Mapping of a dengue virus neutralizing epitope critical for the infectivity of all serotypes: insight into the neutralization mechanism

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Introduction

Dengue virus infections are a growing public health concern and strategies to control the spread of the virus are urgently needed. The murine monoclonal antibody 4E11 might be of interest, since it neutralizes dengue viruses of all serotypes by binding to the 296–400 segment of the major dengue virus envelope glycoprotein (DE). When phage-displayed peptide libraries were screened by affinity for 4E11, phage clone C1 was selected with a 50% frequency. C1 shared three of nine residues with DE306–314 and showed significant reactivity to 4E11 in ELISA. C1-induced antibodies cross-reacted with DE296–400 in mice, suggesting that it was a structural equivalent of the native epitope of 4E11 on DE. Accordingly, 4E11 bound to the DE306–314 synthetic peptide and this reaction was inhibited by DE296–400. Moreover, DE306–314 could block dengue virus infection of target cells in an in vitro assay. A three-dimensional model of DE revealed that the three amino acids shared by DE296–400 and C1 were exposed to the solvent and suggested that most of the amino acids comprising the 4E11 epitope were located in the DE306–314 region. Since 4E11 blocked the binding of DE296–400 to heparin, which is a highly sulfated heparan sulfate (HSHS) molecule, 4E11 may act by neutralizing the interaction of DE306–314 with target cell-displayed HSHS. Our data suggest that the DE306–314 segment is critical for the infectivity of all dengue virus serotypes and that molecules that block the binding of DE306–314 to HSHS may be antiviral reagents of therapeutic interest.
been shown to be the target of a number of neutralizing antibodies (Mégré et al., 1992), suggesting that the interaction between this region of DE and target cells could mediate virus entry. Interestingly, the murine monoclonal antibody 4E11, which was originally raised against dengue virus type 1, can neutralize target cell infection by serotypes 1, 2, 3 and 4 at an IC_{50} of 0.3–2 µM (Thullier et al., 1999). Previous studies demonstrated that 4E11 binds to the disulfide-bridged segment of DE_{296–314} (Mégré et al., 1992) with a nanomolar dissociation constant (Thullier et al., 1999). Identification of the 4E11 epitope on DE may help to identify ways of blocking target cell entry by all four virus serotypes. Moreover, since 4E11 shows sequence similarities with antibodies that bind to influenza and Hantaan viruses (Thullier et al., 1999), identification of such epitopes may help to define general ways of blocking target cell entry by other viruses.

Although the three-dimensional structure of DE is not known, the crystal structure of the highly homologous tick-borne encephalitis flavivirus envelope protein (TBEE) has been resolved (Rey et al., 1995). TBEE is a flat, elongated dimeric protein that extends in a direction parallel to the virus membrane. The monomer folds into three distinct domains, the central region (domain 1) being flanked by a dimerization region (domain 2) and a C-terminal immunoglobulin-like region (domain 3). The crystal structure of TBEE may be useful to model the three-dimensional structure of DE and help to identify residues that are accessible to neutralizing antibodies such as 4E11.

Dengue virus infectivity is mediated by the binding of its envelope protein to highly sulfated heparan sulfate (HSHS) molecules present on the surface of target cells (Chen et al., 1997). Recently, HSHS molecules were shown to initiate dengue virus infection of hepatocytes (Hilgard & Stockert, 2000). This mechanism does not seem to be a particular feature of dengue virus, since heparan sulfates also mediate human immunodeficiency virus (HIV) infection (Patel et al., 1993) and act as primary receptors for herpes virus entry into target cells (Chen et al., 1997; Herold et al., 1995; WuDunn & Spear, 1989). Following interaction with HSHS, herpes virus binds to a secondary receptor (Flynn & Ryan, 1996), which may participate in the internalization process. These data suggest that virus binding to HSHS may promote the interaction of the virus particles with high specificity cell receptors. Since the physico-chemical compositions of the DE_{254–310} and DE_{288–411} segments of DE were typical of HSHS-binding molecules, these segments were proposed to be the regions of the virus that interact with target cell heparan sulfates (Chen et al., 1997).

