The antigenic structure of dengue type 1 virus envelope and NS1 proteins expressed in *Escherichia coli*

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The antigenic structures of the envelope protein, E, and the non-structural protein, NS1, of dengue type 1 virus (DEN1) have been studied in the form of recombinant fusion proteins expressed in *Escherichia coli*. Deletion analysis was used to identify two distinct antigenic domains in E that reacted with subsets of antiviral monoclonal antibodies (MAbs). Domain I of E extends from amino acid residues (aa) 76 to 93 of E; domain II extends from aa 293 to 402 and contains an essential disulphide bridge. MAbs also reacted with several determinants clustered near the N terminus of the NS1 protein (aa 57 to 126). Recombinant fusion proteins containing *E. coli* trpE sequences and most of the sequences for either E or NS1 were immunogenic in mice. The antibodies elicited by the E fusion protein reacted with a portion of the protein containing domain II, whereas antibodies elicited by the NS1 fusion protein did not react with the antigenic determinants defined by our MAbs.

**Introduction**

Dengue virus infection is a substantial world-health threat, causing dengue fever and the more severe illnesses, dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS). The frequency of human dengue disease is estimated to be in the order of hundreds of thousands of cases per year (Halstead, 1988; Shope, 1980; Monath, 1986). The dengue viruses can be divided into four serotypes, DEN1, DEN2, DEN3 and DEN4, which show immunological cross-reactivity but can be distinguished by plaque reduction neutralization tests (Russell & Nisilak, 1967) and monoclonal antibodies (MAbs) (Henchal *et al.*, 1982; Gentry *et al.*, 1982; Monath *et al.*, 1986).

The structural glycoprotein E and the non-structural glycoprotein NS1 are the most likely targets for the development of a subunit vaccine. The dengue virus E protein contains serotype-specific antigenic determinants and determinants important in virus neutralization (Gentry *et al.*, 1982) and E-specific MAbs are capable of passively protecting mice from dengue infection (Kaufman *et al.*, 1987). The E protein deduced from the nucleotide sequence of DEN1 is a polypeptide of 495 amino acids (aa) with a calculated Mr of 53·8K and two potential N-linked glycosylation sites (Mason *et al.*, 1987b). The DEN1 NS1 protein sequence predicted on the basis of the nucleotide sequence of DEN1 cDNA with the amino- and carboxy-terminal ends deduced by alignment with the amino acid sequences established for the NS1 proteins from other flaviviruses (Mason *et al.*, 1987b, Wright *et al.*, 1989) contains 352 aa. Since mice are protected from a lethal DEN2 challenge by either passive administration of complement-fixing anti-NS1 MAbs or immunization with purified DEN2 NS1 (Schlesinger *et al.*, 1987), there appears to be potential for an NS1-based dengue vaccine.

In this study, we have expressed fragments of DEN1 cDNA in *Escherichia coli* to assess the utility of a prokaryotic expression system for the production of dengue antigens and to facilitate the molecular genetic analysis of E and NS1 antigenic structures. Derivatives of the E and NS1 proteins, expressed as trpE fusion proteins, were tested for their reactivity with a panel of MAbs raised to DEN1. The results indicate that a subset of epitopes presented during viral infection, including at least one neutralizing epitope, are formed in these recombinant antigens. The approximate locations of these epitopes within the E and NS1 proteins were deduced by deletion analysis of the recombinant antigens.
Methods

Strains, cell lines and virus stocks. The E. coli plasmids containing DEN1 cDNA and the DEN1 virus stocks have been described (Mason et al., 1987b). Plaque reduction neutralization tests were performed in BHK21-15 cells as described by Morens et al. (1985) using fourfold dilutions of antibody starting with 1:10. The pATH expression plasmids (Dieckmann & Tzagoloff, 1985) were obtained from Dr T.J. Koerner.

