Molecular Characterization of a Neutralizing Domain of the Japanese Encephalitis Virus Structural Glycoprotein

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SUMMARY

Expression of antigenic fragments of the Japanese encephalitis virus envelope protein (E) in Escherichia coli has been used to define the boundaries of an antigenic domain that contains the binding sites for 10 anti-E monoclonal antibodies (MAbs). All of these antibodies neutralized the virus in vitro and some of them passively protected mice from a fatal virus challenge. We have shown previously that nine of these antibodies react with the antigenic determinants encoded by a 405 bp fragment of viral cDNA. To determine the amino acid sequences of specific determinants, truncated polypeptides were expressed as fusion proteins in E. coli following progressive Bal 31 exonuclease digestion of the 5' and 3' ends of the cDNA fragment. Examination of the immunoreactivity of these polypeptides revealed that the region from methionine 303 to tryptophan 396 was the shortest sequence capable of reacting with any of the 10 MAbs or with a polyclonal, antiviral hyperimmune mouse ascitic fluid. Biochemical tests showed that an intramolecular disulphide cross-linkage between cysteine 304 and cysteine 335 of the E protein sequence was required for presentation of the binding site(s) for these MAbs. Although this 95 amino acid antigenic domain appeared to be capable of forming several conformational neutralizing epitopes, it was not an effective immunogen for inducing neutralizing or protective antibodies in mice.

INTRODUCTION

The flavivirus envelope glycoprotein (E) is a major target of the host antiviral immune response. The E glycoprotein contains antigenic determinants for neutralizing monoclonal antibodies (MAbs) (see Roehrig, 1986 for review), and purified E protein from Japanese encephalitis (JE) virus (Takegami et al., 1982) and tick-borne encephalitis (TBE) virus (Heinz et al., 1984a) have been shown to elicit neutralizing antibodies when injected into mice. Different antigenic regions on the E protein have been inferred from a wide variety of biochemical and immunological tests using both MAbs and polyclonal antisera (see Roehrig, 1986 for review). Competitive binding of MAbs has been used to identify the presence of multiple conformationally distinct epitopes on the E protein of several different flaviviruses [dengue 2 (DEN-2): Henchal et al., 1985; Kaufman et al., 1987; JE: Kimura-Kuroda & Yasui, 1983, 1986; Murray Valley encephalitis (MVE): Hawkes et al., 1988; Saint Louis encephalitis (SLE): Roehrig et al., 1983; yellow fever (YF): Cammack & Gould, 1986; TBE: Heinz et al., 1983a, b, 1984b]. Although these results have demonstrated the existence of spatially separable epitopes,
they did not identify the amino acid sequences that form these antigenic determinants. In the case of YF virus, characterization of mutant viruses that escape neutralization by a MAb revealed a neutralizing epitope containing amino acid residues 71 and 72 of the E protein (Lobigs et al., 1987).

The primary structure of the E protein for 10 flaviviruses has been deduced from cloning and sequencing of viral cDNAs [YF: Rice et al., 1985; West Nile (WN): Wengler et al., 1985; MVE: Dalgarno et al., 1986; JE: Sumiyoshi et al., 1986; McAda et al., 1987; DEN-1: Mason et al., 1987b; DEN-2: Deubel et al., 1986; Hahn et al., 1988; Gruenberg et al., 1988; DEN-4: Zhao et al., 1986; SLE: Trent et al., 1987; Kunjin (KUN): Coia et al., 1988; TBE: Mandl et al., 1988]. There is significant heterogeneity in the E protein sequences among these viruses, as indicated by overall relatedness values of 45 to 80%. However, the hydrophilicity profiles for these proteins (Kyte & Doolittle, 1982) are nearly superimposable, suggesting that the different E proteins may have similar secondary and tertiary structures. In this regard, it is therefore noteworthy that each protein contains 12 conserved Cys residues, all of which appear to participate in specific intramolecular disulphide bonds. The positions of the six disulphides have been precisely mapped in the E protein of WN virus (Nowak & Wengler, 1987). The data suggest that highly conserved disulphide bridges are principal elements in the three-dimensional structure of flavivirus E proteins.

