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Structural analysis of a dengue cross-reactive antibody complexed with envelope domain III reveals the molecular basis of cross-reactivity[1]

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Abstract

Dengue virus infections are still increasing at an alarming rate in tropical and subtropical countries underlying the need for a dengue vaccine. Although it is relatively easy to generate antibody responses to dengue virus, low avidity or low concentrations of antibody may enhance infection of Fc receptor-bearing cells with clinical impact, posing a challenge to vaccine production. In this paper we report the characterization of a monoclonal antibody, 2H12, which is cross-reactive to all four serotypes in the dengue virus group. Crystal structures of 2H12-Fab in complex with domain III of the envelope protein from three dengue serotypes have been determined. 2H12 binds to the highly conserved AB loop of domain III of the envelope protein that is poorly accessible in the mature virion. 2H12 neutralization varied between dengue serotypes and strains; in particular, dengue serotype 2 was not neutralized. As the 2H12 binding epitope was conserved, this variation in neutralization highlights differences between dengue serotypes and suggests that significant conformational changes in the virus must take place for antibody binding. Surprisingly, 2H12 facilitated little or no enhancement of infection. These data provide a structural basis for understanding antibody neutralization and enhancement of infection, which is crucial for the development of future dengue vaccines.

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Introduction

Dengue is a mosquito-borne infection of the tropics and subtropics (1, 2). Some 2.5 billion people are at risk and 50-100 million are infected annually. Most infections are either asymptomatic or result in dengue fever (DF[2]), a relatively mild illness. However, a much more severe form, dengue haemorrhagic fever (DHF[3]), develops in 1-5% of infections; this can be life threatening. The incidence of dengue is increasing at an alarming rate and epidemics can severely disrupt healthcare systems in developing countries (1, 2). Although treatment has reduced the mortality rate, there is still an urgent need for a vaccine.

Dengue viruses have been divided into four serotypes differing in overall amino acid sequence by 30% or more (3, 4). Infection with one serotype does not give life long protection against the other serotypes (5), and a hallmark of dengue infection is that DHF is more likely to occur following a secondary infection with a heterotypic serotype, rather than following a primary infection (6). Halstead proposed antibody-dependent enhancement to explain this paradox whereby an acquired humoral response to the first virus could drive a more severe clinical outcome upon a secondary exposure (7, 8). There is now good evidence that cross-reactive poorly neutralizing antibodies can drive infection of Fc receptor-bearing cells, such as monocytes, leading to increased infection and virus production (7-12).

Dengue virus has three structural proteins; capsid (C) that encloses the positive strand genome; precursor membrane protein (prM) and envelope (E), both of which are components of the virion envelope structure. Antibodies to prM are generally poorly neutralizing but potent enhancers of infection (13, 14), whereas antibodies against E show more potent neutralizing activity (15-17). E is composed of three domains (ED): I-III[4] (18). EDI and EDII are formed by discontinuous folds at the membrane proximal N-terminus of the protein; EDII contains the fusion loop. Antibodies that target the highly conserved fusion loop are usually flavivirus cross-reactive (19, 20) but, due to the epitope's inaccessibility on infectious virions, they mostly bind with low avidity and exhibit weak neutralization (20). Recently, however, a flavivirus cross-reactive mAb 2A10G6 that binds to a newly identified epitope within the fusion loop was shown to be broadly cross-neutralizing and cross-protective (21). EDIII is thought to be involved in host cell interaction (22-24), binding to heparan sulfate (25) and/or other as yet poorly characterized receptor(s) (24). In mice, monoclonal antibodies specific to EDIII are potent neutralizers of dengue virus (26-35), and neutralize more strongly than EDI- or EDII-specific antibodies (33). As a result, EDIII has been considered as a potential immunogen for new subunit vaccines (36-40). EDIII is a target of both serotype-specific (16, 26, 27, 32-34, 41, 42) and dengue cross-reactive (28, 30-34, 43) neutralizing antibodies, though the latter tend to neutralize more weakly (28, 34).

Here we report a mouse monoclonal antibody 2H12 that cross-reacts with the four serotypes of the dengue group and which neutralizes Den1, 3 and 4. Crystal structures of 2H12 Fab with recombinant EDIII were determined at resolutions of 1.7 Å, 1.8 Å and 3.0 Å for Den1, Den3 and Den4, respectively. They show that the antibody has a conserved mode of binding and contacts a highly conserved epitope in the AB loop of EDIII, which is largely buried in the mature virion structure, implying that gross conformational changes occur in the surface architecture of the virion upon antibody binding. This binding is temperature dependent, and different across the serotypes, implying that the stability of the virus is a key factor in virus neutralisation.

[2]DF: dengue fever
[3]DHF: dengue haemorrhagic fever
[4]EDIII: envelope domain III

Materials and Methods

Expression and purification of recombinant EDIII

EDIII (aa 295-401) of dengue virus serotype 1-4, strains Hawaii, 16681, H87 and H241 were expressed in *E. coli* and purified as described previously (44). In brief, the EDIII proteins were expressed in inclusion bodies, and refolded in 100 mM Tris HCl, 500 mM L-arginine-HCl, 0.2 mM EDTA, 3.7 mM Cystamine, 100 μM PMSF, 6.6 mM β-mercaptoethylamine. Refolded protein was purified in PBS through a size exclusion column (26/60 Superdex 75, GE Healthcare).

