on the DENV-2 and -3 ED3s (Fig. 1b and Gromowski et al., 2008).

Discussion

Successful development of a tetravalent vaccine for DEN depends on better knowledge on the interactions between DENV complex-specific MAbs and the complex-reactive epitopes. With this in mind, we wanted to determine the physical location and characteristics of the complex-reactive epitopes on the structures of these ED3s. We also want to understand the determining factors that define the energetics of MAbs interacting with the ED3s. We have previously reported the characterization of a panel of DENV complex-specific MAbs with DENV-2 ED3 (Gromowski et al., 2008) and in this study we did a similar characterization of the physical binding and biological properties of this panel of MAbs with DENV-3 ED3. The binding affinity of MAbs to the DENV-2 rED3 was about 5- to 17-fold tighter compared to the binding affinity for the DENV-3 rED3, depending on the MAb. In accordance with these results, the $PRNT_{50}$ concentrations for DENV-2 were about 20- to 50-fold lower, depending on the MAb, compared to the $PRNT_{50}$ concentrations with DENV-3. This is in agreement with results that were previously reported by Gromowski et al. (2008) using this panel of DENV complex-reactive MAbs and further demonstrates that there is an excellent correlation between Kd and $PRNT_{50}$ for DENV ED3-specific MAbs. This relationship was also seen in the HI assays, where the HI titer with DENV-2 was about 30- to 50-fold higher compared to DENV-3, depending on the MAb. Overall, all four MAbs had greater binding affinity (6- to 17-fold higher) and neutralizing activity (20- to 71-fold higher) with DENV-2 compared to DENV-3 for the four MAbs studied. Furthermore, when the physical binding was normalized, the four MAbs neutralized DENV-3 weaker compared to DENV-2 (see Table 1). Thus, for both DENV-2 and DENV-3

the efficiency of neutralization of this panel of MAbs does not quantitatively track the affinity of MAb binding to ED3, implying that neutralization is a composite of more complex reactions than simply binding to ED3.

In this study, we identified five residues (L306, K308, G381, I387, and W389) that were critical for binding all four DENV complex-reactive MAbs to the DENV-3 ED3. In comparison, Gromowski et al. (2008) reported that K310 was the only shared critical residue required for binding to the DENV-2 ED3 using this same panel of MAbs. Thus, each species of DENV has unique residues that are critical to the recognition of even the same MAbs. This conclusion is consistent with the report by Sukupolvi-Petty et al. (2007) that residue K310 was a shared critical residue on the DENV-2 ED3 for two DENV subcomplex-reactive MAbs. Moreover, Lisova et al. (2007) characterized a DENV complex-reactive epitope on ED3 of DENV-1, recognized by MAb 4E11, which was found to consist of residues K307, L308, E309, K310, E311, V312, L387, L389, and W391.

A comparison of critical residues located within the DENV complex-reactive antigenic site on the DENV-2 and DENV-3 ED3s revealed that the location and biochemical properties of four of the shared critical residues are conserved. These residues are K308, V310, I387, and W389 for DENV-3, with the equivalent residues for DENV-2 being K310, I312, L389, and W391, respectively (see Figs. 1c and d). Since the structural fold of ED3 of DENV-2 and DENV-3 are essentially identical (RMSD of the Cα atoms is 0.84) (Modis et al. 2005) this result indicates that the physical locations of the complex-species epitope are very similar, if not identical. This may be one explanation for why the panel of MAbs characterized in this study is capable of recognizing both DENV-2 and DENV-3. If the interfacial interactions between ED3 and MAb define the energetics of MAb binding, then one can expect a difference in the side-chain in the corresponding residue would lead to the differences in Kd values between DENV-2 and DENV-3 that we have observed (Table 1, columns 2 and 3). However, an unexpected result is the additional number of residues whose mutation can lead to significant changes in binding affinity. These additional critical residues included V305, L306, E309, G381, A384, and K386, and were not critical residues in the DENV complex-reactive antigenic site on the DENV-2 ED3. In particular, the biochemical properties of residues K307, E383, and Q386 for DENV-2 (V305, G381, and A384, respectively for DENV-3) are significantly different and this may explain differences in the reactivity of DENV complex-reactive MAbs with the DENV-2 and -3 ED3s. These results imply that although the ED3s assume an essentially identical fold, the differences in the amino acid sequences define a different network of interactions among various residues. The consequence of this difference in sequence leads to the unique set of residues that defines the energetics for MAb binding.

