On a mouse monoclonal antibody that neutralizes all four dengue virus serotypes

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The flavivirus envelope glycoprotein (E) is responsible for viral attachment and entry by membrane fusion. Its ectodomain is the primary target of the humoral immune response. In particular, the C-terminal Ig-like domain III of E, which is exposed at the surface of the viral particle, forms an attractive antigen for raising protective monoclonal antibodies (mAb). 9F12, a mouse mAb raised against a dengue virus (DENV) serotype 2 recombinant domain III, cross-reacts with corresponding domains from the other three DENV serotypes and also with West Nile virus. mAb 9F12 binds with nanomolar affinity to a conserved epitope that maps to the viral surface comprising residues 305, 307, 310 and 330 of the E protein. mAb 9F12 neutralizes all four DENV serotypes in plaque reduction assays. We expressed a single-chain Fv from 9F12 that retains the binding activity of the parent mAb. Adsorption and fusion inhibition assays indicate that mAb 9F12 prevents early steps of viral entry. Its virus inhibition activity and broad cross-reactivity makes mAb 9F12 a suitable candidate for optimization and humanization into a therapeutic antibody to treat severe infections by dengue.

INTRODUCTION

Dengue virus (DENV), a member of the family Flaviviridae, is responsible for over 20 000 deaths per year. Neither a licensed drug nor a vaccine has been approved to treat severe conditions caused by infection with DENV like dengue haemorrhagic fever. Other flaviviruses such as West Nile, yellow fever or Japanese encephalitis viruses are important human pathogens of global concern (Gubler, 2006; Halstead, 2007; Keller et al., 2006). The flavivirus envelope glycoprotein (E), which forms an icosahedral scaffold at the virion surface, is the primary determinant of host-cell tropism and the target of neutralizing antibodies (Rey et al., 1995; Roehrig et al., 2004). The E protein was found to be structurally similar to the Semliki Forest alphavirus E1 protein, leading to the concept of class II viral fusion glycoproteins (Lescar et al., 2001). X-ray crystallographic studies of soluble fragments of the E protein from DENV-2 and 3, tick-borne encephalitis and West Nile virus revealed a well-conserved elongated structure (Kanai et al., 2006; Modis et al., 2003, 2005; Nybakken et al., 2006; Rey et al., 1995). The molecule consists of three domains. The central domain I bears predominantly serotype-specific non-neutralizing epitopes, the homodimerization domain II contains the fusion loop at its extremity (Allison et al., 2001) and can elicit both neutralizing and non-neutralizing monoclonal antibodies (mAbs; Crill & Roehrig, 2001). Domain III, which is involved in receptor binding (Allison et al., 2001; Bressanelli et al., 2004; Heinz & Allison, 2003; Rey et al., 1995), is connected to a C-terminal stem anchored to the viral membrane and adopts an immunoglobulin constant domain fold. It contains three regions centred at amino acids 307, 330 and 384 (DENV-2 numbering) with the corresponding residues projecting from the surface of the mature virion and thus largely accessible to antibodies. At endosomal pH, the virion undergoes structural rearrangements leading to homotrimerization of the E protein (Modis et al., 2004). This extensive reorientation of domain III suggests another strategy to design either
therapeutic antibodies or small molecules that would interfere with conformational changes required for membrane fusion. A survey of neutralizing mAbs (Crill & Roehrig, 2001) indicated that mAbs that bind to domain III are efficient antagonists of virus adsorption to Vero cells. Some potent inhibitory mAbs were found to block infection at a post-attachment stage (Nybakken et al., 2005). Moreover, immunization with domain III alone confers protection to mice from a lethal challenge with West Nile virus (WNV) (Chu et al., 2005; Martina et al., 2008). Here we present the functional and structural characterization of 9F12, a mouse mAb raised against a recombinant domain III from DENV-2 with interesting cross-neutralizing capacity towards five DENV strains.