Hybridoma production

The 2H12 hybridomas were produced following standard protocols previously described (45). BALB/c female mice were immunized with 20 μg recombinant EDIII serotype 2 strain 16681 in complete Freund's adjuvant, and boosted at fortnightly intervals with 20 μg protein in incomplete Freund's adjuvant. Three days prior to sacrifice, the mice were immunized once more, intravenously. The splenocytes were fused with NS1 myeloma cells. After 3 rounds of single cell cloning, mAb 2H12 was purified from the hybridoma supernatant by protein G affinity. This study was carried out in strict accordance with the recommendations in the United Kingdom Coordinating Committee on Cancer Research Guidelines for the Welfare of Animals. All animal procedures have been approved by the Institutional Review Committee and the Home Office UK, under the project title "Manipulation of immunity to transplanted normal and tumour grafts" (70/6874).

Virus production

C6/36 cells were infected with dengue virus serotype 1 to 4 (Den1 strain Hawaii, 02-0435 and 1-0372, Den2 strain 16681, NGC and 31-178, Den3 strain H87, 01-0017 and 2-1969-9 and Den4 strain H241, 1-0093 and 1-0544). Supernatants were collected, clarified, aliquoted and stored at -80 °C.

Dot blot

EDIII protein or UV-inactivated virus supernatant was dotted onto nitrocellulose membrane and allowed to dry. BSA was used as an irrelevant antigen for the recombinant protein and supernatant from mock-infected cells was used as a negative control for the virus blot. The membranes were blocked with PBS containing 0.1 % Tween (PBS-T), containing an additional 5 % milk for 1 h at 37 °C. The membranes were then incubated overnight at 4 °C with purified mAb 2H12 (5 μg/ml) in 5 % milk. After washing with PBS-T, the membranes were incubated with anti-mouse IgG-HRP-conjugated antibody (Sigma-Aldrich) for 1 h at 37 °C. Dots were visualized using ECL Plus (PerkinElmer).

Dissociation constants (K_D) of 2H12 antibody

The K_D of mAb 2H12 against EDIII was calculated as described previously (44). In brief, recombinant EDIII protein was coated onto plates and a direct ELISA with various concentrations of 2H12 was performed. BSA was used as an irrelevant antigen, and the values observed against this were subtracted from the values detected against the EDIII. For each antigen, the % maximum OD was calculated and the dissociation constants were determined (Prism Software). The virus-specific K_D values were determined by capture ELISA. Plates were coated with cross-reactive human anti-dengue mAb 751.B3 (generated in our lab) overnight at 4 °C. Plates were rinsed 3 times with PBS-T and were blocked for 1 h at 37 °C with 200 μl of blocking buffer (3 % BSA in PBS). Plates were incubated for 1 h at 37 °C with supernatant from virus- or mock-infected C6/36 cells diluted in dilution buffer (0.5 % BSA in PBS-T), washed and incubated with 50 μl of 2-fold serial dilutions of mAb

2H12 for 1 h at 37 °C. The plates were then incubated for 1 h at 37 °C in 50 μl of anti-mouse IgG-alkaline phosphatase conjugated secondary antibody (Sigma-Aldrich), followed by p-nitrophenyl phosphate substrate (SIGMA FAST, Sigma-Aldrich). The reaction was quenched, and the virus inactivated, by incubation with 0.4 M NaOH for 20 min at room temperature. Background mock readings were subtracted. Where a plateau was observed, the % maximum OD was calculated and the dissociation constants were determined as the concentration of antibody required for 50% binding (Prism Software).

Focus Reduction Neutralization Test

Micro-FRNTs were conducted on Vero cells as described previously (46). The $FRNT_{50}$ titer was defined as the concentration of antibody that reduced the number of foci by 50 %.

Temperature ELISA

Supernatants from dengue- or mock-infected C6/36 cells were captured onto plates coated with 2H12 and incubated for 1 h at 4 °C, 30 °C or 37 °C. Plates were then incubated with pooled convalescent dengue hyperimmune serum (PCS) (hemagglutination titre ≥ 1/25600), to detect bound virus, followed by alkaline phosphatase-conjugated anti-human IgG. The reaction was developed by the addition of p-nitrophenyl phosphate substrate, and quenched with 0.4 M NaOH for 20 min at room temperature. The absorbance was read at 405 nm.

Antibody-dependent enhancement assay

Ten-fold serial dilutions of mAb 2H12 were incubated with an equal volume of virus for 1 h at 37 °C, at an MOI of 0.2. The anti-envelope mAb 4G2 was used as a positive control. The antibody:virus mixture was transferred onto U937 cells in a 24-well plate and incubated at 37 °C for 4 days (47). Supernatants were harvested and their viral titre assessed by a focus-forming assay on Vero cells. The infected culture supernatants were serially diluted and incubated with Vero cells in a 96-well plate for 2 h at 37 °C. The monolayers were then overlaid with 1.5 % carboxymethylcellulose, in EMEM tissue culture medium, and incubated at 37 °C for 3 days. Virus foci were stained with mAb 4G2, followed by peroxidase-conjugated anti-mouse Ig and visualized by the addition of DAB substrate. The infecting fold enhancement is calculated by dividing foci-forming unit (ffu)/ml in a presence of antibody by ffu/ml in an absence of antibody.

Hybridoma sequencing

The sequences of the 2H12 heavy and light chain variable regions were determined using 5′-Rapid Amplification of cDNA Ends (5′-RACE, Invitrogen) (48). In brief, the mRNA was extracted from the 2H12 hybridoma cells and cDNA was created using a poly T primer. Gene specific primers, complementary to the CH1 region of each chain, were then used as 3′ primers to amplify the variable regions from the cDNA.