Preparation of pATH-DEN1 recombinants. The recombinant pATH clones pMA2 and pMA1, containing the DEN1 E and NS1 sequences respectively, were constructed by inserting fragments of cloned DEN1 cDNA into the appropriate pATH expression plasmid to create an in-frame fusion with the trpE gene. Specifically, pMA2 was constructed by cloning a 1240 bp EcoRI-XbaI fragment of DEN1 cDNA into EcoRI- and XbaI-digested pATH1. Subclones of pMA2 and pMA1 that were missing C-terminal sequences were constructed by digestion of the plasmid DNA with DNase I in the presence of MnCl₂ (Mason et al., 1987a) under conditions that resulted in one or fewer double-strand cuts per plasmid. The digested ends were repaired with Klenow polymerase and ligated to non-phosphorylated oligonucleotide linkers (decamers) containing a BamHI site (New England Biolabs). The linearized plasmid DNA was purified, annealed by heating and slow cooling and used to transform competent E. coli strain HB101 cells (Mason et al., 1989).

Deletions extending from the 3' end of the protein coding sequences were created by excising the cDNA between the linker insertion site and a downstream site in the polylinker of the pATH vector. Selected recombinants were digested with Bal 31 exonuclease to extend deletions from the site of linker insertion. Briefly, the plasmid DNA was digested first with BamHI, then with Bal 31 (IBI) and finally with EcoRI to release the DEN1 cDNA fragments. These fragments were purified from agarose gels and ligated between the EcoRI and Smal sites in the polylinker of the pATH vector to recreate the in-frame fusion to trpE.

Deletions from the 5' end of the viral coding sequences were constructed by isolating the BamHI cDNA fragments extending from the linker insertion downstream to the pATH polylinker and inserting these fragments behind the BamHI site in three different pATH vectors, each representing one of the three possible translational reading frames. Recombinants with in-frame fusions were identified by their ability to express trpE fusion proteins of the appropriate size. Additional deletions were created by Bal 31 digestion as described above. The exact coding regions of all deletions used to define the boundaries of the antigenic domains were established by DNA sequencing using the dideoxynucleotide chain termination method (Sanger et al., 1977, 1980).

Preparation and analysis of trpE fusion proteins. Inclusion bodies containing the trpE fusion proteins were isolated from E. coli HB101 cells containing the recombinant pATH plasmids as described by Mason et al. (1989). The methods for SDS-PAGE, electrophoretic purification of fusion proteins, Western blot analysis and the reduction and alkylation of disulphide bridges have been described previously (Mason et al., 1989).

Immunological reagents. Polyclonal antiserum to DEN1 virus was prepared by the repeated immunization of adult outbred mice with DEN1-infected suckling mouse brain suspension and antibodies were obtained in the form of hyperimmune mouse ascitic fluids (HMAF) (Brandt et al., 1967). A library of 60 MAbs to DEN1 was prepared and partially characterized by Henchal et al. (1982) and the antibodies were generously provided by Dr M. K. Gentry. Nine of these MAbs were selected for further analysis because of their strong reactivity with the recombinant fusion proteins in ELISA (Engvall, 1980). A tenth MAb (D1-4E5-6-5), which was raised to DEN2, was selected for our studies because of its ability to confer passive immunity to DEN2 infection (Kaufman et al., 1987). These MAbs represent a subset of epitopes that were either linear or readily renatured following denaturation (Western blot analyses) and were expressed in proteins synthesized by E. coli. Additional properties of these MAbs are listed in Table 1. Isotype analyses were performed as described by Harlow & Lane (1988). All ascitic fluids contained IgG1 (consistent with the production of non-specific G1 by the myeloma fusion partner), therefore the isotype of each DEN MAb is reported as either the other isotype present in the ascitic fluid or as IgG1 if no other isotype was detected. Polyclonal antisera to recombinant fusion proteins were prepared as described by Mason et al. (1989).