**METHODS**

**Cells and viruses.** BHK-21 (ATCC: CCL-10) and C6/36 cells (ATCC: CRL-1660) were grown in RPMI 1640 medium containing 10% inactivated fetal calf serum (fCS). Vero cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 7% fCS. Strains of DENV-1 (Hawaii; ATCC: VR-71), DENV-2 (New Guinea C; ATCC: VR-1255 and TSV01 (McBride & Vassudevan, 1995)), DENV-3 (H87; ATCC: VR-1256) and DENV-4 (H241; ATCC: VR1490) were propagated at 28 °C either in C6/36 or BHK-21 cells, supplemented with 5% fCS. The hybridoma 4G2 (ATCC: HB-112) was grown in HybridCare (ATCC: 46-X) supplemented with 8–10% fCS. Hybridoma. mAb 9F12 was obtained by hyper-immunization of BALB/c mice with DENV-2 TSV01 domain III, using standard protocols (Clancy et al., 2007). Mouse hybridoma cells secreting 9F12 were initially grown in Hybrid-Care medium in NUNC flasks and upon confluence were switched to RPMI 1640 supplemented with 10% fCS. Cell supernatants were centrifuged at 800 g for 5 min at 22 °C and stored at −20 °C in 1 M Tris/HCl (pH 8.0).

**Cloning, expression and purification of recombinant domains III from DENV-1–4.** The primers used for PCR amplifications are listed in Table 1. PCR products were digested and ligated into the Pet16b vector (Novagen). Transformed *Escherichia coli* BL-21(DE3) cells were grown at 37 °C until an OD _600_ of 0.8 was reached and protein expression was induced with 1 mM IPTG for 6 h at 30 °C. Cells harvested by centrifugation at 5000 g for 20 min at 4 °C were resuspended in a buffer containing 50 mM Tris/HCl (pH 8.5), 200 mM NaCl, 1% Nonidet-P40 and 1% sodium deoxycholate and resuspended in a buffer containing 50 mM Tris/HCl (pH 8.5), 200 mM NaCl, 10 mM EDTA, 5 mM reduced/oxidized glutathione and 50 mM L-arginine. Refolded proteins concentrated by ultrafiltration (Amicon) were purified on a Superdex 75 (HR 10/30) column (GE Healthcare) in 12 mM Tris/HCl (pH 8.0), 250 mM NaCl, 0.1 mM EDTA and 3 mM DTT to prevent intermolecular disulfide bond formation. Proper folding was assessed by circular dichroism (CD) spectroscopy (Chu et al., 2005).

**Plaque reduction neutralization assays.** The protocol follows that of Thullier et al. (1999). Briefly, all plaque reduction neutralization test 50% (PRNT50) assays were carried out with BHK-21 cell lines at 37 °C. Serial dilutions of mAb 9F12 were mixed with 100 p.f.u. DENV ml⁻¹ and incubated for 2 h at 4 °C. mAb and virus were incubated with BHK-21 cells at 37 °C for 2 h. After incubation, the mixture was replaced with RPMI 1640 with 1% carboxy methyl cellulose, and appropriate mAb dilution and plates were placed in a 5% CO₂ incubator for 4–5 days for DENV-2, 5 days for DENV-4 and DENV-3, and 6 days for DENV-1. After fixing with 4% formaldehyde and staining with 1% methyl violet, plaques were manually counted and the neutralization capacity was estimated as the mAb concentration causing a 50% reduction in p.f.u.

**Adsorption assays using cell-based flavivirus immuno-detection (CFI).** A 96-well micro culture dish was dispensed with 2 x 10⁷ Vero cells (100 μl) in DMEM supplemented with 2% fCS and incubated overnight at 37°C in a 5% CO₂ incubator. For the preadsorption assay, tenfold dilutions of either mAb 9F12 or 4G2 was mixed with an equal volume of 2 x 10⁴ p.f.u. of DENV-2 virus and incubated at 4 °C for 1 h. The virus and mAb mixture was added to the confluent cell surface and incubated at 4 °C for 1 h for the virus to get adsorbed onto the cell surface. Negative control received serum free (sf) DMEM in place of the mAb. Cells were washed three times.

**Table 1. Summary of domain III constructs**

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</tr>
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*The XhoI (forward) and ClaI (reverse) restriction sites are underlined.
with sf DMEM at 4°C. After incubation with DMEM for 2 days, cells were mixed with the 1:20 diluted 4G2 antibody for immuno-detection. The immune reaction was probed with anti-mouse HRP conjugate, tetramethyl benzidine and stopped with 0.5 N sulphuric acid; absorption was then measured at 450 nm. The presence of a uniform number of cells per well was confirmed by staining with propidium iodide (PI) (Sigma) in PBS for 10 min and the fluorescent signal was read at 537–617 nm in a Tecan plate reader. For the postadsorption assay, a virus dilution containing 1 × 10^6 p.f.u. of DENV-2 was added to the cells directly and incubated at 4°C for 1 h followed by washes with sf DMEM at 4°C to remove unadsorbed virus. The mAb dilutions were added to cell surface with the adsorbed virus and incubated for 1 h at 4°C. The rest of the assay to detect DENV-2 NGC was as described for preadsorption.