Crystallographic structure determination

Purified 2H12 Fab, prepared by papain digestion of mAb, was incubated with EDIII for 1 h at room temperature followed by purification by size exclusion chromatography. The protein complexes were concentrated, and set-up in crystallization experiments, using the sitting vapour diffusion method (49). The complexes were mixed with 10% glycerol prior to crystallization to slow nucleation and improve crystal size, as initial crystallization trials resulted in prevailing microcrystals. All crystals used for data collection grew at 21 °C with 20 % w/v PEG3350 used as precipitant and various salts (0.2 M potassium thiocyanate for 2H12-EDIIID1 and 2H12-EDIIID4, 0.2 M di-hydrogen phosphate for 2H12-EDIIID3). Protein crystals were cryoprotected with 20 % glycerol. X-ray data were collected at 100 K at Diamond Light Source (Beamline I04), as a series of 1 degree oscillations. Diffraction

data were processed using either HKL2000 (50) or xia2 (51). The structures of the complexes were solved by molecular replacement using Phaser (52). For each Fab-antigen complex the structure was determined using three search models: one for the constant region of Fab (CH1 and CL), one for the variable region of Fab (VH and VL) and one for EDIII antigen. The Fab was divided into two search models due to flexibility of the elbow between variable and constant domains (53). The complex of 2H12-EDIIID3 was solved using component coordinates (pdb code 1A3R and 1UZG (residues 296-394)) for the Fab and EDIIID3, respectively). The refined structure of 2H12-EDIIID3 was used as a search model for molecular replacement of 2H12-EDIIID1 and 2H12-EDIIID4. The complex structure was again separated into three search models comprising the Fab constant domains, the Fab variable domains and EDIII.

Atomic positions and their associated B-factors were refined using Refmac 5 (54-56) and Buster (57), and were rebuilt in Coot (58). Non-crystallographic local structure similarity restraints were used during the refinement of all structures. The quality of the models was analysed with MolProbity (59), and the binding surfaces were analysed with PISA (http://www.ebi.ac.uk/msd-srv/prot_int/pistart.html) (60) (see Supp. Table III for details).

Footnote

The coordinates presented in this article have been submitted to the Protein Data Bank (http://www.rcsb.org/pdb/home/home.do) with accession numbers 4al8, 4ala, 4am0. The EDIII sequences of Den1 strain 02-0435 and 1-0372, Den2 strain 31-178, Den3 strain 01-0017 and 2-1969-9 and Den4 strain 1-0093 and 1-0544, used in this study have been submitted to Genbank (http://www.ncbi.nlm.nih.gov/genbank/) with accession numbers JQ740878, JQ740879, EU482654.1, JQ740880, JQ74088, JQ740882 and JQ740883

Results

Characterisation of mAb 2H12

Monoclonal antibody 2H12 was produced from BALB/c mice immunized with recombinant EDIII from dengue serotype 2 (EDIIID2) strain 16681**.** 2H12, an IgG2b antibody, was found to be cross-reactive against the four dengue virus serotypes by dot blot, recognizing both the recombinant EDIII antigen (Fig. 1A) and the whole virus (Fig. 1B).

The neutralizing effects of 2H12 were tested on three strains of each dengue serotype. Neutralization was observed against all three strains of serotypes 1 and 4, and against a single strain 01-0017 of Den3 (Fig. 2A-D) but not against the three strains of serotype 2. The mean (n = 2-3) concentration of antibody required for 50 % neutralization was between 0.56 and 54 nM for Den1, 29 nM for Den3 strain 01-0017 and 145 nM for Den4 strain H241. This was somewhat weaker than the 4G2 positive control (Fig. 2E) and weaker than a Den2-specific anti-EDIII mouse monoclonal antibody 3H5 (1.2-4.6 nM) (28, 34).

In addition, antibody-dependent enhancement assays were performed on U937 cells, a human monocytic cell line expressing FcγRII (61, 62), which binds mouse IgG2b antibodies (61). In the absence of antibody, these cells show a very low level of infection (0.01-1% infected cells at MOI=1 or 10-100 ffu of progeny/ml). To our surprise, the presence of 2H12 resulted in little or no enhancement of infection in U937 cells (Fig. 2F). In comparison, the control mAb 4G2 exhibited enhancements of between 50- and 2500-fold (Fig. 2G).

The avidity of full-length 2H12 for EDIII was determined by ELISA with immobilized recombinant EDIII protein (Fig. 1C). The binding avidity of 2H12 for EDIII of the four serotypes was very similar, with K_D values of 0.45, 0.42, 0.47 and 0.42 nM for EDIIID1, D2, D3 and D4, respectively.

Finally, ELISA assays were performed on whole virus particles for all four serotypes, using 2H12 antibody (Fig. 1D). Unlike for the recombinant EDIII, there were differences in the measured K_D values. The strongest binding was against serotype 1 (1.90 nM), followed by serotype 4 (13.81 nM). The binding against serotypes 2 and 3 did not reach saturation and so the K_D were estimated at >125 nM and >62.5 nM, respectively. The similar binding affinities of 2H12 for EDIII, and the differences in binding for whole virus particles, suggests that the differences in neutralization are due to differences in some property of the virions.

The structures of 2H12-EDIII complexes

Fab fragments of 2H12 were mixed with EDIII from the four serotypes and used in crystallization trials. Dengue strains Hawaii, 16681, H87 and H241 were used for serotypes 1, 2, 3 and 4, respectively. Three of four 2H12-EDIII complexes yielded crystal structures; EDIIID1, D3 and D4. Unfortunately, all diffracting crystals of the putative 2H12-EDIIID2 complex were composed of Fab alone, despite 2H12 forming a stable complex prior to crystallization (data not shown).