Table 1. Characteristics of the DEN1 MAbs

<table>
<thead>
<tr>
<th>Hybridoma*</th>
<th>Specificity†</th>
<th>PRNT‡</th>
<th>Isotype</th>
<th>Cross-reactivity§</th>
</tr>
</thead>
<tbody>
<tr>
<td>D2-8C2</td>
<td>E</td>
<td>&lt;1:10</td>
<td>IgG1</td>
<td>DEN1, DEN2</td>
</tr>
<tr>
<td>D2-8F9</td>
<td>E</td>
<td>&lt;1:10</td>
<td>IgG2a</td>
<td>DEN1, WN, SLE</td>
</tr>
<tr>
<td>D2-9D12</td>
<td>E</td>
<td>1:40</td>
<td>IgG1</td>
<td>DEN1, DEN2, DEN3</td>
</tr>
<tr>
<td>D1-4E5-6-5</td>
<td>E</td>
<td>&lt;1:10</td>
<td>IgG2a</td>
<td>DEN2</td>
</tr>
<tr>
<td>D2-13D4</td>
<td>E</td>
<td>&lt;1:10</td>
<td>IgG2a</td>
<td>DEN1, DEN2, DEN3, DEN4</td>
</tr>
<tr>
<td>D2-13B6</td>
<td>NS1</td>
<td>ND</td>
<td>IgG2a</td>
<td>DEN1, DEN3, DEN4</td>
</tr>
<tr>
<td>D2-13A1</td>
<td>NS1</td>
<td>ND</td>
<td>IgG2a</td>
<td>DEN1</td>
</tr>
<tr>
<td>D2-15F3</td>
<td>NS1</td>
<td>ND</td>
<td>IgG2a</td>
<td>DEN1, DEN4</td>
</tr>
<tr>
<td>D2-5C11</td>
<td>NS1</td>
<td>ND</td>
<td>IgG2a</td>
<td>DEN1</td>
</tr>
<tr>
<td>D2-7E11</td>
<td>NS1</td>
<td>ND</td>
<td>IgG2a</td>
<td>DEN1, DEN2, DEN3, DEN4, JEV, WN, MVE, SLE, YF, KUN</td>
</tr>
</tbody>
</table>

* Several of these MAbs have been described previously: D2-9D12, D2-5C11, D2-15F3 (Henchal et al., 1982); D1-4E5-6-5 (Kaufman et al., 1987); D2-9D12 (Henchal et al., 1985); D1-4E5-6-5 is a subclone of D1-4E5 used by Henchal et al. (1985) (E. Henchal, personal communication.)
† Determined by Western blot analysis of DEN1-infected C6/36 cells.
‡ Ascitic fluid dilution yielding ≥ 50% plaque reduction.
§ Determined in an immunofluorescence assay (Tesh, 1979) using acetone-fixed, flavivirus-infected C6/36 cells and 1:100 dilutions of each ascitic fluid. Flaviviruses tested were DEN1, DEN2, DEN3, DEN4, JEV, WN, MVE, SLE, YF and Kunjin (KUN).
ND, Not determined.
ELISA of antisera to DEN1-trpE fusion proteins. An ELISA procedure with truncated DEN1-trpE fusion proteins as the immobilized test antigens was used to evaluate which DEN1 sequences in the full-length fusion proteins were immunogenic in mice. In order to use trpE fusion proteins for both immunization and as the target for antibody binding in ELISA, it was necessary to eliminate the background signal due to antibodies to determinants on the trpE-encoded portion of the fusion proteins. This was accomplished by preincubating the test antisera with a non-viral trpE fusion protein that competitively blocked antibodies to the trpE moiety. Briefly, the antisera raised to the DEN1 fusion proteins were diluted to the final test concentration in Tris-buffered saline solution containing 1% bovine serum albumin and approximately 25 μg/ml of an inclusion body fraction isolated from E. coli cells expressing a yeast protein fused to trpE. This mixture was incubated for 2 h at room temperature and then used directly in an ELISA (Engvall, 1980) with various fusion proteins as the immobilized antigen.

