Epitope Mapping of Japanese Encephalitis Virus Envelope Protein Using Monoclonal Antibodies against an Indian Strain

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SUMMARY
A panel of monoclonal antibodies (MAbs) raised against an Indian strain of Japanese encephalitis (JE) virus was used to map topographically the epitopes on the envelope protein. Two separate clusters of epitopes were revealed. On the basis of reactivity in haemagglutination inhibition (HI), neutralization (NT), passive protection and antibody-dependent plaque enhancement (ADPE) assays with the MAbs, five functional domains (A, B, C, D and E) were delineated. The flavivirus cross-reactive domain for HI (A) was distinct. The JE virus-specific domain for HI (B) was in continuum with those domains representing non-HI JE virus-specific MAbs (C) and flavivirus cross-reactive MAbs (D). Domain E, which mapped close to domain D was represented by two MAbs that reacted with both JE virus and uninfected cell nuclei. Four conclusions can be drawn. (i) Two distinct antigenic domains were associated with HI, (ii) HI and NT in vivo or in vitro were dissociated functions, (iii) ADPE activity was solely linked with the A domain and (iv) all MAbs reacting with epitopes in the B domain had HI/NT/protective activity but failed to show ADPE. The B domain might therefore be considered the most suitable for development of synthetic or genetically engineered vaccines.

INTRODUCTION
Japanese encephalitis (JE) virus belongs to the family Flaviviridae, consisting of some 66 antigenically related viruses (Westaway et al., 1985). It has been gaining importance as a human pathogen with an increase in the frequency of epidemics (Monath, 1986). The viral envelope (E) protein is largely responsible for antigenic cross-reactivity within the family (reviewed by Porterfield, 1980). Monoclonal antibodies (MAbs) have been used to identify physical and functional domains within the E protein of several flaviviruses (Henchal et al., 1985; Kobayashi et al., 1985; Heinz, 1986). Recently, on the basis of genome sequencing and MAb analysis (McAda et al., 1987; Mason et al., 1987), a cluster of epitopes with neutralizing activity and antigenic cross-reactivity was localized in 30% of the genomic sequence of the JE virus E protein.

We have previously produced and characterized a panel of MAbs against JE virus that exhibit either antigenic specificity or cross-reactivity and also show activity in some or all of the following tests: haemagglutination inhibition (HI), neutralization (NT) in vivo or in vitro, and immune enhancement of infectivity (Kedarnath et al., 1986; Cecilia & Ghosh, 1988). These MAbs have now been used to map antigenic sites on the E protein of JE virus and to assign biological functions to the mapped domains.

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METHODS

Viruses. Three isolates of JE virus, which were antigenically identical when tested with 16 different MAbs, were used. They were chosen because of their suitability in the individual tests. JE-733913, a human brain isolate from West Bengal, India, was used for production of MAbs and for HI tests. JE-P20778, a human brain isolate from Vellore, India, was used for ELISAs. JE-Nakayama (from the Rockefeller Foundation) was used for all other tests. The details of these viruses and their antigenicity were given by Kedarnath et al. (1986). Infected mouse brain pools were used for passive protection experiments and in vivo NT tests. For the in vitro NT test virus pools were prepared in PS (porcine kidney stable) cell cultures maintained in Earle's MEM with 10% goat serum. For ELISA, JE virus (P20778) grown in PS cell culture, was purified by sucrose density gradient (10 to 40% w/v) centrifugation (80000 g for 2.5 h). The fractions with highest haemagglutination (HA) activity and infectivity were pooled, centrifuged at 100000 g for 4 h and used at a concentration of 1 µg/well.

NT tests. NT tests were carried out in vivo and in vitro. For the in vitro test, twofold dilutions of MAbs were incubated with TCID₅₀ of virus for 1 h at 37 °C. The mixture was then assayed for virus infectivity in PS cells. The antibody titre was the reciprocal of the dilution producing a 50% reduction of infectivity. For the in vivo test a 1:50 dilution of the MAb was tested against a 100 LD₅₀ dose of virus inoculated intraperitoneally (i.p.) in 2-day-old infant mice.

Passive protection. Ten-day-old infant mice were inoculated i.p. with 0.1 ml of monoclonal ascitic fluid. After 24 h JE virus was inoculated i.p. as the challenge virus in two doses. A high dose of 100 LD₅₀ killed all the control mice and a low dose of 5 LD₅₀ killed 75 to 90% of the non-immunized mice. The percentage survival was recorded after 20 days.

Titration of MAbs. Tenfold dilutions of MAbs (ascitic fluid) were added to microtitre plates coated with purified JE virus at a concentration of 1 µg/well. After 2 h the wells were washed and incubated with goat anti-mouse-horseradish peroxidase (HRPO) conjugate (Sigma) for 2 h at a predetermined optimal concentration. This was followed by the addition of substrate, o-phenylenediamine (Sigma) and urea peroxide in phosphate-citrate buffer pH 5.0. The reaction was terminated with 2 M-sulphuric acid and the absorbance at 490 nm was measured using an automated Titertek ELISA plate reader.