In this work, we have shown that DE_{296–400} can directly and specifically bind HSHS in vitro. Since the binding of 4E11 to DE_{296–400} suppressed this interaction, we postulated that the 4E11 epitope on DE may contain amino acids that are critical for HSHS binding. Affinity screening of phage-displayed random nonapeptides for 4E11 permitted the selection of peptides that specifically bound 4E11 and presented sequence similarities with DE_{296–400}. A synthetic peptide, DE_{306–314}, reacted specifically with 4E11 and blocked dengue virus infection in vitro. These data, combined with the analysis of the three-dimensional structure of DE, indicated that a limited set of residues that are contained within DE_{296–314} and well-exposed to the solvent in the folded molecule represent most of the antibody binding site. Our results suggest that the interaction between the identified residues and the HSHS molecules on target cells may be critical for the infectivity of all dengue virus serotypes.

### Methods

**Reagents.** Hybridoma cells producing 4E11, raised against the envelope protein of dengue virus type 1, were kindly provided by D. Morens (Morens & Halstead, 1990). The unconstrained and constrained phage-displayed nonapeptide libraries (pVIII-9aa and pVIII-Cys-9aa) were provided by F. Cortese (IRBM, Pomezia, Italy). DE_{296–400} was expressed in bacteria as a fusion protein, MaLE-DE_{296–400}, as described previously (Mégré et al., 1992). This fusion protein reacted in ELISA with both 4E11 and anti-MaLE antibodies. Heparin (sodium salt) was obtained from Sigma and peptides were synthesised by Neosystem.

**Biopanning.** Affinity screening of phage-displayed peptide libraries for 4E11 binding was conducted essentially as described by Felici et al. (1995). Briefly, oxirane acrylic beads (Sigma) were coated with 10 nM 4E11, blocked with UV-killed M13KO7 helper phage (5×10^{10} particles/ml) and incubated with phage suspensions (10^{11} particles). Bound phages were eluted with 0.1 M HCl, pH 2.2, and 1 mg/ml BSA buffer. Eluates were neutralized and titred as transducing units (TU). XLI-Blue cells were infected with phage eluates, plated and grown overnight at 37 °C. The next day, cells were scraped and a small sample was used to inoculate a liquid culture. The culture was then super-infected with M13KO7 helper phage, supplemented with IPTG (0.1 mM final concentration) and grown with strong agitation for 5 h at 37 °C. Phage particles were isolated by PEG precipitation and resuspended in TBS (50 mM Tris–HCl, pH 7.5, and 150 mM NaCl).

**Colonie blotting.** After super-infection with helper phage, a culture sample was centrifuged to eliminate excess helper phage and the pellet was resuspended in 1 ml of culture medium. Aliquots were spread onto agar plates and grown overnight at 37 °C. Colonies were then transferred onto C-super Hybrid nitrocellulose filters (Amersham). Membranes were blocked with PBS containing 3% BSA and 0.1% Tween and phages were screened as described previously (Christian et al., 1992). 4E11 (3 mg/ml) and peroxidase-conjugated anti-mouse antiserum (1 mg/ml) (Biosys) were diluted 1:2000 in PBS containing 1% BSA and 0.1% Tween. Colour development of positive clones was performed with the ECL detection kit (Amersham).

**DNA sequence analysis.** Selected clones were sequenced by the chain termination method (Sanger et al., 1977) using a T7 DNA polymerase kit (Amersham). Sequences were then analysed with GAP software (Genetics Computer Group, Wisconsin, WI, USA). The method of Needleman & Wuns (1970) was used to find an optimal alignment between the peptides and DE.

**Immunization of mice.** CsCl-purified phage samples were injected into four 2-month-old Biozzi mice (Biozzi et al., 1979; Demangel et al., 1996, 1998). Intraperitoneal injections of 2×10^{11} TU without adjuvant were carried out on days 0, 21, 42 and 79. Animals injected with wild-
type phage particles were used as controls. Mice were bled on days 31, 52 and 90 and the avidity of the immune sera was assessed according to Friguet et al. (1985).