Results

Expression of E protein sequences in E. coli and localization of antigenic domains

In pMA2, the DEN1 E sequence is fused in frame to trpE at the first codon of E (Mason et al., 1987b) and extends downstream for 412 of the 495 codons of E. The size of the fusion protein expressed from pMA2 agreed well with the predicted molecular mass of 85K for a polypeptide containing 355 residues specified by the trpE gene and the first 412 aa of E. The fusion protein reacted with polyclonal antiviral antibodies (HMAF) and five anti-E MAbs in ELISA and Western blot assays.

Deletions were constructed in pMA2 to generate fusion proteins lacking sequences from either the amino-terminal end or the carboxy-terminal end of the E protein sequence. In all cases, BamHI linker insertions in pMA2 were used to excise progressively longer segments of the E coding region (see Methods). The fusion proteins truncated by the removal of sequences from the 3' end of the E coding region are shown in Fig. 1(a) and the reactivities of these truncated proteins with antibodies in the polyclonal HMAF are shown in Fig. 1(b). Progressive deletion of the carboxy-terminal region was accompanied by a gradual loss of immunoreactivity with the antiviral HMAF, suggesting that the protein contains several different antigenic domains. The presence of distinct antigenic domains in E was shown more directly by the patterns of reactivity between the five MAbs and the proteins expressed by the deleted plasmids. Based on the reactivities of two of these five MAbs (Table 2), domain I was localized to a region between aa residues 76 and 93 of E (Fig. 2). The reactivities of the remaining three antibodies (Table 2) were used to locate domain II in the relatively large region of E between aa 293 and 402 (Fig. 2). Previously, competitive binding assays were used to show that two of these domain II MAbs (D1-4E5-6-5 and D2-9D12) bind to topologically related sites on the surface of the DEN2 virion (Henchal et al., 1985).

A disulphide bridge is an important feature in the antigenic structure of domain II

Domain II in the E protein of DEN1 is very similar in length and position to a disulphide-dependent antigenic domain we described previously for the E protein of Japanese encephalitis virus (JEV) (Mason et al., 1989). There are two highly conserved cysteine residues at positions 302 and 333 within domain II of the DEN1 E
protein (Mason et al., 1987b) and it is likely that these cysteines form a disulphide bridge in the native E protein (Nowak & Wengler, 1987). As shown in Fig. 3, binding of domain II MABs was essentially eliminated by reduction and alkylation of the fusion proteins, indicating an important role for a disulphide bridge in the antigenic structure of domain II of the DEN1 E protein. As treatment of the fusion proteins with reducing agent (dithiothreitol) alone had minimal effects on immunoreactivity, it appears that the essential disulphide bridge in domain II can form during sample preparation and analysis. Furthermore, the 50% reduction in domain II immunoreactivity after fusion proteins were treated with alkylation agent (iodoacetamide) alone suggests that these fusion proteins as-isolated contained many free sulphhydrals, which is not unusual for proteins extracted from E. coli inclusion bodies (Tsuji et al., 1987).

Reduction, alkylation, and reduction plus alkylation of fusion proteins containing domain I did not affect the binding of the two domain I MABs (results not shown).

![Fig. 2. Schematic diagram of the recombinant proteins used to define antigenic domains I and II within the DEN1 E protein. Sequences encoded by individual plasmids are shown below the DEN1 E protein (the exact amino acid residues expressed by each recombinant protein are shown in Table 2). The deduced positions of antigenic domain I (defined by MABs D2-8C2 and D2-8B9) and domain II (defined by MABs D2-9D12, D1-4E5-6-5 and D2-13D-4) are aa 76 to 93 and 293 to 402 respectively.](image)

![Fig. 3. Immunoreactivity of reduced, reduced and alkylated, or alkylated preparations of the E-trpE fusion protein expressed from pMA2. Samples of the fusion protein were reduced with dithiothreitol (DTT) and/or alkylated with iodoacetamide (IAA) and then used as the immobilized antigen in ELISA with each of the three domain II E MABs (data shown are the averages of the values obtained for the three MABs). (a) Crude fusion protein from the insoluble cell fraction of E. coli harbouring pMA2. (b) Electrophoretically purified pMA2 fusion protein.](image)