The crystals of 2H12-EDIIID1 and 2H12-EDIIID3 diffracted to 1.7 Å and 1.8 Å, respectively (Fig. 3A; Table I) and each contained one copy of the Fab:EDIII complex. The crystals of 2H12-EDIIID4 diffracted to a lower resolution (3.0 Å) and contained four copies of the Fab:EDIII complex. The structures of the complexes were solved by molecular replacement and refined with residuals R =17.1, R_{free}=19.8, R=18.4, R_{free}=21.7 and R=19.6, R_{free}=25.2 for serotypes 1, 3 and 4 respectively (Table I). The stereochemistry of the refined models was excellent, with residues in favoured region/outliers of the Ramachandran plot: 98.3%/0.00% for 2H12-EDIIID1, 97.9%/0.21% for 2H12-EDIIID3 and 95.4%/1.00% for 2H12-EDIIID4.

Binding of the Fab did not significantly change the overall structure of EDIII (Fig. 3), with root mean square deviations (r.m.s.d.) of Cα's of 1.23 Å, 0.92 Å and 1.58 Å for EDIIID1, EDIIID3 and EDIIID4, respectively, compared to the published atomic models (pdb codes 3IRC – EDIIID1, 1UZG – EDIIID3 and 2H0P – EDIIID4). The main conformational change occurred in the AB loop, with Gln316 shifted by 2.98 Å, 3.00 Å and 3.44 Å in EDIIID1, EDIIID3 and EDIIID4 respectively. Moreover, the structures of the three EDIII proteins are very similar, when compared to each other, with r.m.s.d. of Cα's of 0.84 - 1.18 Å (Supp. Table I), despite 34 to 43 % difference between the amino acid sequences.

The elbow angle of the bound Fab (the angle between constant and variable domains) does, however, differ between the three complexes and is 172°, 155° and 144° for 2H12-EDIIID1, 2H12-EDIIID3 and 2H12-EDIIID4, respectively (Supp. Fig. 1A), perhaps reflecting the different crystal packing of the three complexes. Despite the differences in the elbow angle, the variable (VHVL) and constant (CH1CL) regions of the Fab structures are very similar in the three complexes (Supp. Table I).

The 2H12 epitope

Two structures of Fabs complexed to EDIII of flaviviruses have previously been solved. E16 is a murine mAb that binds to the so-called lateral ridge epitope of WNV EDIII, defined by four discontinuous regions, including the N-terminal linker region (residues 302 to 309) and three strand-connecting loops, namely, BC (residues 330 to 333), DE (residues 365 to 368), and FG (residues 389 to 391) (Fig. 4A) (63, 64); it is thought to be prototypic for a number of high avidity and potently neutralizing antibodies to both dengue virus and WNV. Antibody 1A1D-2, specific for dengue serotypes 1-3, binds to an epitope on the A strand of EDIII (30, 34). The 2H12 epitope footprints on the EDIII proteins are presented as a

gradient of buried surface area of residues in Fig. 3B. Between these 3 serotypes, the mode of engagement of 2H12 Fab is very similar, with only minor differences in the contact surface area. For the 2H12-EDIIID1, D3 and D4 complexes, the surface area of interaction of EDIII was 549, 567 and 521 $Å^2$, respectively, and 652, 448 and 394 $Å^2$ of 2H12. This interface area is significantly smaller than that observed for 1A1D-2 (30) and E16 (64) (905 $Å^2$ and 782 $Å^2$, respectively).

EDIII binds to 2H12 Fab predominantly via the AB loop (residues 314-317), which is inserted into a groove formed between the heavy and light chains of Fab. Residues from strands A and E and the D-Dx loop are also involved in contacts with the CDR of 2H12 (Fig. 4A). Antibody-antigen binding is mediated by hydrogen bonds and van der Waals interactions formed between seven fully conserved residues (Supp. Table II; Fig. 4A): Lys310, Glu314, Thr315, Gln316, His317, Ile352 and Glu368, and four CDRs: three from the heavy chain and one from the light chain (residues 33-35, 57-59, 100-101 of the heavy chain and residues 94-96 of the light chain). An example of the interface between EDIIID1 (Gln316/His317) and 2H12 are presented in Supp. Fig. 1B. In addition, the structures of 2H12-EDIIID1 and 2H12-EDIIID3 determined at higher resolutions also indicate possible water-mediated hydrogen bonds engaging Val312, Gln/Lys323 and Asn366 and van der Waals interactions mediated by Ala/Ser313, Val/Leu321 and Ala/Ser354 (Supp. Table II). However the relatively large distances between these residues (> 3.5Å) suggests that their contribution to the energy of binding may be limited; furthermore as the avidity of 2H12 to EDIII is very similar across all 4 serotypes (Fig. 1C.) differences in these water mediated interactions is unlikely to differentially modulate the strength of the interaction.

Although 2H12 has an epitope distinct from 1A1D-2 (Fig. 4A and 4B), their epitopes overlap by one residue, Lys310 (30, 34). The residue has been shared by several anti EDIII mAbs which recognize the strand A such as 1A1D-2, 9D12 and 4E11 (17, 28, 32, 34, 43, 65). This residue forms a hydrogen bond, via the ε-NH3(+) group, with the carbonyl oxygen of Val58 of 2H12 heavy chain and the carbonyl oxygen of Lys30 or Asp52 of 1A1D-2 heavy chain (30).

When modeled onto the structure of mature dengue virions, the epitope footprint of 2H12 revealed the likely basis for its poor neutralization. The epitope is not accessible on the surface of the virus, but is buried at the dimer interface between envelope domains I and III (Fig. 5A and B; Supp. Table III). Only a small area of the epitope is exposed on the model of the pre-fusion mature virion (Fig. 5C and 5D). Furthermore, the residues involved in the core AB loop epitope, which are buried in the mature virus, are not solvent accessible on the trimeric prM-E spikes of the immature virus (pdb code 3C5X, 3C6D) or the trimeric E spikes in the post-fusion conformation (pdb code 1OK8, 3G7T) (Supp. Table III).