The method followed for biotinylation of MAbs was a modification of that described earlier (Yolken et al., 1983). Monoclonal IgG antibodies were purified by passing the ascitic fluid through a Protein-A-Sepharose CL-4B column while IgM was then dialysed overnight against 0.1 M-carbonate buffer pH 8.0. N-Hydroxysuccinimidobiotin (Pierce Chemicals, Rockford, Ill., U.S.A.), dissolved in DMSO at a concentration of 1 mg/ml, was added at a ratio of 0.15 mg of the biotin derivative to 1 mg of Ig, and the reaction was allowed to proceed for 4 h at room temperature with constant stirring. To remove free biotin, the mixture was dialysed against 0.01 M-phosphate-buffered saline pH 7.2.

Competitive binding assay (CBA). For competitive binding ELISAs, the competing MAb (in most cases purified IgG) was added in serial 10-fold dilutions to antigen-coated wells and allowed to react for 2 h. The wells were washed and the second biotinylated MAb was added at a predetermined optimal concentration and incubated for 90 min. Percentage binding was calculated on the basis of the absorbance readings in the presence of control ascitic fluid. A decrease in binding to 60% was considered to represent significant competition, and an increase to 200% was considered significant enhancement.

RESULTS

Characterization of MAbs

A summary of the modified nomenclature and characterization of the MAbs is given in Table 1. All MAbs were detected by ELISA with purified intact JE virus. Thus they are considered to be specific for the envelope protein. Of the 16 MAbs (against strain 733913) selected for epitope mapping, nine were HI-positive and seven were HI-negative. Of the nine HI-positive MAbs, four were JE strain-specific (Hs-1 to -4) and five were flavivirus cross-reactive (Hx-1 to -5). Among the seven HI-negative MAbs, two were JE virus-specific (NHs-1 and -2), three were flavivirus cross-reactive (NHx-1 to -3) and the remaining two were designated autoreactive (NHA-1 and -2) since they reacted with the nuclei of infected/uninfected cells of PS, Vero and HDCS cultures in immunofluorescence tests (data not shown) as well as reacting with JE virus in ELISAs.

The titres of the monoclonal ascitic fluids in ELISA varied between 10² to 10¹⁰, and in general the clones could be grouped as high (NHs) or medium (mainly HI-positive) secretors.

The NT activity was determined both in vitro and in vivo. The MAbs that showed NT activity in vitro were all positive in vitro but the reverse was not the case. All Hs MAbs showed high NT
Epitope mapping of JE virus

Table 1. Biological properties of JE MAbs

<table>
<thead>
<tr>
<th>New (and old) nomenclature</th>
<th>ELISA*</th>
<th>HI*</th>
<th>Neutralization*</th>
<th>ADPE*</th>
<th>Passive protection</th>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>100 LD$_{50}$</td>
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<tr>
<td>HI-reactive MAbs</td>
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<tr>
<td>Flavivirus cross-reactive domain A</td>
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<td></td>
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<tr>
<td>Hx-1 (VF6G5)</td>
<td>6.0</td>
<td>4.7</td>
<td>3.82</td>
<td>2+†</td>
<td>5.0</td>
</tr>
<tr>
<td>Hx-2 (VF6E3)</td>
<td>6.0</td>
<td>3.8</td>
<td>3.39</td>
<td>2+</td>
<td>5.7</td>
</tr>
<tr>
<td>Hx-3 (IIB1B7)</td>
<td>5.7</td>
<td>3.8</td>
<td>&lt;1.00</td>
<td>-</td>
<td>5.0</td>
</tr>
<tr>
<td>Hx-4 (VF6G3)</td>
<td>5.4</td>
<td>4.7</td>
<td>ND‡</td>
<td>2+</td>
<td>7.0</td>
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<tr>
<td>Hx-5 (IIB1E7)</td>
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<td>3.8</td>
<td>&lt;1.00</td>
<td>-</td>
<td>1.7</td>
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<td>Hs-1 (VG6H1)</td>
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<tr>
<td>NHA-2 (IH7G6)</td>
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<td>&lt;1.0</td>
<td>&lt;1.0</td>
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</table>

* Titres are expressed as 1/log$_{10}$ endpoint antibody dilution showing signal : noise ratio of >2 in ELISA, inhibition of 8 to 16 HA units in HI test, 50% inhibition of c.p.e. in the in vitro NT test or twofold enhancement in the number of plaques in the ADPE assay.
† 4+, 3+, 2+ and - indicate 80 to 100%, 60 to 80%, 40 to 60% and <40% protection respectively.
‡ ND, Not determined.

Activity both in vivo and in vitro. Of the Hx MAbs, Hx-1, -2 and -4 showed lower NT activity in vivo, and Hx-3 and -5 showed none. NHs-1 and -2, NHx-1 and -3 and NHA-1 showed NT activity only in vitro. In general, ability to inhibit HA was associated with ability to neutralize infectivity in vivo.