ELISA. The binding of DE\textsubscript{296-400} to HSHS was tested in an ELISA assay using the recombinant MalE–DE\textsubscript{296-400} protein. Microtitre plates were coated with heparin (0.5 µg/ml in 0.1 M carbonate buffer, pH 9.6) for 2 h at 37 °C. These conditions allowed maximal heparin binding, as determined by the use of an anti-heparin monoclonal antibody (Chemicon) (data not shown). After washing, plates were saturated with PBS containing 0.1% Tween and 0.5% gelatin for 1 h at 37 °C. MalE–DE\textsubscript{296-400} or MalE was then incubated at concentrations ranging from 50 to 0.78 µg/ml for 20 min at 4 °C. After washing, complexes were incubated with anti-MalE rabbit antibodies for 2 h at 37 °C, then with a β-galactosidase goat anti-rabbit immunoglobulin conjugate for 1 h at 37 °C. To test for E11 inhibition of MalE–DE\textsubscript{296-400} binding to heparin, MalE–DE\textsubscript{296-400} (25 µg/ml) was incubated overnight at 4 °C with serial dilutions of E11 and then added to heparin-coated plates, as described above.

ELISA measuring the binding of phage-displayed peptides to E11 and anti-C1 immune sera antibodies to MalE–DE\textsubscript{296-400} was conducted as described previously (Demangel et al., 1996; Lafaye et al., 1995).

To examine the reactivity of synthetic peptides, microtitre plates (Maxisorp, Nunc) were coated with poly(lysine) (Sigma) in 0.1 M carbonate buffer, pH 9.6, for 2 h at 37 °C. After washing in PBS containing 0.1% Tween and 0.5% gelatin for 1 h at 37 °C, plates were then washed and incubated with peptides (1 µg/ml in PBS containing 1 mM EDTA) overnight at 4 °C. Coated plates were washed, saturated with 50 mM gelidine in PBS–EDTA for 1 h and incubated with serial dilutions of E11 for 2 h at 37 °C. After further washing steps, complexes were revealed with β-galactosidase-conjugated goat anti-mouse immunoglobulin. For affinity measurements, twofold dilutions of synthetic peptides were pre-incubated with 0.1 µg/ml E11 overnight at 4 °C. The mixtures were then incubated for 20 min at 4 °C on plates coated with peptides and complexes were revealed as described above.

To test for synthetic peptide inhibition of MalE–DE\textsubscript{296-400} binding to E11, microtitre plates were coated overnight at 4 °C with MalE–DE\textsubscript{296-400} (1 µg/ml in PBS) and subsequently washed. E11 (0.1 µg/ml) was pre-incubated overnight at 4 °C with a serial dilution of peptides. Antibody–peptide mixtures (100 µl) were then incubated in the coated plates for 20 min at 4 °C. After washing, complexes were revealed with β-galactosidase goat anti-mouse immunoglobulin conjugate.

Statistical analysis. A two-tailed Student’s t-test was used to compare the ELISA signals of peptide binding to E11. Calculated P values of < 0.05 were considered to be statistically different.

Plaque-reduction neutralization test (PRNT). Twofold serial dilutions of mouse immune sera or synthetic peptide (1 mg/ml) were incubated overnight at 4 °C with 100 p.f.u. of dengue virus type 1, strain Hawaii, 1944. Mixtures were then incubated on a monolayer of Vero cells, in duplicate, for 2 h, covered with 1% carboxymethylcellulose in Iscove’s medium and re-incubated at 37 °C in a 5% CO\textsubscript{2} incubator for 6 days. Infected cells were then fixed and detected with anti-dengue virus antibodies (Ref. 9402, Laboratoire des Arbovirus, Pasteur Institute, Paris) that bind all dengue virus serotypes, followed by horseradish peroxidase-conjugated goat anti-mouse immunoglobulin. (Despres et al., 1996).

Molecular modelling. Sequences of the envelope proteins from dengue virus and tick-borne encephalitis virus, DE and TBE, show 40% identity (Rey et al., 1995). Although the crystal structure of TBE (PDB 1SVB) is known, that of DE has not yet been solved. A structural model of DE (serotype 2) based on the crystal structure of TBE has been described previously, but this model ends at Gly\textsuperscript{255} of DE due to the addition of four residues in the DE sequence (Chen et al., 1997). Therefore, we analysed our data directly on the structure of TBE, which ends at Lys\textsuperscript{288}, using the WHAT IF program (Vriend, 1990). Accessible surface area (asa) was calculated with the ACCESS routine using a 1 Å radius probe and expressed as a percentage of the asa of the same residue (X) in a Gly–X–Gly tripeptide using the VACACC subroutine.