Table 2. Summary of reactivity of MABs and HMAF with recombinant E proteins

<table>
<thead>
<tr>
<th>Antibody binding*</th>
<th>Domain I MABs</th>
<th>Domain II MABs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clone†</td>
<td>Region (aa)‡</td>
<td>D2-8C2</td>
</tr>
<tr>
<td>pMA2</td>
<td>1-412</td>
<td>+</td>
</tr>
<tr>
<td>pMS70</td>
<td>1-402</td>
<td>+</td>
</tr>
<tr>
<td>pMS69</td>
<td>1-386</td>
<td>+</td>
</tr>
<tr>
<td>pMS71</td>
<td>1-91</td>
<td>-</td>
</tr>
<tr>
<td>pMT1</td>
<td>293-412</td>
<td>-</td>
</tr>
<tr>
<td>pMT2</td>
<td>317-412</td>
<td>-</td>
</tr>
<tr>
<td>pMT4</td>
<td>76-412</td>
<td>+</td>
</tr>
<tr>
<td>pMT5</td>
<td>91-412</td>
<td>−</td>
</tr>
</tbody>
</table>

* Binding of individual MABs and the polyclonal anti-DEN1 HMAF with the recombinant proteins was determined by immunoblot: +, strong positive reaction; −, undetectable reaction; +/−, weak reaction.
† Identity of the pATH-DEN1 E recombinant.
‡ Amino acid residues of the E protein expressed by each clone.
Antigenic structure of DEN1 E and NS1

Expression of NS1 protein sequences in E. coli and localization of antigenic domains

The NS1 fusion protein expressed from pMA1 contains aa 57 to 352 of the 352 aa NS1 protein and aa 1 to 99 of NS2A. This fusion protein accumulated only at low levels in induced cells and its mobility in SDS-PAGE corresponded to a polypeptide of 80K, less than the predicted value of 83K. These properties can probably be attributed to the extremely hydrophobic domains of NS2A (Mason et al., 1987b) which could affect the synthesis, stability and SDS-binding of this particular fusion protein. The fusion protein specified by pMA1 reacted with antiviral antibodies present in HMAF and with five anti-NS1 MAbs in ELISA and Western blot analyses.

The epitopes for the five anti-NS1 MAbs were localized on subdomains of the NS1 protein by a deletion analysis similar to that used for the E protein. The results of these analyses, summarized in Table 3 and Fig. 4, show that a fusion protein expressing a short section of NS1 (aa 57 to 126; clone pMS11) contained the binding sites for all five MAbs and was strongly reactive with HMAF. Analysis of further deletions distinguished multiple antigenic determinants in this region. Binding sites for two of the MAbs were contained in aa 57 to 103 of NS1 (clone pMS12), but this region lacked detectable binding sites for antibodies in HMAF. Interestingly, of these two MAbs, D2-13B6 did not bind to a sequence beginning three residues downstream at residue 60 and D2-13A1 did not bind to a sequence beginning at aa 69. The three other MAbs all reacted similarly with the fusion proteins we tested. Their binding sites, along with the sites for most of the reactive antibodies in HMAF, were contained in the 45 aa stretch between residues 81 and 126 of the NS1 protein.

Immunogenicity of E and NS1 fusion proteins

The trpE fusion proteins specified by pMA1 (296 C-terminal NS1 aa plus 99 aa of NS2A) and pMA2 (412 N-terminal E aa) were used to immunize BALB/c mice. The resulting antibodies recognized the authentic viral proteins in Western blot analyses (data not shown). In order to determine which domains of the fusion proteins had triggered the immune response in the mice, we tested the antisera for binding to our existing battery of fusion proteins in a modified ELISA (see Methods). The results indicated that the antibodies elicited by immunization with the pMA2 fusion protein reacted equally well with fusion proteins containing either domain II alone, or domains I and II together (Fig. 5). Furthermore, the pMA2 antibodies appeared to be specific for DEN1 because they did not react with a fusion protein expressing most of the E protein-coding region of JEV. In contrast, the immune response against the pMA1 fusion protein was targeted mainly to C-terminal sequences of NS1 that were not included in the antigenic domains defined by our anti-NS1 MAbs (Fig. 5).