We have expressed JE virus cDNA in Escherichia coli to localize antigenic determinants within the E protein and to test the immunogenicity of recombinant forms of viral proteins. In previous work, we showed that fusion proteins specified by Δgt1 recombinants contained determinants recognized by antiviral antibodies (Mason et al., 1987a). One of these proteins was of particular interest because it contained only 135 of the 500 amino acid residues of the E protein and still retained reactivity with a panel of nine antiviral MABs (Mason et al., 1987a). To map the boundaries of the immunoreactive protein fragment more precisely, we used Bal 31 exonuclease to create deletions from the 5' and 3' ends of the cDNA fragment encoding the antigen. We report here that a 95 amino acid fragment was the minimal structure capable of binding any of 10 MABs and that the immunoreactivity of this fragment was dependent on formation of disulphide bonds. Although a recombinant fusion protein containing the 95 amino acid sequence reacts with neutralizing and protective MABs in direct binding assays, it failed to induce a neutralizing immune response when injected into mice. These results further document the importance of tertiary structure in the antigenic properties of the flavivirus E glycoprotein.

### METHODS

**Plasmids, bacterial strains, cell lines, virus stocks.** The JE-Δgt1 recombinant J7-1 (Mason et al., 1987a) and the E. coli strain HB101 (Bouy & Roulland-Dussioix, 1969) have been described. The E. coli plasmid pATH11 is a derivative of the plasmid described by Dieckmann & Tzagoloff (1985) and was supplied by Dr T. J. Koerner. Vero cells were grown at 37 °C in Eagle's MEM supplemented with 5% heat-inactivated foetal bovine serum and antibiotics. The passage history of the Nakayama strain of JE virus used in these experiments has been described (McAda et al., 1987).

**Construction of JE–pATH11 recombinant plasmids.** The recombinant plasmid p280-414 was generated by ligating the EcoRI/SalI JE virus cDNA fragment of 4 clone J7-1 (Mason et al., 1987a) into EcoRI/SacI-cut pATH11 plasmid DNA. Deletions from the 3' end of the JE virus cDNA fragment contained in p280-414 were created by digestion with Bal 31 exonuclease. Briefly, the plasmid DNA was cut at a unique HindIII site at the 3' end of the JE sequence, treated with Bal 31 exonuclease (New England Biolabs), and then digested with EcoRI. The shortened cDNA fragments were gel-purified and ligated into EcoRI/Smal-digested pATH11 DNA. Similar methods were used to produce 5' end deletions of the JE virus cDNA fragment contained in p280-414. Plasmid DNA cut at the unique EcoRI site of the JE virus sequence was treated with Bal 31 exonuclease and the ends were repaired with Klenow polymerase. The resulting DNA was ligated to phosphorylated oligonucleotide linkers containing an EcoRI restriction site and digested to completion with EcoRI and HindIII. The JE virus cDNA containing fragments were gel-purified and ligated to EcoRI/HindIII-cut pATH11 DNA. To identify the one in three plasmids that contained in-frame trpE-JE fusions, individual transformants obtained from the ligated population were screened for their ability to produce inclusion bodies containing the trpE fusion proteins. This screening was based on the knowledge that the trpE protein produced by the parent pATH11 plasmid remained soluble, whereas JE–trpE fusion proteins were found in inclusion bodies. An ELISA (see below) using antibodies generated to non-
viral trpE fusion proteins was used to detect the presence of fusion protein antigens in the insoluble fractions isolated from small-scale cultures of individual plasmid-containing transformants (see below). As expected, about one-third of the transformants produced trpE proteins that could be recovered in the insoluble cell fraction. In all cases, in-frame fusions were confirmed by determining the sizes of the fusion proteins and by nucleotide sequencing of the JE virus cDNA inserts.

cDNA fragments derived from selected fragments were subcloned into the M13 phage vector (Yanisch-Perron et al., 1985) and sequenced by standard techniques (Sanger et al., 1977, 1980).

Preparation of JE-trpE fusion proteins. JE-trpE fusion proteins were isolated in the form of insoluble fractions produced in tryptophan-starved E. coli HB101 cells harbouring the recombinant pATH plasmids. Fusion protein synthesis was initiated by the addition of 10 μg/ml of indoleacrylic acid (Sigma) to early log phase cells grown in M9 minimal medium at 30 °C in the presence of 100 μg/ml ampicillin. After growth for 5 to 7 h, the cells were harvested by centrifugation, and the insoluble fraction was isolated as described by Kleid et al. (1981).