Results

E11 blocks the binding of DE\textsubscript{296-400} to model HSHS molecules

Since HSHS molecules displayed by target cells may be the primary receptors for dengue virus, we examined whether they showed reactivity to DE\textsubscript{296-400}. Soluble heparin is a close structural homologue of HSHS (Lyon et al., 1994) and is able to inhibit dengue virus infectivity in vitro (Chen et al., 1997). Thus, we tested if heparin reacted with a recombinant form of DE\textsubscript{296-400} (MalE–DE\textsubscript{296-400}). MalE–DE\textsubscript{296-400} significantly bound heparin in ELISA, whereas MalE did not show any reactivity (data not shown), suggesting that the binding of DE to heparin was mediated by the DE\textsubscript{296-400} fragment.

As E11 is directed against DE\textsubscript{296-400} we then examined if E11 binding to DE modified this interaction. Fig. 1 shows that E11 could significantly suppress DE\textsubscript{296-400} binding to immobilized heparin; complete inhibition of binding was obtained with a 0.5 molar ratio of E11 to MalE–DE\textsubscript{296-400}. Since E11 is an IgG, this ratio corresponds to equimolar amounts of antibody binding sites and target. This result could suggest that E11 may block virus entry into target cells by blocking the binding of DE\textsubscript{296-400} to heparan sulfate groups.

Phage clones selected from the unconstrained nonpeptide library bind E11 specifically

To identify the binding site of E11 on DE, two phage-displayed random nonpeptide libraries were affinity screened for the antibody. In the first one, peptides were flanked by two cysteine residues that favour their cyclization (constrained library), whereas the second one displayed unconstrained peptides (Felici et al., 1995). To follow the enrichment in positive clones during the selection process, the proportion of colonies that produced phage particles that bound E11 was determined after each round of panning. After the third selection cycle, fewer than 10% of colonies from the constrained library were positive in immunoblotting, compared to more than half of the colonies from the unconstrained library (data not shown). We therefore decided to proceed only with the unconstrained library.

When 24 clones from the third selection cycle of the unconstrained library were sequenced (Table 1), clone C1 was found 12 times (f = 1/2), C9 was found twice (f = 1/12) and all of the other clones were found only once (f = 1/24). This suggested that C1 was the phage clone that had the highest affinity for E11.
Fig. 1. 4E11 blocks the binding of DE296–400 to model heparan sulfate groups. The percentage of inhibition of DE296–400 binding to immobilized heparin in the presence of various ratios of 4E11 to DE296–400 is shown.

C1 shows sequence identity with DE304–314

To determine if these clones showed similarities with DE, we compared their amino acid sequences with the sequence of DE296–400 from dengue virus type 1 (Mason et al., 1987). Table 1 shows that the 12 different peptide sequences could be grouped into four sets of three. Three clones (C1, C6 and C11) showed sequence identity with DE304–311, three clones (C34, C39 and C4) with DE318–338, and three clones (C9, C33 and C41) with DE380–399, whereas the last three clones (C18, C19 and C21) did not share significant identity with DE296–400.

From these data, it appeared that three regions, DE311–321, DE318–338 and DE380–399, may be important in binding to 4E11. To examine their relative importance, we measured the binding of a representative phage clone of the three identified groups (C1, C34 and C33) to immobilized 4E11 in ELISA (Fig. 2). Only C1 showed a significant signal, suggesting that the DE311–321 fragment of DE played a major role in binding to 4E11 and that C1 mimicked the 4E11 epitope in DE304–314.

Table 1. Sequence analysis of the peptide clones selected by affinity for 4E11

The fragments of TBEE (strain Neudorff) or DE (serotype 1, strain Nauru Island, 1974) that match the identified peptides are shown, as well as the solvent accessible surface area (asa) of the TBEE residues. Synthetic peptides are indicated in bold.