Table 3. Summary of reactivity of MAbs and HMAF with recombinant NS1 proteins

<table>
<thead>
<tr>
<th>Clone†</th>
<th>Region (aa)‡</th>
<th>D2-13B6</th>
<th>D2-13A1</th>
<th>D2-15F3</th>
<th>D2-5C11</th>
<th>D2-7E11</th>
<th>HMAF</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMA1</td>
<td>57–352</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>pMS11</td>
<td>57–126</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>pMS12</td>
<td>57–103</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>pMT10</td>
<td>81–352</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>pMT11</td>
<td>104–352</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+/-</td>
</tr>
<tr>
<td>pMT12</td>
<td>60–352</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>pMT13</td>
<td>69–352</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* Binding of individual MAbs and the polyclonal anti-DEN1 HMAF with the recombinant proteins was determined by immunoblot: +, strong positive reaction; –, undetectable reaction; +/-, weak reaction.
† Identity of the pATH-DEN1 NS1 recombinant.
‡ Amino acid residues of the NS1 protein expressed by each clone.
Fig. 5. Antibody binding analysis to determine the regions of the E and NS1 fusion proteins that were immunogenic in mice. Mice were immunized with electrophoretically purified preparations of either the E fusion protein expressed from pMA2 or the NS1 fusion protein expressed from pMA1 (see Methods). The resulting antisera were diluted with a non-viral trpE fusion protein and used in an ELISA with wells coated with different E or NS1 fusion proteins as antigens (see Methods). (a) Binding of antibodies elicited by the E fusion protein to the following test antigens: (1) the pMA2 fusion protein (aa 1 to 412 of E); (2) a fusion protein expressing aa 196 to 412 of the DEN1 E protein; (3) a fusion protein expressing aa 97 to 500 of the JEV E protein. (b) Binding of antibodies elicited by the NS1 fusion protein to the following test antigens: (1) the pMA1 fusion protein (aa 57 to 352 of NS1 and aa 1 to 99 of NS2A); (2) the pMS11 fusion protein (aa 57 to 103 of NS1); (3) the pMS12 fusion protein (aa 57 to 126 of NS1); (4) the pMT11 fusion protein (aa 104 to 352 of NS1 and aa 1 to 99 of NS2A).

Discussion

We have shown that at least two distinct antigenic domains on the E protein of DEN1 can be efficiently expressed in E. coli. Antigenic domain I binds two anti-E MAbs and extends between aa 76 and 93, and antigenic domain II binds three MAbs and lies between aa 293 and 402. The sequence of domain I is well conserved among the sequenced dengue viruses, but it has a relatively low relatedness to other flaviviruses. This antigenic domain is interesting because it is located within the disulphide-rich region I of the E protein (as defined by Nowak & Wengler, 1987) and is next to a segment of 14 aa that is absolutely conserved among many different flaviviruses [DEN1, DEN2, DEN4, JEV, Murray Valley encephalitis (MVE), Saint Louis encephalitis (SLE), West Nile (WN) and yellow fever (YF); Mason et al., 1987b]. Despite the location of domain I, the epitopes for the two domain I MAbs we tested were not disulphide bridge-dependent and neither MAb neutralized DEN1 in vitro. Epitopes defined by neutralization escape mutants of YF (Lobigs et al., 1987) and tick-borne encephalitis (TBE) (Mandl et al., 1989) viruses are located in a region immediately preceding domain I in the DEN1 E protein. The juxtaposition of these epitopes with the epitopes in domain I is particularly interesting as our analyses were restricted to the subset of epitopes efficiently expressed in E. coli.