The effect of temperature on binding

The relatively “inaccessible” epitope for mAb 1A1D-2 can be exposed by a change in virus conformation or “virion breathing", which has been shown to be temperature sensitive (30). We therefore tested the effect of temperature on 2H12 binding (Fig 2H). For all serotypes, a reduction in temperature resulted in a reduction in binding, with around 30% lower binding to Den1, 3 and 4 at 4 °C. Interestingly the binding to Den2 was the most temperature sensitive with a 73 % reduction in binding at 4 °C (Fig. 2H). Of note 2H12 binding to Den1 was around two fold higher than to the other serotypes.

Discussion

Much of the current interest in dengue virus is driven by the underlying need for a vaccine or effective treatment. Although antivirals have a role to play, the development of effective

vaccines is key to the prevention and spread of this important human pathogen. Having a structural basis for understanding antibody neutralization, as well as correlating these effects with antibody dependent enhancement, will be crucial in the development of dengue vaccines.

In this paper, we have functionally characterised a monoclonal antibody, 2H12, raised in mice against EDIII of dengue virus serotype 2. 2H12 can bind to the four serotypes. The neutralising potential of 2H12 is lower than a number of other DIII antibodies (27, 28, 34), but in contrast it showed no antibody dependent enhancement activity. Alongside these functional data, we have reported the structures of the 2H12 Fab complexed with EDIII, revealing the core epitope underlying recognition by this cross-reactive anti-dengue monoclonal antibody. This epitope is largely buried in the interface between EDIII and EDI of the pre-fusion mature virus (as determined by cryo-EM), with only 18% of the epitope surface exposed to solvent (see Fig. 5). All seven of the contact residues for 2H12 are conserved between the four virus serotypes, despite the significant sequence and functional differences between serotypes. The epitope centres on a core ETQH motif within the conserved six amino acid sequence 314ETQHGT319, in the AB loop of dengue EDIII. This region has been suggested previously to contain binding epitopes, and was detected through yeast display mapping of a panel of EDIII antibodies (34). Although these antibodies were cross-reactive, and in some cases also targeted WNV, they were generally poor neutralizers, consistent with our findings for 2H12.

In addition Lys310, which forms direct hydrogen bonds with Val58 of the heavy chain, and forms part of the strictly conserved epitope, may significantly contribute to neutralization by 2H12. Interestingly Lys310 also contributes to the epitopes recognised by other known cross-reactive anti EDIII antibodies that recognize strand A of EDIII, such as 1A1D-2, 4E11, 9D12 and WN E114, suggesting that Lys310 is an important residue for this antibody group (30, 34, 67).

The neutralizing potency of 2H12 is low compared to other mAb binding to EDIII, particularly those binding to the lateral ridge such as E16 that targets WNV (64). Furthermore, despite having a conserved epitope, 2H12 neutralized Den1 and 4 and one strain of Den3, but showed no neutralisation of Den2. Similarly, recent studies showed that neutralizing capacity varies between virus strains of the same serotype (26, 32, 33, 35), despite the epitopes being well conserved and, in some cases, binding being similar (32). The difference in 2H12 virus binding and neutralisation contrasts with the very similar binding affinity of 2H12 to the EDIII, despite 28 to 46% differences between serotypes for this domain, and must reflect differences in the structural stability and dynamics of the dengue virions (see Fig. 5) .

The fact that 2H12 can bind to the virion implies there must be conditions under which the buried AB loop epitope becomes accessible to the antibody. It is established that icosahedral viruses, such as picornaviruses and nodaviruses are dynamic particles and can undergo ‘breathing’ motions (away from low energy structures determined by crystallography) (68, 69). Further insight into conformational breathing in flaviviruses has come from structures of Fabs E16 and 1A1D-2 complexed to WNV and dengue (respectively). The structure of E16 Fab bound to WNV revealed that its lateral ridge is fully exposed (in 120 out of the 180 copies in the virus) and needs little or no conformational change in the intact virion for binding (63, 64). In contrast, 18 % of the binding surface of 1A1D-2 is obscured in the mature virion structure, and full saturation of dengue virus by 1A1D-2 Fab only occurred on raising the temperature to 37 °C (30). A cryo-EM structure of this complex revealed that binding of 120 copies of 1A1D-2 Fab resulted in all 180 copies of E undergoing major conformational rearrangements, with complete distortion of the virus compared to its native

pre-fusion form. This conformational change is temperature sensitive and it was proposed that increased “breathing” of the virus capsid would allow the exposure of buried epitopes (30).

The binding of 2H12 was also shown to be temperature sensitive. This temperature dependence differed between serotypes, much like neutralizing ability, with significantly poorer binding to Den2 occurring at lower temperatures. This temperature dependent difference in antibody binding between Den2 and the other serotypes is intriguing and may reflect a more rigid Den2 capsid, and possibly explains the inability of 2H12 to neutralise this serotype.

A model in which the virus “breathes” between the pre-fusion mature structure and various antigenically altered forms, when antibody binds, may also provide an explanation for the lack of ADE observed for 2H12. ADE occurs by uptake of antibody bound virus by Fc receptor-bearing cells. If the structural distortions that are locked in place by Fab binding are sufficient to prevent the rearrangements required for fusion, ADE will not occur. Alternatively, at low Ab concentrations (the point at which ADE usually occurs), antibody occupancy may not be sufficient to facilitate ADE. Recently, it has been shown that receptor tripartite motif-containing 21 (TRIM21) interacts with the Fc part of antibody bound to adenovirus leading to virus degradation inside cells. This suggests that potentially TRIM21 may have a role to play in neutralization and ADE of dengue virus (70).