<table>
<thead>
<tr>
<th>Group</th>
<th>Origin</th>
<th>Sequence</th>
<th>Synthetic peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>asa (%)...</td>
<td>66 40 5 56 22 60 55 48 5 51 51</td>
<td>T K F T W K R A P T D</td>
</tr>
<tr>
<td></td>
<td>TBEE310–320</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>DE304–311</td>
<td>G S F K L E K E V A E</td>
<td>P1</td>
</tr>
<tr>
<td></td>
<td>C1</td>
<td>W S L F L N H A E</td>
<td>P1</td>
</tr>
<tr>
<td></td>
<td>C6</td>
<td>P W L K Y A H E A</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C11</td>
<td>Y T W R W D S K L</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>TBEE310–320</td>
<td>A M L I T P N P T I</td>
<td>P2</td>
</tr>
<tr>
<td></td>
<td>DE318–338</td>
<td>G R L I T A N P I V</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C34</td>
<td>M R I H I A V L</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C39</td>
<td>K W L T T H D G T</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C4</td>
<td>R F L P Y Y E I P</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>TBEE318–326</td>
<td>N I I Y V G – – – – E L S H Q W F Q K G</td>
<td>P3</td>
</tr>
<tr>
<td></td>
<td>DE380–399</td>
<td>S Y I V G A G E K A L K L S W F K K G</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C9</td>
<td>L N H G H R Q L V</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C33</td>
<td>K F L L M P S Q</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C41</td>
<td>W K W R Y F S S Q</td>
<td></td>
</tr>
<tr>
<td>Non-homologous sequences</td>
<td>C18</td>
<td>V N W H W T V T V</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C19</td>
<td>R W I S I K E H A</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C21</td>
<td>W W W Q T F D A R</td>
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DE296–400, specifically binds 4E11 in ELISA. Immobilized synthetic DE296–400 matching with C1 (P1) peptides corresponding to C1-displayed peptide (P1) and to regions of DE296–400 matching with C1 (P1), C34 (P2) and C33 (P3) were tested for binding to 4E11. An irrelevant peptide (control) was also included.

The C1 homologous fragment in DE reacts significantly with 4E11

To further investigate the role of DE_{304–314} in the interaction between DE and 4E11, two nonapeptides were chemically synthesized. The first one (P1, WSLFLNHAEL) was identical to the C1-displayed peptide, whereas the second one (P’1, FKLEKEVAEL) corresponded to the segment of DE_{304–314} that was homologous to P1 (Table 1). A peptide of irrelevant sequence (ASPGLRRPS) was used as a negative control.

4E11 bound significantly to immobilized MalE–DE_{296–400} in ELISA (P = 0.011) and with a higher reactivity than the irrelevant peptide (Fig. 3). However, soluble P1 only weakly competed with immobilized MalE–DE_{296–400} for the binding to 4E11 (25 % inhibition at 5 × 10^{-4} M) (Fig. 4). Accordingly, the dissociation constant (K_d) between P1 and 4E11 was 10^{-4} M, which is indicative of a weak affinity. 4E11 bound significantly to immobilized P’1 in ELISA (P = 0.007) and with a higher reactivity than P1 (Fig. 3). Soluble P’1 strongly competed with immobilized MalE–DE_{296–400} for the binding to 4E11 (Fig. 4). The K_d between P’1 and 4E11 was 3 × 10^{-6} M, indicating that P’1 had a higher affinity for 4E11 than P1.

To investigate the roles of DE_{356–358} and DE_{384–399} in the recognition of DE by 4E11, peptides P2 and P3 (RLITANPIV and VGAGEKALKLSWFKGG, respectively) were also synthesized. P2 and P3 did not react with immobilized 4E11 (Fig. 3) and did not compete with immobilized DE_{296–400} for the binding of 4E11 (data not shown). These data suggest that DE_{356–358} and DE_{384–399} do not contribute to a large extent to the binding of 4E11 to DE and that most of the residues that interact with 4E11 may be contained within DE_{304–314}.

C1 is an immunological equivalent of the 4E11 epitope in DE

Since C1 and its homologous fragment in DE were antigenic, we also examined if they were immunogenic. C1 phage particles were injected into four Biozzi mice as described previously (Demangel et al., 1996) and the reactivity of the resulting immune sera for MalE–DE_{296–400} was examined. One mouse immunized with C1 showed an anti-MalE–DE_{296–400} response, as measured by a direct ELISA (data not shown). Although the avidity of this immune sera for MalE–DE_{296–400} was in the nanomolar range (4 × 10^{-10} M), as measured by PRNT, it was not associated with neutralizing properties and the immune response declined after the second immunization.