**Membrane fusion inhibition assay.** A fusion inhibition assay based on syncytia formation by C6/36 Aedes albopictus cells at low pH (Randolph & Stollar, 1990) was used for screening fusion inhibitors. The antibody 4G2 was used as a positive control as it prevents syncytia formation (Summers et al., 1989). Assays were carried out in triplicate both for 4G2 and 9F12. Briefly, a 96-well micro culture dish was dispensed with a mixture of 1.5 × 10^5 C6/36 cells and DENV-2 at an m.o.i. of 0.1 per 100 μl in RPMI 1640 supplemented with 5% ICSF and incubated for 72 h at 28°C in an air-tight humidified container. Both 9F12 and 4G2, diluted to a final concentration of 10 μM in 95 μl of sf RPMI 1640, were added to the wells and incubated for 1 h at 28°C. Subsequently, 5 μl of 0.5 M (N-morpholino)ethanesulphonic acid (pH 5.0) was added to the wells and incubated at 37°C for 1 h to induce syncytia formation. The low pH solution was finally replaced with 0.025 mg PI ml^-1 solution made in sf RPMI 1640 and incubated at 28°C for 30 min in order to stain non-viable cells/syncytia. Plates read at ×10 magnification were visualized with a Nikon fluorescence microscope.

**Cloning, expression and purification of single chain antibody variable fragment (scFv) 9F12.** A total of 10^6 cells of the mAb 9F12 hybridoma were used for RNA extraction using TRIzol reagents. The V_H chain was amplified with the forward primer (5’-CCAGTTCCGAGCTCGTGATGACACAGTCTCCA-3’) and reverse primer (5’-GCGCCGTCTAGAATTAACACTCATTCCTGTTGAA-3’) and the V_L chain was amplified with a light chain primer mix 9F12 and 4G2, to a final concentration of 10 μM in 95 μl of sf RPMI 1640, were added to the wells and incubated for 1 h at 28°C. Subsequently, 5 μl of 0.5 M (N-morpholino)ethanesulphonic acid (pH 5.0) was added to the wells and incubated at 37°C for 1 h to induce syncytia formation. The low pH solution was finally replaced with 0.025 mg PI ml^-1 solution made in sf RPMI 1640 and incubated at 28°C for 30 min in order to stain non-viable cells/syncytia. Plates read at ×10 magnification were visualized with a Nikon fluorescence microscope.

**Measurement of binding affinities.** Binding affinities of mAb 9F12 or scFv9F12 for various DENV domain III serotypes were determined by surface plasmon resonance (SPR) at 25°C using a Biacore 3000 instrument (GE Healthcare). Each domain III was covalently conjugated with alkaline phosphatase for 1 h at 22°C, followed by addition of p-nitrophenyl phosphate (Pierce), and the optical density was measured at 405 nm (OD405).

**Epitope mapping.** Yeast surface display of DENV-2 domain III mutants was determined. The DNA fragment encoding amino acid residues 294–409 (domain III) of DENV-2 E protein was expressed on the surface of yeast as an Aga2 fusion protein as described previously (Sukulpolvi-Petty et al., 2007). DENV-2 domain III mutants that were generated as part of a random library by error-prone mutagenesis in the pYD1 vector were expressed on the surface of yeast as described previously (Sukulpolvi-Petty et al., 2007). Wild-type or mutant DENV-2 domain III displayed on yeast were harvested, washed with PBS supplemented with BSA (1 mg ml^-1) and stained with 50 μl diluted mAbs (9F12, 3H5-1). After 30 min incubation on ice, yeast were washed in PBS with BSA and then stained with a goat anti-mouse IgG secondary antibody conjugated to Alexa Fluor 647 (Molecular Probes, Invitrogen). After fixation with 1% paraformaldehyde in PBS, yeast cells were analysed on a FACS Scan flow cytometer (Becton Dickinson) using FlowJo software (Tree Star). A set of 11 overlapping peptides, each comprising 10–12 amino acids from DENV-2 domain III was synthesized and purified by HPLC. ELISA was performed following the protocol of Thullier et al. (2001). Briefly, microtitre plates were coated with poly-(L-lysine) (Sigma) in 0.1 M bicarbonate buffer (pH 9.6) followed by incubation with 0.1% glutaraldehyde in PBS. Wells were coated with 100 μl of peptides at concentrations ranging from 10^-4 to 10^-7 M followed by blocking with 50 mM glycine in PBS-EDTA. A volume of 100 μl mAb 9F12 at 10^-8 M was distributed in the wells.