Overall, this study demonstrates that the location of the DENV complex-reactive antigenic site on ED3 for DENV-2 and -3 is conserved, as are several critical residues for MAb binding. However, the data indicate that unique critical epitope residues on ED3 may be important for each of the four DENVs. The results of this study, therefore, provide insight into how DENV complex-reactive MAbs are capable of recognizing multiple DENV ED3s. In addition, we give evidence that structural and biochemical differences within the DENV complex-reactive antigenic site on the DENV-2 and -3 ED3s correlates with differences in binding affinity and neutralization efficacy.

Materials and methods

Cells and viruses

Monkey kidney Vero cells were maintained in Dulbecco's modified essential media (DMEM) supplemented with 8% fetal bovine serum (FBS) and were incubated at 37 °C in a 5% CO_2 incubator. Mosquito C6/36 cells were maintained at 28 °C in DMEM containing 10% FBS and supplemented with tryptose phosphate buffer. DENV-3 strain H87 was used in this study. It was passaged in C6/36 cells and was used as antigen for the hemagglutination inhibition (HI) assays and virus for plaque reduction neutralization tests ($PRNT_{50}$) to characterize the DENV complex-reactive MAbs.

Cloning, expression and purification of recombinant DENV-3 ED3

The DENV-3 rED3 was constructed as previously described (Gromowski and Barrett, 2007). Briefly, the ED3 region of the DENV-3 strain H87 was reverse transcription-PCR amplified for cloning and expression as maltose binding protein (MBP) fusions using the pMal-c2x system (New England Biolabs, Beverly, MA). RNA was extracted from virus-infected cell culture supernatant using the Qiagen Viral RNA Extraction Kit (Qiagen). Reverse transcription-PCR was undertaken using the Titan Kit (Roche). The primers used (Forward: 5′-CGAGGGAAGGATTTCAAAGGGGATGAGCTATGCAATG-3′ and Reverse: 5′-GCCAAGCTTTCATCCCTTCCTGTACCAGTTGATTTTCA-3′) were designed for cloning into the pMal-c2x vector and contained XmnI and HindIII ED3 restriction sites, respectively. The recombinant ED3 (rED3) proteins were expressed in *Escherichia coli* as fusion protein with MBP as the fusion partner. Expression and purification were undertaken by following the manufacturer's instructions and as previously described (Beasley and Barrett, 2002).

Dengue-complex specific monoclonal antibodies (MAbs)

Four commercially available MAbs were used in this study: MDVP-55A (Immunology Consultants Laboratory, Inc.), GTX29202 (GeneTex), MA1-27093 (Affinity BioReagents), and 20-783-74014 (GenWay). These MAbs were affinity purified IgG2a mouse MAbs and have been previously reported by Gromowski et al. (2008).

Mutagenesis of recombinant DENV-3 ED3

Site-directed mutagenesis of the DENV-3 H87 ED3 gene fragment in the pMal-c2x vector was undertaken using the Quickchange kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions.

Indirect ELISA

An indirect ELISA was used to investigate the binding of the DENVs MAbs to DENV-3 strain H87 rED3 as described by Gromowski and Barrett (2007) and the data shown as apparent Kd value since they have been determined by an ELISA technique.

Affinity measurements by indirect ELISA

A total of 27 single amino acid substitutions were selected and evaluated using an indirect ELISA as previously described (Gromowski and Barrett, 2007).

HI assay

The HI assays were performed as previously described (Clarke and Casals, 1958). HI assays were carried out at pH 6.0 with goose erythrocytes using four hemagglutinating units of DENV-3 strain H87. The titer was defined as the maximum dilution at which hemagglutination was completely inhibited and MAb titrations were carried out in serial two-fold steps starting at 200 nM.

Plaque reduction neutralization test ($PRNT_{50}$)

The four MAbs were diluted to 1000 nM in PBS. Two-fold serial dilutions were prepared and mixed with an equal volume of DENV-3

(~50 pfu/well). Virus and MAb mixtures were incubated for 1 h at room temperature. Subsequently, the virus-MAb mixture was transferred to ~80% confluent Vero cells in 6-well plates. After 1 h of incubation at room temperature, cell monolayers were overlaid with MEM containing 2% FBS and 1% Agar, and incubated at 37 °C. Plaques were observed until visualizing by staining with neutral red. $PRNT_{50}$ data were converted to percent neutralization relative to controls in the absence of MAb and $PRNT_{50}$ concentrations were calculated by doing a non-linear regression analysis using Sigmaplot (Version 9.01, Systat Software, Inc., CA) as previously described (Gromowski and Barrett, 2007).

Statistical analysis

The Kd values are presented as mean ± standard error of the mean (SEM) and were analyzed using SigmaStat (Version 3.1, Systat Software, Inc., CA).

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