The C1 homologous fragment in DE neutralizes dengue virus

When tested by PRNT, P’1 at 0.5 mg/ml showed 50 % inhibition of dengue virus target cell infection. A negative control peptide (ALQPRYL) did not show any inhibition (Fig. 5).

Structural properties of the 4E11 epitope

Analysis of our results in the light of the crystal structure of TBEE showed the following points (Rey et al., 1995). DE_{304–314} is homologous to TBEE_{310–320}. The residues of TBEE_{311–320} are in an extended conformation with a bulge at positions Lys^{315}–Arg^{316}. The residues of TBEE_{310–320} are well exposed to the solvent (> 40 % exposure) in the dimeric form of TBEE, except for the residues Phe^{312}, Trp^{314} and Pro^{318} (Table 1). Thus, the corresponding residues of DE could be accessible to the solvent and 4E11 in the isolated dimeric form of DE.

The arrangement of the TBEE dimers at the surface of the virions is not known. However, the positions at the C-terminal end of the crystallized soluble fragment, the carbohydrate that modifies residues 154–156, and mutational analysis altering the binding of neutralizing antibodies have indicated which face of the dimer is oriented towards the outside of the virion.
Fig. 5. In each well, cells were infected with 100 p.f.u. of dengue virus type 1, incubated previously with a peptide (P'1 or a negative control). The virus alone control was not pre-incubated with a peptide and wells labelled 1/2 and 1/4 were infected with 50 and 25 p.f.u. respectively. Infected cells were revealed with anti-dengue virus antibodies and are represented in dark grey. When compared to the virus alone control, 0.5 mg/ml of P'1 peptide reduced the number of infected cells by half.

Fig. 6. Location of residues TBEE310–320, homologous to residues DE304–314, in the crystal structure of TBEE. Residues TBEE310–320 are represented in dark grey and the carbohydrate-modifying TBEE molecules are shown in mid grey (arrows). The residues beyond position TBEE395 are not visible in the crystal structure. The top panel shows a dimer viewed from the side (normal to the molecular dyad). The bulge formed by Lys315–Arg316 and the hydrophobic interactions between Trp314, the Ca and Cb of Ala317 and the Cg and Cd of Pro318 are clearly visible. The bottom panel shows the dimer viewed along the molecular dyad, from a direction corresponding to the outside of the virus particle.

Discussion

We have screened a phage-displayed nonapeptide library by affinity for 4E11, a dengue virus neutralizing antibody. The selected peptides showed sequence similarities with three

and which face is oriented towards the lipid bilayer (Fig. 6). TBEE310–316 would thus be on the outside half of TBEE in the virion. The fact that mutations of residues TBEE384 and TBEE387 affect the binding of antibodies to the virion has shown that the corresponding lateral side of domain III is accessible (Rey et al., 1995). Therefore, residues TBEE317–320, which are close to residues TBEE384 and TBEE387 in the structure, could also be displayed on the virion surface, indicating that the whole DE304–314 segment would be accessible to 4E11.
distinct regions of DE, DE$_{294-314}$, DE$_{249-328}$ and DE$_{280-399}$. Phage clone C1 was selected with the highest frequency (50%) and showed the strongest sequence identity with DE (33.3% identity with DE$_{296-314}$). C1 specifically reacted with 4E11 in ELISA and competed with DE$_{296-409}$ for binding to the antibody. These results suggested that the C1-displayed peptide represented a structural equivalent of the native epitope of 4E11 on DE. This conclusion was confirmed by experiments showing that C1 could induce, in mice, immune sera of $4 \times 10^{-10}$ M avidity for MalE–DE$_{296-409}$. The fact that only one of the four mice generated antibodies reacting with MalE–DE$_{296-409}$ and that the immune response of the positive mouse decreased after the second immunization can be explained by the fact that the immune response directed against the phage coat protein hides the anti-nonapeptide response, as described previously (Demangel et al., 1998). We could not show any direct binding of the two other identified DE regions to 4E11 at this stage.