Purification of fusion proteins. JE-trpE fusion proteins were purified from the insoluble cell fractions by electrophoresis from SDS-containing polyacrylamide gels. The samples were prepared for electrophoresis by resuspending the insoluble fractions in gel electrophoresis sample buffer (Laemmli, 1970) containing 1% SDS (Sigma), 1% 2-mercaptoethanol (Sigma), and 1 mM-phenylmethylsulphonyl fluoride (Sigma) followed by heating at 70 °C for 15 min. brief sonication and clarification by microcentrifugation. The fusion proteins were located in preparative gels by staining part of the gel with Coomassie Brilliant Blue and the protein bands were excised from the unstained portion of the gel. The slices were inserted into dialysis bags containing approximately 20 volumes of 80 mM-Tris, 12.5 mM-glycine, 20% methanol, and the protein was electroeluted by immersing the bags in an electrophoresis chamber containing the same buffer and applying a voltage drop of 8 V/cm for 2 h at room temperature. After removal of the gel slices, the protein was dialysed for 72 h at room temperature against several changes of 100 mM-NH₄HCO₃ and then lyophilized. Protein concentration was determined by the method of Lowry et al. (1951).

Immunological reagents. Polyclonal anti-JE virus antibodies were elicited by the repeated immunization of adult outbred mice with JE virus-infected suckling mouse brain and antibodies were obtained in the hyperimmune mouse ascitic fluids (HMAF) (Brandt et al., 1967).

Hybridoma cells that secreted JE virus-specific MAbs were developed from mice immunized with antigen derived from the Nakayama strain of JE virus. BALB/c mice (Jackson Laboratories) were initially immunized intraperitoneally with a suckling mouse brain suspension of virus. The subsequent booster injections administered varied in three different fusion experiments. For fusion J1, the mouse brain suspension was injected once intraperitoneally; for fusion J2, purified virion treated with Triton X-100 was injected twice subcutaneously; for fusion J3, purified virion was injected twice subcutaneously. JE virions were purified by potassium tartrate-glycerol density gradient centrifugation followed by rate-zonal centrifugation in sucrose gradients as previously described (Repik et al., 1983). For each group of animals, the last booster injection was performed 3 days before fusion, and hybridomas were generated as described by Gentry et al. (1982). Antibody-secreting cell lines were identified by indirect solid phase-radioimmunoassay using a JE virus-infected Vero cell extract and 125I-labelled anti-mouse immunoglobulin. Cell lines were cloned on soft agarose (Gentry, 1985) and antibody-containing ascitic fluids were generated as described by Rener (1985). The production and characterization of monoclonal antibody D1-4G2 has been previously described (Gentry et al., 1982).

Ascitic fluids containing polyclonal antibodies to the JE-trpE fusion proteins were produced in BALB/c mice (Charles River Laboratories) immunized with electrophoretically purified fusion proteins. Before injection the proteins were dissolved in phosphate-buffered saline or 8 M-urea. The immunization schedule was as follows: 42-day-old mice were given a primary injection of 25 μg of protein emulsified with Freund's complete adjuvant, a 25 μg booster injection was given 21 days later in incomplete adjuvant, and a second 25 μg boost was given 21 days later without adjuvant. Ascitic fluids were generated in immunized mice by injection of sarcoma 180/TG cells on the 3rd day following the final boost (Sartorelli et al., 1986).

Passive protection by monoclonal antibodies. The ability of MAbs to protect mice passively against a JE virus challenge was tested in C57BL/6 mice (Jackson Laboratories). Four-week-old mice in groups of 10 were injected intraperitoneally with 0.1 ml of MAb-containing ascitic fluids. The animals were challenged 24 h later by intraperitoneal injection of approximately 1000 LD50 units of JE virus (Beijing strain) and observed for 25 days.

Under these challenge conditions, the polyclonal anti-JE virus HMAF protected nine out of 10 mice whereas only three out of 10 mice injected with non-immune ascitic fluid survived.