Antigenic domain II of E (aa 293 to 402) is much longer than antigenic domain I and is disulphide bridge-dependent. In these respects, domain II is similar to a domain we have identified in JEV (Mason et al., 1989) and to domain B of TBE virus which has been described by Heinz and coworkers (Winkler et al., 1987; Mandl et al., 1989). One of the domain II MAbs neutralized DEN1 virus in vitro (D2-9D12, see Table 1), providing additional evidence that the DEN1 domain II is similar to the corresponding domain of the JEV E protein which was recognized by 10 different neutralizing MAbs (Mason et al., 1989). A second domain II MAb, D1-4E5-6-5, did not neutralize DEN1 in our tests, but others have shown that this antibody neutralizes DEN2 in vitro and passively protects mice from lethal DEN2 challenge (Kaufman et al., 1987). Consistent with its failure to neutralize DEN1 this antibody also failed to react with DEN1 in immunofluorescence assays (Table 1) but, interestingly, it reacts with other flaviviruses in dot immunobinding assays (Kaufman et al., 1987) and with the DEN1 E protein and the DEN1–trpE fusion proteins in Western blot assays (Tables 1 and 2). The localization of the epitopes for two dengue virus-neutralizing antibodies to domain II (referred to as domain B by Heinz and coworkers) further highlights the importance of this region of the E protein for conferring immunity to flavivirus infection.

Currently there is no other information available on the localization of epitopes within the primary structure of the dengue virus E protein. However, based on recent progress in the mapping of monoclonal antibody escape mutants for other flaviviruses (Lobigs et al., 1987; Mandl et al., 1989) we anticipate that it will be possible to identify and map additional neutralization epitopes within the primary structure of the E protein.

We have detected multiple antigenic determinants in the segment of NS1 between amino acid residues 57 and 126. Two of the NS1 MAbs reacted with sites between aa 57 and 103, a segment that was not reactive with antibodies in the polyclonal HMAF. An overlapping segment from residues 81 to 126 contained the epitopes for three of the anti-NS1 MAbs and was strongly reactive with the HMAF. In sequence comparison among a large number of flaviviruses, this 68 aa antigenic region (aa 57 to 126) is not as highly conserved as the NS1 sequence.
overall. Nevertheless, 15 of these 68 aa are identical in the NS1 sequences of DEN1, JEV, MVE, SLE, WN and YF viruses, suggesting the presence of important conserved features in this region.

The expression of viral antigens in E. coli has been used by Putnak et al. (1988) to study the antigenic structure of the NS1 protein of DEN2. These studies showed that polyclonal anti-DEN2 antibodies were strongly reactive with fusion proteins containing sequences from the N-terminal end of the DEN2 NS1 protein, whereas the epitopes for both of the DEN2 MAbs studied were located in the C-terminal third of the NS1 sequence. Thus, our results obtained with DEN1 and the results of Putnak et al. (1988) obtained with DEN2 are consistent with respect to the binding sites for the polyclonal antibodies but differ with respect to the location of the epitopes for the NS1 MAbs. There is no obvious explanation for this difference, but it is most likely due to the methods used to generate and select the MAbs rather than a fundamental difference in the antigenic structures of the NS1 proteins from the two dengue virus serotypes.

The pMA2 (E) and pMA1 (NS1) fusion proteins were strongly immunogenic in mice, eliciting antibodies that reacted with the authentic viral proteins in Western blots. In the case of the E protein, the antibodies raised against the pMA2 fusion protein reacted poorly with domain I and reacted well with fusion proteins containing domain II. In the case of the NS1 protein, the majority of the antibodies elicited by the fusion protein reacted with determinants in the C-terminal region of NS1 and not with the N-terminal antigenic region defined by the five NS1 MAbs. With regard to this latter finding, it is worth noting that rabbits immunized with immunopurified authentic DEN2 NS1 apparently also produced antibodies to the C-terminal region of the NS1 protein (Putnak et al., 1988).

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References


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