Recent studies suggest that the EDIII-specific response in humans following infection actually plays less of a role than first thought (44, 71-73). Although the EDIII-specific antibody response can be easily measured in human serum, depletion of these antibodies does not reduce the neutralizing (44, 73) or enhancing (44) capacity of the serum. Despite this, EDIII remains an attractive vaccine target because antibodies that target certain regions are often of high avidity and can be potently neutralizing. When sequential immunizations are carried out in mice with recombinant EDIII alone, the neutralizing response is considerably weaker than that induced against the intact virion (74). However, when EDIII is used to boost responses following priming with the intact virion, neutralizing responses are significantly higher than using the intact virion alone (74). EDIII will therefore likely make an excellent boosting antigen by strengthening the high avidity and functionally relevant response that is primed by the virion.

2H12 was generated by immunization with recombinant EDIII. Although its epitope is not fully exposed on the intact virion, 2H12 is likely to be typical of some antibodies induced by an EDIII subunit vaccine. An epitope such as this, although weakly neutralizing, may well be beneficial in vaccines, as it does not facilitate ADE. It is hoped that structural studies, such as ours, may further our understanding of cross-neutralisation as well as the structural basis for ADE, critical for the design of effective vaccines in the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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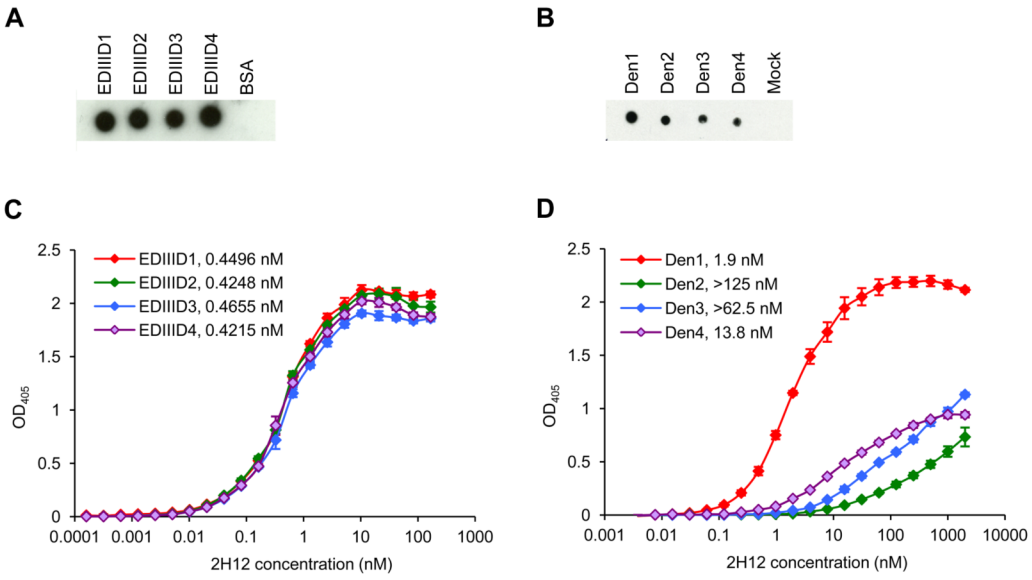

Figure 1. Binding properties of mAb 2H12

Dot blots against (A) recombinant EDIII from all four serotypes and (B) UV-inactivated supernatants from C6/36 cells mock-infected or infected with Den1 to 4. C) 2H12 ELISA against immobilised EDIII. D) 2H12 ELISA against live virus, captured by immobilised cross-reactive human mAb 751.B3. Saturation levels were used to determine maximum binding and, from this, K_D values were calculated; these are depicted in the key. Graphs depict the mean OD405 of 4 independent antibody dilution curves, within a single experiment. Error bars represent standard deviations. The graphs are representative of 3 separate experiments.

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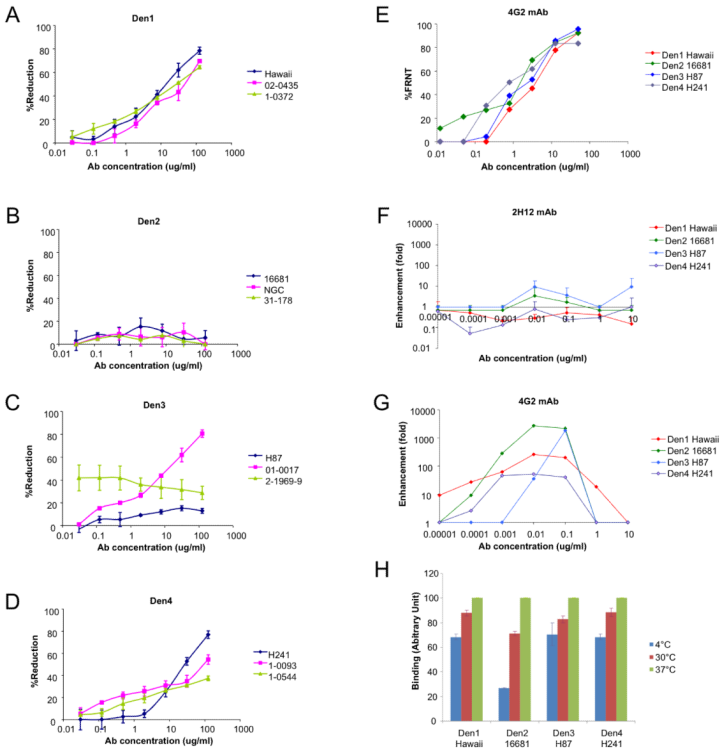