**RESULTS**

**Purification and characterization of recombinant domain III**

Domain III from DENV-1–4 was expressed at high level as inclusion bodies that required refolding. Domain III from WNV was expressed and purified as described by Chu et al. (2005). Typically, bacterial culture of 1 l resulted in 6–8 mg of pure recombinant proteins with ~30% refolding efficiency, measured according to Cowieson et al. (2008). Typical SDS-PAGE of purified domain III from DENV-1–4 is shown in Fig. 1(a). For unknown reasons, the stability of DENV-4 domain III was much poorer. The identity of the expressed proteins was confirmed by mass spectrometry (not shown). CD spectroscopy was used to assess the folding of recombinant domain III which elutes as monomers as judged by gel filtration profiles. An example is shown for DENV-2 domain III (Fig. 1b) indicating proper folding with a predominantly β-sheet structure, in agreement with the known 3D structure (Volk et al., 2004). An alignment of the amino-acid sequences of the various domains III studied in this work is presented in Fig. 1(c),
Preliminary characterization of mouse mAb 9F12

As part of an effort to raise antibodies against all four DENV serotypes, we immunized BALB/c mice with 50 µg purified domain III from DENV-2 (TSV01 isolate) using standard protocols. A panel of mAbs that reacted with DENV-2 domain III were identified using ELISA and 9F12 was selected for further studies because it cross-reacted with domain III from DENV-1–4 and WNV in a Western blot assay under denaturing conditions, and also in a dot-blot immunoassay under non-denaturing conditions. mAb 9F12 belongs to the IgG1 subtype with kappa light chain.

In vitro neutralization of DENV by mAb 9F12

Given its broad cross-reactivity towards various recombinant domain III proteins, we tested the neutralizing activity of mAb 9F12 against all four DENV serotypes in a plaque reduction neutralization test assay. Interestingly, purified mAb 9F12 neutralized all five DENV strains tested, belonging to serotypes DENV-1–4. The PRNT₅₀ ranged from 2 × 10⁻⁷ to 2 × 10⁻⁸ M for DENV-2, 4 and 1, 3, respectively. A representative assay plate is shown in (Fig. 2a). The commercially available mouse mAb 4G2 was used as the positive control and naive mouse serum was used as negative control. The 50% neutralization point for the various dengue strains tested varied between 14 ± 0.3 nM and 130 ± 0.8 nM of 9F12. The neutralizing capacity of mAb 9F12 appears to be comparable to that of mAb 4G2 (positive control). Fab fragments prepared by papain cleavage and recombinant scFv9F12 were also tested by PRNT₅₀ and showed comparable inhibitory activities against all five strains tested (data not shown), a result that might relate to the fact that scFv9F12 tends to dimerize.

Mechanism of virus neutralization

Cell-based infection assays (Fig. 2b and c) were based on the fact that nuclei of fused C6/36 cells are stained by PI whilst intact cells are not permeable.

These assays suggest that mAb 9F12 hinders an early step in the virus life cycle, most likely viral adsorption and entry, rather than some post cell entry stages like membrane fusion between the endosomal membrane and viral lipid bilayer; in the absence of antibodies or in the presence of mAb 9F12, infected C6/36 cells are stained by PI, demonstrating membrane fusion and syncytia formation (Fig. 2b, panels 2 and 4). By contrast, uninfected cells or cells protected by mAb 4G2 remain intact and are not permeable (Fig. 2b, panels 1 and 3). mAb 4G2 recognizes the fusion loop at the extremity of domain II of E protein from all four serotypes and prevents syncytia formation. 9F12 on the other hand shows drastically reduced protection when it is introduced after virus adsorption, whilst the protection conferred by mAb 4G2 remains largely unaffected (Fig. 2c).
Sequencing of mAb 9F12, cloning and purification of scFv9F12