The synthetic peptide P1, corresponding to the C1 phage-displayed peptide, had a lower reactivity with 4E11 than C1 in ELISA. Such differences of reactivity between the synthetic and phage-displayed forms of a peptide suggest that the phage context could indirectly contribute to antibody binding (Demangel et al., 1996). The P’1 peptide corresponding to DE$_{296-314}$ bound 4E11 with a stronger reactivity than P1, as shown by competition experiments with MalE–DE$_{296-409}$ and affinity measurements ($K_d = 3 \mu$M), showing that most of the 4E11 epitope was located in the linear DE$_{296-314}$ segment. This location was also consistent with the structural model of DE, which showed that DE$_{296-314}$ could form one of the strands of a β-barrel, and may explain the lack of reactivity of the cyclized peptides displayed by constrained libraries.

Since HSHS molecules mediate the binding of dengue virus to host cells in vitro, these molecules are likely to play a critical role in virus infectivity. This is consistent with the observation that the absence or the removal of HSHS from the cell surface leads to cell resistance to virus infection. DE$_{284-310}$ and DE$_{285-411}$ have been proposed, based on sequence similarities with other heparan sulfate-binding proteins, to bind HSHS (Chen et al., 1997). Interestingly, the predicted epitope for 4E11, DE$_{296-314}$, overlaps with DE$_{284-310}$ and DE$_{285-399}$ which has sequence similarities with three of the selected phage-displayed peptides, is partially contained by DE$_{285-411}$, the other potential HSHS-binding region. The structural model based on the sequence identity between TBEE and DE indicates that DE$_{296-314}$ and DE$_{291-398}$ could form a sheet of two parallel β-strands that are well exposed at the surface of the molecule and rich in lysine residues. These positively charged β-strands could be involved in the interaction between the virus and HSHS on the surface of the host cell. Our demonstration of a direct competition between 4E11 and heparin for binding to DE$_{296-409}$ and of virus neutralization in the presence of DE$_{296-314}$ suggests that 4E11 inhibits dengue virus infection by blocking DE$_{296-314}$ binding to target cell HSHS. Using a similar ELISA, no competition for heparin binding could be demonstrated between DE$_{296-314}$ and DE$_{296-409}$, but the affinity of DE$_{296-314}$ could be lower for heparin than for HSHS molecules on the surface of living cells. Alternatively, the high density of immobilized heparin molecules necessary to obtain a readable signal after DE$_{296-409}$ binding could have lowered the sensitivity of the test when using a synthetic peptide for competition.

Interestingly, DE$_{310-314}$ is well conserved between the four dengue virus serotypes. All four serotypes share a consensus motif, KEΦAE (where Φ is a hydrophobic residue), which could account for the cross-reactivity of 4E11. The sequence variability at positions DE$_{307}$ and DE$_{309}$ could explain the differential neutralization efficacies of 4E11 on the four serotypes.

The importance of heparan sulfates or heparin for interactions between cells and viruses has been established for pseudorabies virus (Trybala et al., 1998), HIV (Patel et al., 1993) and herpes simplex virus (Wu Dunn & Spear, 1989). The neutralizing activity of dextran sulfate against hepatitis C virus, a member of the family Flaviviridae, has also been reported (Cribier et al., 1998). In an HIV infection model, the finding that dextran sulfate shows neutralizing activity led to the correct hypothesis that this virus binds to heparan sulfates on target cells (Callahan et al., 1991). HSHS binding could be the first step of the interaction between target cell and virus and may act by maximizing the probability of binding to a more specific secondary receptor before internalization (Flynn & Ryan, 1996). Chemically modified heparins of minimal anticoagulant properties are potent inhibitors of HIV in vitro (Lopalco et al., 1994). Such reagents may have therapeutic interest in vivo against HIV infection. Our data indicate that molecules that block the binding of dengue virus to cell surface heparan sulfates may also be useful as anti-dengue virus infection therapies and may efficiently block all serotypes of the virus. These data could also be relevant to the design of a vaccine.

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References


Biology

Virology

Journal of Molecular Immunology

1 with mimotopes selected from a phage-displayed peptide library. Immune response against the hepatitis C virus adsorption to peripheral blood mononuclear cells by bacteriophage.

BIJC

Anti-HIV type 1


Impact of dengue virus infection and its control.


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