Figure 2. Functional properties of mAb 2H12

A-E) Neutralisation assays of 2H12 on three dengue strains from Den1, 2, 3 or 4, expressed as FRNT on infected Vero cells, mAb 4G2, which cross-reacts against the envelope of all 4 serotypes, was used as a positive control F-G) Antibody-dependent enhancement assays on U937 cells. Four days after infection, the U937 supernatants were harvested and their viral titre assessed by a focus-forming assay on Vero cells. The fold increase in infection, compared to the level observed in the absence of antibody, was calculated, mAb 4G2, was used as a positive control. The graphs depict the mean of 2-3 experiments. Error bars represent standard errors. H) Temperature sensitivity of 2H12 binding. ELISA plates were coated with 2H12 and incubated with the indicated viral serotypes for 1hr at 4, 30 and 37 °C. Virus binding was revealed by dengue immune serum followed by AP-conjugated anti-human-IgG. The binding unit was calculated by OD_{405} value at 37°C

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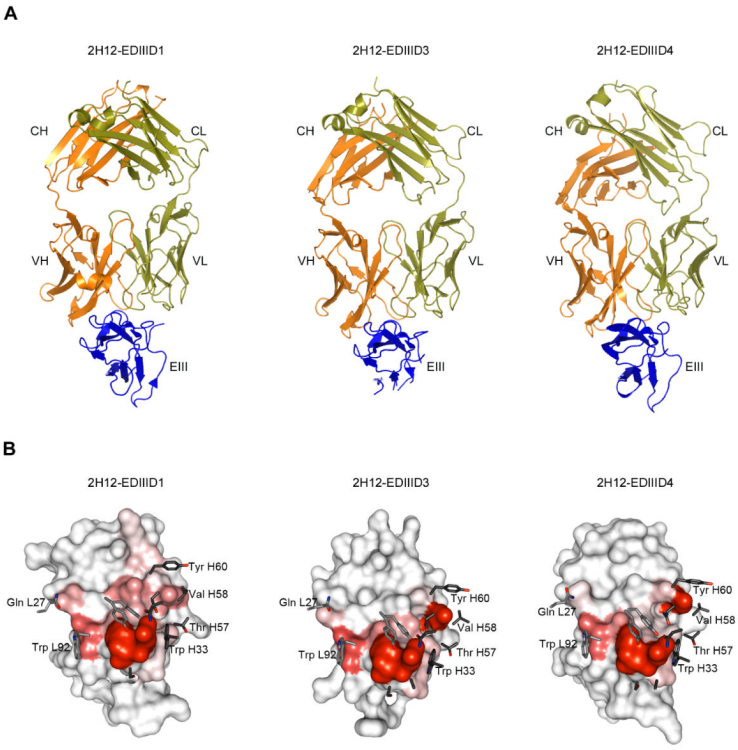

Figure 3. Crystal structure of the 2H12 Fab complexed with EDIII

A) Cartoon representations of 2H12 Fab complexed with EDIIID1, EDIIID3 and EDIIID4. Light chain variable and constant domains are shown in olive, heavy chain variable and first constant domain are shown in orange, EDIII is shown in blue. B) 2H12 epitope footprint in complexes 2H12-EIIID1, 2H12-EDIIID3 and 2H12-EDIIID4. Surface buried residues are depicted as a gradient of red (fully buried) and white (not buried). 2H12 paratope is shown as dark gray and light gray representing heavy chain and light chain residues involved in antigen binding. In this view, the front of EDIII is facing EDI, whereas the bottom faces the viral membrane. Water-mediated hydrogen bonds were excluded from this model.

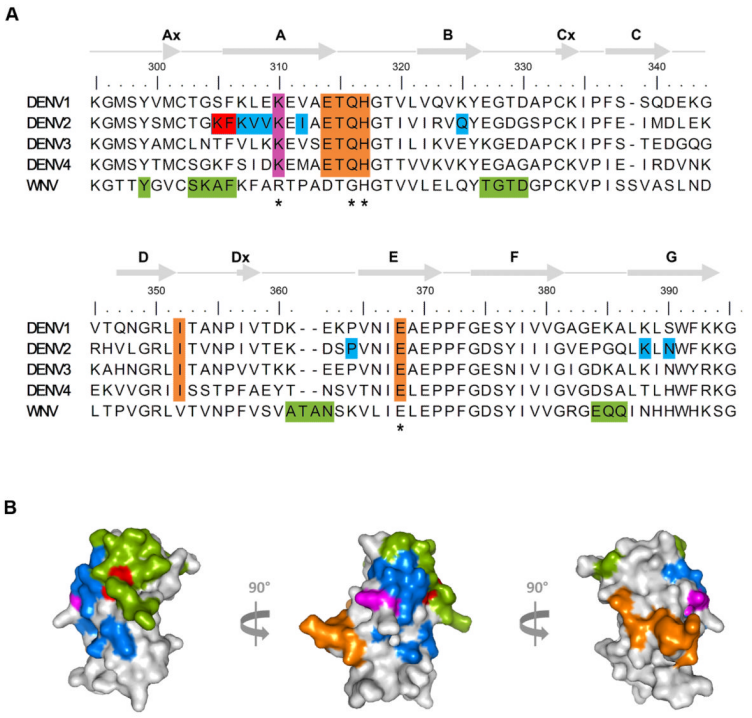

Figure 4. Epitope mapping of the 2H12 Fab complexed with EDIII

A) Sequence alignment of four dengue serotypes EDIII and West Nile virus EDIII. The sequences of EDIII from Den1 (strain Hawaii), Den2 (strain 16681), Den3 (strain H87), Den4 (strain H241) and WNV (strain NY99) are shown. Secondary structure elements of Den1 EDIII from the 2H12-EDIIID1 complex (assigned with dssp (75)) are shown with arrows (β-strands) and are labelled according to Rey *et al.*, 1995 (76). The epitopes identified in Fab-EDIII complex crystals are highlighted. Cross-reactive antibody 2H12 recognizes residues highlighted in orange in all four dengue virus serotypes. Subcomplex specific 1A1D-2 mAb (pdb code 2R29, complex with EDIIID2) binds residues in blue. Type-specific E16 (pdb code 1ZTX, complex with WNV EDIII) binds residues in green. The overlapping residues between 1A1D-2 and E16 epitope are shown in red, and between 1A1D-2 and 2H12 are shown in magenta. Asterisks denote hydrogen bonds. B) MAbs E16, 1A1D-2 and 2H12 epitopes were mapped onto EDIIID1 structure. Colours are as described in (A).