Evaluation of the p280-414 fusion protein as a protective immunogen. Groups of 20 4-week-old C57BL/6 mice were immunized on days 0, 3 and 21 with 50 μg of fusion protein from recombinant p280-414, 50 μg of the trpE fusion protein containing non-viral sequences, or one-tenth of a human dose (corresponding to less than 8 μg of protein) of a commercially available, formalin-inactivated mouse brain JE virus vaccine (Biken). Immunizations were by intramuscular injection in the presence of Freund's adjuvant (complete for dose one, incomplete for doses two and three). On day 28 the mice were injected intraperitoneally with 0.1 ml of a 1:10 dilution of freshly harvested
suckling mouse brain infected with the Nakayama strain of JE virus (corresponding to a challenge dose of about $10^8$ p.f.u. of virus), and mice were observed for 21 days.

Serological tests. Plaque reduction neutralization titers (PRNT) were determined using twofold dilutions of ascitic fluids containing MAbs (Tesh & Duboise, 1987). Haemagglutination-inhibition assay (HAI; Clark & Casals, 1958), fluorescent antibody assay (Tesh, 1979) and the ELISA (Engvall, 1980) were performed using standard techniques. Isotype determination was done by indirect solid-phase radioimmunoassay using goat anti-mouse immunoglobulin (Rothman et al., 1988).

Preparation of JE virus E protein. Partially purified virions were used as the source of the JE virus E protein for ELISA and Western blot analyses. JE virions in Vero cell culture fluid were precipitated by incubation in 2.5% NaCl and 7% PEG for 5 h at 4 °C (Repik et al., 1983) followed by centrifugation for 30 min at 12000 g at 4 °C. The virus particles were resuspended in STE buffer (100 mM-NaCl, 10 mM-Tris-HCl pH 7.75, 1 mM-EDTA), large particulate matter was removed, and the virions were pelleted by centrifugation for 60 min at 110000 g. Virus preparations were stored at −70 °C until use.

Reduction and alkylation of authentic and recombinant antigens. Partially purified virions or electroeluted fusion proteins (containing approximately 100 to 200 μg/ml protein) were dissolved in STE buffer, containing 1 mM-PMSF and 0.1% SDS, heated for 30 min at 60 °C, and clarified by microcentrifugation. The samples were reduced by incubation for 30 min at 60 °C in the presence of 2 mM-dithiothreitol (Sigma). The free sulphydryl groups were then alkylated by incubation for 30 min at 60 °C in the presence of 8 mM-iodoacetamide (Sigma).

PAGE and immunoblotting. Proteins were resolved by PAGE in the presence of SDS (Laemmli, 1970) and transferred to nitrocellulose filter paper as described by Towbin et al. (1979). The immunoblots (Western blots) were processed as described by Mason et al. (1987a).

RESULTS

Characterization of anti-JE virus MAbs

Over 300 antibody-secreting lymphocyte hybridoma lines were obtained from three separate fusion experiments. Of these, 55 cell lines were cloned and amplified in mice to produce ascitic fluid. Ten of these antibodies which were shown to be E-specific on the basis of their strong reactivity to the JE virus E protein on immunoblots (not shown) were selected for further characterization. Their functional properties were examined further using assays for plaque reduction neutralization, haemagglutination-inhibition and passive protection. In addition, an indirect fluorescent antibody assay was used to test these 10 antibodies for cross-reactivity with other flaviviruses, including members of the JE-WN subgroup, the dengue viruses, and YF. The properties of the 10 JE MAbs and the flavivirus group-reactive MAb D1-4G2 (Gentry et al., 1982) are summarized in Table 1. Interestingly, all of the JE virus MAbs showed neutralization activity, although none of them showed appreciable haemagglutination-inhibition activity, in striking contrast to the D1-4G2 MAb. The JE virus MAbs were different in their functional properties, indicating that these antibodies recognize multiple epitopes on the E protein. Moreover, the epitopes for several of these MAbs were distinguishable in cross-blocking experiments (D. S. Burke, unpublished data).

Expression of E protein sequences in E. coli

The JE virus cDNA fragment from the 7gtl1 recombinant J7-1 (Mason et al., 1987a) was ligated into the pATH vector to produce a plasmid (p280-414) with the sequence for amino acids 280 to 414 of the E protein fused in-frame with the E. coli trpE gene. The fusion protein expressed by this plasmid was estimated to be 51K by SDS–PAGE, which agrees with the calculated value for the protein deduced from the DNA sequence (51.3K). This fusion protein was strongly reactive with anti-JE virus HMAF and each of the 10 anti-JE virus MAbs in both ELISA (Table 2) and immunoblot assays (not shown).