In order to explore the molecular basis of virus neutralization by mAb 9F12, the VH and VL genes of antibody 9F12 were sequenced and a scFv fragment was cloned to facilitate further structural studies. PCR amplification yielded products of 440 and 400 bp for its VH and VL variable domains, respectively. The VH region of 9F12 shows highest sequence identity for the heavy chain of the anti-HIV-1 p24 Fab Fragment Cb41, an antibody exhibiting cross-reactivity and polyspecificity (PDB accession no.: 1CFS) (Keitel et al., 1997). The light chain of 9F12 is most similar to the anti-HIV protease Fab fragment.
Binding affinities of mAb 9F12 and scFv9F12 to various flavivirus domain III

Initial binding assays indicated that mAb 9F12 broadly cross-reacts with various flavivirus domain III antigens. We probed the interaction between mAb 9F12 and several recombinant flavivirus domain III using two independent methods. First, the interactions were assessed using ELISA assays (Fig. 3a). The EC₅₀ values calculated for the interactions with DENV domain III range from 0.17 ± 0.03 nM to 84 nM ± 1.20 nM with the following binding affinities for the antigens: DENV-2=DENV-4>DENV-1>DENV-3. Surprisingly, the apparent EC₅₀ for WNV domain III is comparable to the immunogen DENV-2 (Fig. 3a). The observation of heteroclitic binding for mAb 9F12 (higher affinity for an antigen other than the immunogen) is not unprecedented and has been thoroughly analysed using model antigens such as avian lysozymes (Chitarra et al., 1993; Lescar et al., 1995). It usually occurs when an epitope is shared between evolutionary-related antigens. To confirm these results, scFv9F12 was expressed, purified and its binding affinities measured using SPR, along mAb 9F12, in order to obtain kinetic parameters of the various complexes (Table 2). Due to the high level of non-specific interactions established by domain III proteins with the sensor chip surface, which could not be overcome either by increasing NaCl concentration or by adding carboxy methyl dextran to the sample buffer, we immobilized the antigen on the surface and passed the mAb 9F12 or its scFv as 'analytes'. The binding of the mAb and the scFv was specific. Fig. 3(b) shows examples of sensorgrams obtained for the interactions of domain III of E protein of DENV-1, 2, 3 and WNV with either mAb 9F12 or scFv9F12. An approximation of the active concentrations of mAb 9F12 and scFv9F12 was obtained using initial binding rates to a high level immobilized surface of domain III of the E protein of DENV-2 in which mass transfer limitation was almost complete (Karlsson et al., 1994). Interactions with mAb and scFv were best fitted using the 'bivalent analyte model' where one analyte molecule is assumed to bind to one or two immobilized ligands. Binding to the first ligand is described by a single set of rate constants, so that both sites on the analyte are equivalent. Binding of the second ligand molecule is described by a second set of rate constants, allowing the model to take cooperative effects into account. The resulting affinity (Table 2) is an approximation since it is calculated from the ratio of the kinetic constants kₐ₁/kₐ₂. For the mAb 9F12, this model is an obvious choice. For the scFv, the bivalent analyte model gave a better fit than a 1:1 Langmuir model, indicating that a large part of the scFv9F12 molecules were not monomeric (Dolezal et al., 2000; Kortt et al., 1994). Indeed, scFv9F12 elutes as a dimer in gel filtration analysis (data not shown). As shown in Table 2, mAb 9F12 binds to all domain III proteins from the different serotypes of DENV and also to WNV, but with different affinities. The highest affinities observed were for the interaction between mAb 9F12 and DENV-2 and WNV that all bind with the same order of magnitude. For DENV-1 the affinity is one order of magnitude lower and for DENV-3, two orders of magnitude lower than for the immunogen. The same pattern for the affinities is observed for scFv9F12 as seen in Table 2.