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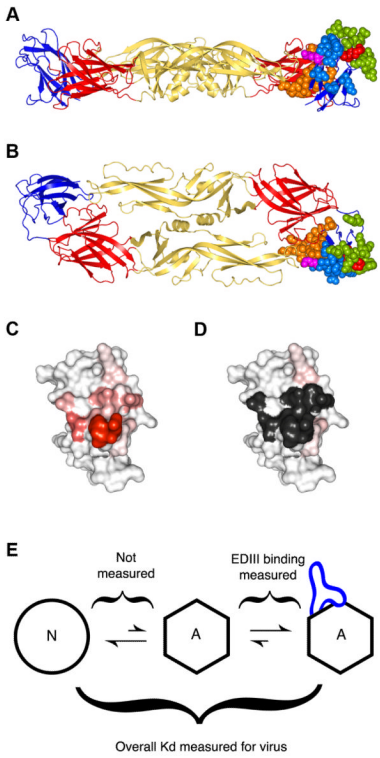

Figure 5. Epitope mapping of 2H12 on full length E protein

A) Side view of the envelope dimer, with the bottom of the dimer facing the viral membrane. A comparison of the 2H12, E16 and 1A1D-2 epitopes on the E dimer (pdb code 1OAN) is shown. Residues recognized by 2H12, E16 and 1A1D-2 are shown as orange, green and blue spheres, respectively. Both E16 and 1A1D-2 recognize residues in red. The residue in magenta (Lys310) is recognized by 1A1D-2 and 2H12. E domain I, domain II and domain III are shown in red, yellow and blue, respectively. B) Top view of (A). C) Surface representation of the 2H12 footprint on the EDIIID1 surface presented as gradient of red (fully buried residues) and white (not buried residues). D) 2H12 epitope accessibility on the E protein in a pre-fusion state. Residues in black are recognized by 2H12 but are buried at the interface of EDIII and EDI (Den2 – 1OAN, Den3 – 1UZG). E) A model of the structural dynamics of dengue virus. Dengue virus in its native state (N) is in dynamic equilibrium with antigenically altered states (A), one of which is bound by 2H12. EDIII binding reflects the binding of Ab to A, whereas binding measurements for virus reflects the change from N to A. The equilibrium between N and A can be shifted by temperature, changing the observed binding of antibody. No ADE is observed with dengue (A) complexed with mAb.

Table I

Data collection and refinement for the 2H12-EDIII complexes.

	2H12-EIIID1	2H12-EIIID3	2H12-EIIID4
Data collection			
Number of images	360	360	180
Space group	P1	$P2_1$	P1
Cell dimensions			
a, b, c (Å)	40.4, 60.6, 62.8	40.6,126.0,53.0	69.9, 92.8, 96.6
α, β, γ(°)	93.3, 102.1, 101.5	90 ,95.7, 90	118.6, 90.3,104.1
Resolution	50.00-1.55(1.58-1.55)	38.46-1.84(1.88-1.84)	55.83-3.02(3.1-3.02)
R_{merge} [a]	0.056(0.487)	0.101(0.795)	0.112(0.475)
I/σ	39.7(3.3)	15.0(2.0)	8.1(1.8)
Completeness [%]	96.9(95.9)	96.8(80.3)	96.1(96.2)
Redundancy	7.1 (5.2)	7.3(6.3)	2.0(2.0)
Refinement			
Resolution Range (Å)	30.0-1.7	20.0-1.8	55.7-3.0
No. reflections	65066	44332	38469
Rxpct [b]/Rfree [c]	17.0/19.8	18.4/21.7	19.6/25.2
Molecules per a.s.u.[d]	1	1	4
No. atoms			
Protein	3990	3817	15830
Water	649	446	0
B-factors			
Protein	30.6	30.8	44.7
Water	40.19	37.95	-
R.m.s. d.			
Bond length (Å)	0.011	0.011	0.010
Bond angles (°)	1.1	1.2	1.3

Numbers in parentheses refer to the relevant outer resolution shell.

r.m.s.d.: root mean square deviation from ideal geometry

[a] $R_{merge} = \Sigma_{hkl} \Sigma_i |I(hkl;i) - \langle I(hkl) \rangle| / \Sigma_{hkl} \Sigma_i I(hkl;i)$, where $I(hkl;i)$ is the intensity of an individual measurement and $\langle I(hkl) \rangle$ is the average intensity from multiple observations.

[b] $R_{xpct} = \Sigma_{hkl} ||F_{obs}| - |F_{xpct}|| / \Sigma_{hkl} |F_{obs}|$, where $|F_{obs}|$ and $|F_{xpct}|$ are the observed structure factor amplitude and the expectation of the model structure factor amplitude, respectively

[c] R_{free} equals the R_{xpct} of the test set as calculated above but using against 5% of the data removed prior to refinement.

[d] a.s.u.- asymmetric unit