Deletion analysis of antibody binding sites

To define the amino acid sequences recognized by the anti-E MAbs more clearly, plasmids were constructed for the expression of different segments of the cDNA insert in p280-414. First, deletions were created that progressively removed residues from the C-terminal end of the E protein sequence expressed in p280-414 (Fig. 1). Secondly, deletions were created in p280-400 that removed amino acid residues from the N-terminal end of the expressed E protein sequence (Fig. 1).
<table>
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<th>HA‡</th>
<th>Isotype</th>
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* Preliminary data on nine of the JE virus MAbs has been presented elsewhere (Mason et al., 1987a), and MAb J3-11G5 has been used to detect JE virus-specific antibodies in human sera using an epitope-blocking immunoassay (Burke et al., 1987).
† Dilution yielding >90% reduction.
‡ Dilution.
§ Number protected/number challenged.
|| Determined by fluorescent antibody assay using flavivirus-infected C6/36 cells, see Methods. All hybridoma ascitic fluids were diluted 1:100: -, non-reactive; +, reactive; ++, strongly reactive. All hybridoma ascitic fluids showed ++ reactivity with JE virus-infected cells.
¶ ND, Not determined.
Table 2. Antibody binding to truncated E protein sequences in ELISA*

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<th>J2-7F12</th>
<th>J3-10E1</th>
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* Values shown were based on the absorbance value of the reaction obtained with each fusion protein relative to the reaction obtained with a trpE fusion protein containing non-viral sequences. Plates were coated with insoluble fractions diluted to yield a fusion protein concentration of 0.25 µg/ml. -, relative A < 0.1; +, relative A > 0.1, < 0.5; ++, relative A > 0.5, < 1.0; ++++, relative A > 1.0.
† Identity of pATH-JE clone expressing the fusion protein; clone numbers correspond to the E protein amino acid residues it specifies.
‡ Non-immune mouse ascitic fluid.

Fig. 1. E protein amino acid residues expressed by the p280-414-derived recombinants. E protein amino acids are shown in upper case, pATH vector-specified amino acids present at the 5' and 3' junction regions of the fusion proteins are shown in lower case (all residues are shown in the standard single letter amino acid code). Numbering indicates the amino acid residue number in the 500 amino acid E protein (McAda et al., 1987).
Fig. 2. Reactivity of MAb J3-11B9 with JE trpE fusion proteins. Protein samples containing from 0.5 to 2 µg of each fusion protein were separated by electrophoresis in a 10% polyacrylamide gel containing SDS and then subjected to immunoblot analysis. The primary antibody was a 1:2000 dilution of an ascitic fluid containing the MAb J3-11B9. The immune complexes were decorated with alkaline phosphatase-conjugated goat anti-mouse IgG and visualized by incubation with 5-bromo-4-chloroindoxyl phosphate and nitro-blue tetrazolium (Blake et al., 1984). The first and last amino acid residues of the E protein sequence expressed in each of the trpE fusion proteins are as follows for lanes 1 to 11: 280–414, 280–400, 280–398, 280–396, 280–393, 280–389, 288–400, 296–400, 303–400, 310–400, respectively. The M, standards shown at the left were bovine serum albumin (66K) and ovalbumin (44K).

Fig. 3. Effect of reduction and alkylation on the reactivity of the E protein on immunoblots. Samples of partially purified virion were prepared for analysis as described in the Methods. The samples were: lanes 1, untreated; lanes 2, reduced; lanes 3, reduced and alkylated. After treatment, the samples were diluted in SDS–PAGE sample buffer without reducing agent and the proteins were separated by electrophoresis in a 12.5% gel. Protein blots were incubated with 1:1000 dilutions of the ascitic fluids for MAbs J3-11B9 or D1-4G2, the anti-JE HMAF, or the hyperimmune ascitic fluid prepared from mice immunized with the E–trpE fusion protein specified by the p280-414 recombinant. The immune complexes were decorated with 125I-labelled goat anti-mouse IgG and the filters were autoradiographed.