Epitope mapping

To map the epitope recognized by mAb 9F12, we used a panel of DENV-2 domain III mutants displayed on the surface of yeast that were previously generated to map a panel of type-specific, subcomplex-specific and cross-reactive mAbs (Sukulpolvi-Petty et al., 2007). 9F12 showed markedly reduced binding with domain III mutations at residues K305, K307, K310 and G330 (Fig. 4a), yet retained binding to variants at residues E383 and P384. This pattern of binding is most consistent with a neutralizing epitope centred on the A-strand of domain III (Fig. 4b). We also synthesized overlapping peptides corresponding to the sequence of DENV-2 and used them for epitope mapping by ELISA. Peptides S298–V309 (SYSMCTGKFKVV) and V324–I335 (VQYEGDGSPKI) were bound most strongly, followed by V309–G318 (VKJEIAETQHG). The other peptides tested along with a peptide from an unrelated protein showed no appreciable binding (Fig. 4b). Mapping these binding results onto the 3D crystal structure of DENV-2 envelope protein suggests that 9F12 most likely binds to an epitope that include basic residues 305, 307 and 310 that project from β-strand A and residues 327–331 at the tip of the BC loop (Fig. 1c and 4b).

DISCUSSION

Several cellular attachment molecules were proposed for DENV, including heparan sulfate (Chen, 1997), DC-SIGN (Pokidysheva et al., 2006; Tassaneetrithep et al., 2003), L-SIGN (Navarro-Sanchez et al., 2003), the mannose-binding receptor on macrophages (Miller et al., 2008) and the laminin-binding protein (Thepparit & Smith, 2004; Tio et al., 2005). Despite this variety of host-cell surface receptors, one viral component consistently shown to bind directly to cellular receptors is the Ig-like domain III of the E protein (Beasley & Barrett, 2002; Hung et al., 2004; Lin & Wu, 2003; Mandl et al., 2000; Thullier et al., 2001). Subtle structural variations at the surface of domain III are thus likely to influence virus interactions with attachment...
molecules (cell tropism) and also with antibodies from the host that define serotypes. Several neutralizing antibodies to flavivirus domain III proteins (Lisova et al., 2007) have already been identified: their epitopes cluster onto the top lateral surface of the Ig-like domain. A structurally well-characterized interaction involves mAb E16 with domain III from WNV (Nybakken et al., 2005). mAb E16 binds to an epitope composed of residues 302–309 (belonging to the NTR and β-strand A) and three loops, BC, DE and FG (Fig. 1c). Neutralizing mAbs, particularly to DENV, were proposed to bind two structurally distinct epitopes centred either on the FG loop, as in the case of mAb 3H5 (Gromowski et al., 2008), or to the more conserved A strand, like mAb 1A1D-2 (Lok et al., 2008). By contrast, several cross-reactive mAbs that bind to residues from the AB loop were found to be poorly neutralizing as this loop projects toward the lipid bilayer in the mature viral particle. The relatively strong neutralizing capacity of mAb 9F12 can thus be partly attributed to the fact that it binds an epitope centred at the solvent-exposed ‘A’ strand and the BC loop (Fig. 4b). Amongst residues that form the epitope recognized by mAb 9F12, K305 and G330 are shared by DENV-2 and 4, K307 by DENV-2 and 1 and WNV and K310 by DENV-1–4 (WNV has an arginine at this position). Thus, the pattern of substitutions between the various DENV serotypes appears grossly consistent with the range of affinity we observe, with DENV-3 having T305, V307 and D330 being recognized with the lowest affinity (Figs 1c and 3). Interestingly, some strains of DENV-2 (e.g. isolate Malaysia M2, Swiss-Prot accession number P14338; see Bhardwaj et al., 2001) have Glu at position 310. Given that a positively charged residue (K or R) is found at position 310 in all the strains we have tested, it will be of great interest to see whether such variant DENV-2 strains can also be neutralized by mAb 9F12.

Of note, mutations of the residues listed above did not affect binding of domain III-lateral ridge antibodies on yeast (Sukulpolvi-Petty et al., 2007). Studies by Gromowski et al. (2008) used analogous mutations with recombinant domain III, suggesting that these substitutions are likely to have only local rather than long-range effects on the structure of domain III. Moreover, in the case of mAbs E16 and 1A1D2, their epitopes were mapped first by yeast surface display and were later confirmed by crystallography (Lok et al., 2008; Oliphant et al., 2005). Whether a larger area of contact between 9F12 with one or more conserved residues at the surface of domain III does favour
cross-reactivity must await further structural studies, and more insight into the interactions of 9F12 with various domain III requires crystallization of the complex.