The minimal amino acid sequence required for expression of the MAb epitopes was inferred from the immunoreactivity of the truncated fusion proteins. The results of ELISA performed with the trpE fusion proteins as the binding target are given in Table 2. All of the MAbs had similar reactivity profiles with the reactivity dependent on the expression of a large part of the E protein (Table 2). By this analysis, the binding sites for all 10 MAbs lie within the JE virus E protein sequence between Met 303 and Lys 398 (McAda et al., 1987). However, eight out of 10 antibodies had significant reactivity with the slightly shorter sequence expressed from p280–396, and one antibody, J3-12H11, showed highly reproducible but weak binding to the protein from p280–393. Immunoblot experiments confirmed the loss of antibody binding with fusion proteins shorter than the 95 amino acid sequence from residues 303 to 398. A representative example of these immunoblot assays is shown in Fig. 2.

Interestingly, the binding of the polyclonal JE virus HMAF to these recombinant proteins was qualitatively the same as that of the MAbs. Specifically, the anti-JE virus HMAF was reactive only with those fusion proteins which contained the entire sequence between amino acid residues 303 and 396 (Table 2).

**Antigenic structure requires disulphide bond formation**

The presence of two cysteines between residues 303 and 396 of the E protein sequence suggested the possibility of a role for disulphide bonding in the antigenic structure of this region. To test this possibility, the reactivities of reduced, reduced and alkylated, and alkylated forms of the authentic E protein and the fusion protein expressed from p280–414 were analysed by ELISA (Table 3) and immunoblotting (Fig. 3). For both the recombinant protein and the authentic viral protein, reduction diminished reactivity with all 10 MAbs, reduction and alkylation eliminated reactivity, and alkylation alone had little effect.
Table 3. Effect of reduction and alkylation on the immunoreactivity of the E protein and the p280-414 fusion protein in ELISA

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Untreated</th>
<th>Reduced</th>
<th>Reduced and alkylated</th>
<th>Alkylated</th>
</tr>
</thead>
<tbody>
<tr>
<td>E Protein†</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JE HMAF</td>
<td>&gt;1.7</td>
<td>0.68</td>
<td>0.52</td>
<td>1.6</td>
</tr>
<tr>
<td>NMAF</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>J2-5A11</td>
<td>&gt;1.7</td>
<td>0.45</td>
<td>-</td>
<td>1.4</td>
</tr>
<tr>
<td>J3-10E1</td>
<td>0.32</td>
<td>0.05</td>
<td>-</td>
<td>0.20</td>
</tr>
<tr>
<td>J3-11B9</td>
<td>&gt;1.7</td>
<td>0.74</td>
<td>-</td>
<td>1.6</td>
</tr>
<tr>
<td>D1-4G2</td>
<td>&gt;1.7</td>
<td>0.03</td>
<td>-</td>
<td>1.6</td>
</tr>
<tr>
<td>F1§</td>
<td>1.3</td>
<td>1.5</td>
<td>1.3</td>
<td>1.1</td>
</tr>
<tr>
<td>F2§</td>
<td>0.21</td>
<td>0.28</td>
<td>0.19</td>
<td>0.19</td>
</tr>
<tr>
<td>F3§</td>
<td>0.14</td>
<td>0.25</td>
<td>0.13</td>
<td>0.15</td>
</tr>
<tr>
<td>p280-414 Fusion protein†</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JE HMAF</td>
<td>0.49</td>
<td>0.26</td>
<td>0.06</td>
<td>0.46</td>
</tr>
<tr>
<td>NMAF</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.02</td>
</tr>
<tr>
<td>J2-5A11</td>
<td>0.67</td>
<td>0.46</td>
<td>0.08</td>
<td>0.62</td>
</tr>
<tr>
<td>J3-10E1</td>
<td>0.06</td>
<td>0.07</td>
<td>-</td>
<td>0.10</td>
</tr>
<tr>
<td>J3-11B9</td>
<td>0.85</td>
<td>0.64</td>
<td>0.16</td>
<td>0.75</td>
</tr>
</tbody>
</table>

* Data shown are the absorbance values relative to wells that did not contain antigen.
† Partially purified E protein preparation, see Methods.
‡ Relative absorbance values less than or equal to 0.01.
§ Ascitic fluids from three BALB/c mice immunized with p280-414 fusion protein.
†定量 Electroeluted, PAGE-purified fusion protein specified by clone p280-414.

The complete loss of antibody binding after reduction and alkylation demonstrates the importance of a disulphide bridge between Cys 304 and Cys 335 in the structure of the antigenic domain recognized by the 10 MAbs. The incomplete loss of reactivity with reduction alone shows that this disulphide bond can re-form following reduction. It is noteworthy that the flavivirus group-reactive MAb D1-4G2 did not bind to either the reduced or the reduced and alkylated E protein (Table 3, Fig. 3), showing that the epitope for this antibody was irreversibly lost upon reduction.

Characterization of antibodies elicited by the p280-414 fusion protein

The fusion protein expressed by recombinant p280-414 was strongly immunogenic when the purified protein was used to immunize mice. The antibodies in ascitic fluids from these mice were reactive with partially purified virions by ELISA, and they were shown to be specific for the E protein by both immunoblotting (see Fig. 3) and radioimmunoprecipitation (data not shown) of proteins extracted from JE virus-infected cells. These antibodies were, however, qualitatively different from antibodies in JE virus HMAF and MAbs obtained from virus-infected mice. The antibodies raised in infected animals had sharply reduced reactivity with the reduced and alkylated E protein (Fig. 3, Table 3), and all of them were able to neutralize the virus in in vitro plaque reduction assays (Table 1). In contrast, the antibodies to the fusion protein were strongly reactive with the reduced and alkylated E protein (Fig. 3, Table 3) and showed no plaque reduction neutralization activity at 1:10 dilutions of the immune ascitic fluids. Since the p280-414 fusion protein reacted well with neutralizing MAbs, the failure of the fusion protein to elicit a measurable neutralization titre indicates that the recombinant protein was not an effective immunogen for the neutralization epitopes.

Animal protection assays

The immune response to the fusion protein specified by the p280-414 recombinant was further evaluated in a virus challenge experiment with C57BL/6 mice. Although immunization with the fusion protein produced antisera that reacted in ELISA with viral antigen, the antisera did not
Fig. 4. Schematic representation of the 95 amino acid antigenic domain in the E protein. This diagram is adapted from Nowak & Wengler (1987) and is based on the assumption that the disulphide bonds in JE virus are the same as those believed to occur among the conserved Cys residues in the E protein of WN virus. The antigenic domain is indicated by the shading which extends from residue 303 to residue 398. The positions of the intra-chain disulphide bridges are indicated with a solid line connecting Cys residues; the potential N-linked glycosylation site at residue 104 is indicated by a solid diamond.

Table 4. Protection of mice from virus challenge

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>Biken vaccine</th>
<th>p280-414 fusion protein</th>
<th>Non-viral trPE fusion protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Survivors</td>
<td>19</td>
<td>7*</td>
<td>3</td>
</tr>
<tr>
<td>Died</td>
<td>0</td>
<td>12</td>
<td>17</td>
</tr>
</tbody>
</table>

* No significant protection; chi-square analysis yields $P = 0.23$.

exhibit any plaque reduction neutralization activity, and there was no beneficial effect on either the survival time (data not shown) or, as shown in Table 4, the morbidity following the challenge of the immunized animals with lethal doses of the virus. The results clearly show the potency of the peripheral challenge in non-immune animals and the efficacy of the commercial vaccine derived from formalin-inactivated virus (Biken).
DISCUSSION

Several lines of evidence suggest that most, if not all, of the neutralization epitopes on flaviviruses are localized on the E structural glycoprotein. A number of MAbs against the E protein of JE virus, all of which neutralize the virus, react with E protein sequences expressed in *E. coli* (Mason et al., 1987a), and we have shown that these neutralizing MAbs also passively protect mice from a lethal challenge of the virus (Table 1). The ability to express immunoreactive viral polypeptides in *E. coli* has facilitated a molecular biological approach to understanding the structure of the binding sites for this group of neutralizing and protective MAbs.