How does the neutralizing activity of mAb 9F12 compare with previously described dengue neutralizing antibodies? Compared with domain III-specific mAbs described earlier by Sukupolvi-Petty et al. (2007), the neutralizing activity of mAb 9F12 (3.2 × 10⁻⁹ M against DENV-2) is lower than some of the type-specific mAbs like 1F1, 6B6-10, 9A3D-8 or 3H5-1 that have neutralizing activities in the range of 1.2 × 10⁻⁹ to 6.7 × 10⁻¹¹ M range. However, mAb 9F12 appears to be more potent than cross-reactive mAbs like E111 and E114. A recent immuno-pathological study of

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**Table 2.** Kinetic constants and binding affinities of mAb 9F12 and scFv9F12 for the domain III of protein E from the different DENV serotypes and WNV by SPR

$k_D$ is an apparent affinity calculated by the ratio $k_{d1}/k_{a1}$ obtained from the fit with the bivalent analyte model. In all cases, $\chi^2$ was lower than 5% of $R_{\text{max}}$.

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*The constants were evaluated from the sensorgrams depicted in Fig. 3(b), using the bivalent analyte model for both the mAb and the scFv. The BIAevaluation 4.1 software was used for data analysis.

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**Fig. 4.** Epitope mapping. (a) Flow cytometry histograms of mAbs binding to yeast expressing wild-type or mutant domain III. The following antibodies were used as negative and positive staining controls, respectively, for each of the indicated yeast based on data from a prior publication (Sukupolvi-Petty et al., 2007): wild-type, WNV E16 and 3H5-1; K305E, 1A1D-2 and 3H5-1; K307C, 1A1D-2 and 3H5-1; P384A, 3H5-1 and 5A2-7; G330D, 6B6-10 and 3H5-1. In each case, staining with 9F12 is shown in red. The data are representative of three independent experiments. (b) Binding of mAb 9F12 to DENV-2 domain III and peptides covering its sequence. ELISA plates coated either with the immunogen or peptides were incubated with 9F12. The experiment was carried out in triplicate. Error bars, SD. The inset is a model of domain III showing the epitopes recognized by mAb 9F12, based on the yeast surface display results. Residues forming the mAb 9F12 epitope (dotted spheres – K305, K307 and K310) and shared by the mAb 1A1D-2 (Lok et al., 2008) are labelled in blue. G330, which is recognized by mAb 9F12, is marked in black. Loops BC, DE and FG and the N-terminal region (NTR) are coloured in blue. The solvent-inaccessible loop AB (in the context of the viral particle) is marked with a black arrow.
WNV (Oliphant et al., 2007) suggested that highly neutralizing virus-specific antibodies of the IgG subtype do not appear in detectable levels during primary infection of mice until days 10–15, by the time the viraemic phase is completed. Moreover, analysis of convalescent serum samples from WNV-infected humans showed that a significant proportion of individuals never developed antibodies to the neutralizing epitope located on the domain III lateral ridge. Likewise, potent neutralizing human mAbs to domain III are rarely isolated from WNV-infected patients (Throsby et al., 2006). The antibody responses thus appear to be skewed toward the induction of less neutralizing antibodies, particularly to the fusion loop. Overall, the IgG response to domain III appears variable and comprises only a small fraction of the whole antibody response. Thus, for unclear reasons, the development of neutralizing antibodies that bind WNV domain III appears to be an uncommon event in humans, in contrast to experimental infections in C57BL/6J mice. In a preliminary experiment of mouse protection assay with 9F12 and DENV-2 performed in our lab, a significantly reduced amount of the viral NS1 protein was observed, suggesting that the 9F12 antibody may retain neutralization activity in vivo.

CONCLUSION

The availability of a single high affinity antibody that could efficiently neutralize all four DENV serotypes early enough during the viraemic phase may rapidly reduce the viral load and possibly prevent progression to severe vascular leakage syndrome, which occurs at a later phase during the natural history of infection in a subset of dengue patients. In the absence of a fully validated tetravalent vaccine, and given the potential risks posed by antibody-dependent enhancement effects, treatment via passive immunotherapy appears as a possible alternative, provided an early enough diagnostic of infection by dengue can be made. In this respect, the mouse mAb 9F12 that cross-reacts with all four serotypes of DENV, and strongly neutralizes virus infection, is a possible candidate for further rounds of affinity maturation and humanization.

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