Deletion analysis was used to delimit a region of the E protein between residues 303 and 396 that appears to be the minimal structure capable of forming several E protein conformational epitopes. The binding of 10 MAbs and polyclonal antibodies in JE virus HMAF was lost when deletions were constructed to remove amino acids selectively at either end of this 95 amino acid sequence. The individual epitopes for these antibodies were apparently part of a conformational antigenic domain that represents a strong determinant on the surface of the virion. Antibody binding to this domain in both the authentic E protein and one of the recombinant proteins was sensitive to reduction and alkylation of the antigen, implicating a disulphide bridge between Cys residues at positions 304 and 335 of the E protein as an essential element in the antigenic structure of the E protein. Additional support for the importance of this disulphide was provided by the work of Nowak & Wengler (1987), who have shown that the homologous cysteines form a disulphide bridge in the WN virus, and by our observation that antibody binding was lost upon deletion of the Cys at position 304. The 95 amino acid antigenic domain we have located in the JE virus E protein (Fig. 4) spans the R2 and R3 regions defined for the WN virus E protein (Nowak & Wengler, 1987). Although this schematic representation (Fig. 4) suggests that this 95 amino acid domain is located within the E protein, the antibody binding studies presented in this paper as well as protease sensitivity studies performed on WN virus (Wengler et al., 1987; Kimura & Ohyama, 1988) indicate that this region is exposed on the surface of the flavivirus virion.

The antigenic domain we have defined in the JE virus E protein corresponds to similar domains in the E proteins of DEN-1 and TBE viruses. In our companion studies on DEN-1, deletion analysis was used to localize three MAb-binding sites between residues 293 and 401 of the DEN-1 E protein sequence (M. A. Zuegel, P. W. Mason, M. J. Fournier & T. L. Mason, unpublished data). In the case of TBE virus, Winkler et al. (1987) identified an immune-reactive region, designated domain B, that was contained in a 9K tryptic fragment of the E protein. This fragment was stabilized by a disulphide bridge, which can re-form after reduction and SDS denaturation and was required for the binding of several domain B-specific MAbs. Recently, two of these antibodies were used to isolate TBE virus neutralization-escape mutants. Direct sequencing of the mutant viral RNA showed that substitutions in the E protein at positions 389 (Ser to Arg) and 384 (Tyr to His) conferred resistance to MAbs B1 and B4, respectively (F. X. Heinz, personal communication). These amino acid substitutions were presumably near the C-terminal end of the 9K tryptic fragment of TBE virus, and the related positions in JE and DEN-1 viruses are near the C-terminal borders of the antigenic domains defined by our deletion studies. Taken together, these results show that a similar disulphide bridge-stabilized antigenic structure is present on the surface of flaviviruses from three different serological subgroups.

The 95 amino acid antigenic domain that we have expressed in *E. coli* reacted with polyclonal antibodies and MAbs raised in JE virus-infected mice, including antibodies that neutralize the virus. However, when this domain was used as an immunogen in the form of a JE-*trpE* fusion protein, the antibodies produced were not functionally equivalent to those derived from infected animals. In particular, antisera against the fusion proteins had no detectable neutralization titre, and these immunizations provided no protection against a peripheral challenge with a lethal dose of the virus. Furthermore, the anti-E antibodies from these animals reacted strongly with the E protein in ELISA and immunoblot assays, but this reactivity was not affected by reduction and alkylation of the E protein. In contrast, the MAbs and the polyclonal antibodies elicited in
response to the virus were completely non-reactive with the reduced and alkylated 95 amino acid domain. Apparently the immune response to this domain during infection was exclusively against conformation-dependent epitopes whereas the response to the fusion protein was predominantly against different, presumably linear epitopes.

Our results provide new information about the antigenic structure of the flavivirus E structural glycoprotein. In particular, we have delineated the minimal sequence of the binding domain for a group of neutralizing and protective MAbs. An explanation for the failure of this domain to elicit neutralizing antibodies in mice might be that the native antigenic structure does not renature efficiently or is more labile when it is in the context of a JE–trpE fusion protein. Alternative modes of expression might yield a recombinant form of the 95 amino acid domain with immunogenic properties similar to those of the native E protein.

Note added in proof. The data on TBE virus neutralization-escape mutants communicated to us by F. X. Heinz have been published by Mandl et al. (1989; Journal of Virology 63, 564–571).

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REFERENCES


JE virus E protein neutralizing domain


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