Antibody-dependent plaque enhancement (ADPE)

The results of immune enhancement in P388D1 macrophage-like cells have been reported previously (Cecilia & Ghosh, 1988). However they have been included in Table 1 since they are relevant to the analysis. As can be seen, ADPE activity was confined to Hx MAbs.

Passive protection

Protection by monoclonal ascitic fluids was studied against two doses of challenge virus (Table 1). Protection (60 to 100%) was observed against a 100 LD$_{50}$ dose of virus with the administration of Hs-1 to -4 and Hx-2 MAbs, which were neutralizing both in vivo and in vitro. On lowering the dose of virus to 5 LD$_{50}$ Hx-4, NHs-1, NHs-2, NHx-2 and NHX-3 also showed some protection. NHs-1, which has high NT activity in vitro, gave 80 to 100% protection against a low challenge dose.

Competitive binding assay

The results of the reciprocal CBAs carried out with 14 biotinylated MAbs indicated that there could either be inhibition or enhancement of the probing antibody, depending on the epitopes involved. Fig. 1 illustrates this by showing the binding curves of conjugated Hs-1 against several blocking MAbs. Inhibition was either reciprocal, indicative of overlapping sites, or one-way (Fig. 2), indicating adjacency (Yewdell & Gerhard, 1981). Mapping of the epitopes on this
Fig. 1. CBA. Competition binding curves of Hs-1 in the presence of Hs-1 (■), Hs-2 (●), Hs-3 (▲) and Hs-4 (▲) showing inhibition, with Hx-1 (♭), Hx-2 (○), Hx-4 (△) and Hx-5 (□) showing enhancement, and with NHx-3 (○—○) showing no effect.

Fig. 2. Schematic diagram showing results of the CBAs. The interactions between MAbs, i.e. —— reciprocal inhibition, —— one-way inhibition, —→ reciprocal enhancement, and —— one-way enhancement are illustrated. —— indicates that only a one-way reaction has been determined.
basis revealed the presence of two distinct clusters of overlapping and adjacent sites and one isolated epitope (Fig. 3). Each epitope has been represented by a single MAb; the data for other MAbs defining the same epitope have not been included.

Unidirectional and bidirectional enhancement in the binding of the second MAb was observed. Binding of three MAbs, Hs-1 and -2 and NHs-1 was enhanced to a very high degree, almost three- to sixfold by several MAbs, the Hx MAbs being the most effective. Enhancement of binding between MAbs in the same group was observed only in the Hx group.

The clustering of MAbs indicated by the CBA results correlated well with the functional groups assigned on the basis of specificity and HI reactivity. Five functional domains, A, B, C, D and E (Fig. 3), were delineated.

The HI cross-reactive domain (A) was distinct and comprised five epitopes represented by the Hx-1 to -5 MAbs. There was reciprocal inhibition between Hx-1 and -2 and between Hx-3 and each of the other MAbs. There was only one-way inhibition of Hx-2 and Hx-5 by Hx-4.

The HI-specific domain (B) consisting of four epitopes, defined by MAbs Hs-1 to -4, was in continuum with the other domains. These MAbs showed reciprocal blocking, but different epitopes were assigned to them because of a difference in their antibody-binding enhancement characteristics. In addition, Hs-3 was reciprocally blocked by NHs-1 and NHx-2.

The HI-negative JE virus-specific site (C) contained two overlapping epitopes reactive with NHs-1 and NHs-2. The NHs-1 MAb showed reciprocal inhibition with Hs-3.

The non-HI cross-reactive region (D) was represented by three non-overlapping MAbs, NHx-1, -2 and -3. Although there was no internal similarity in the domain, NHx-1 blocked NHs-1 one-way and NHx-2 blocked NHs-1 and Hs-3 reciprocally. The two non-HI cross- and autoreactive epitopes defined by NHA-1 and -2 overlapped and were juxtaposed to the NHx-1 MAb reactive site.
The E protein of flaviviruses, which contains most antigens involved in immunological reactivity (reviewed by Monath, 1985) induces HA-inhibiting, neutralizing and protecting antibodies (reviewed by Roehrig, 1986). Therefore, attempts to identify and characterize epitopes within the E protein that induce a protective response seem justified. Earlier reports on JE virus have distinguished up to eight antigenic determinants on the basis of HI/NT activity, serological specificity and CBAs (Kimura-Kuroda & Yasui, 1983, 1986). Three HI sites were detected, of which the cross-reactive and subgroup-specific sites were distinct. No HI site specific for JE virus was detected. Our results were more in accordance with those of Kobayashi et al. (1985), wherein the JE-specific and flavivirus cross-reactive HI sites were shown to be distinct. We have now revealed additional sites using CBAs, passive protection tests and ADPE assays. In common with other investigators (Heinz, 1986), we have observed extensive overlapping between the sites. Enhancement in the binding of antibodies, both unidirectional and bidirectional, was observed between different groups of MAbs, the Hs being the most enhanceable and the Hx the most enhancing. This has been shown for several flaviviruses including dengue (Henchal et al., 1985), tick-borne encephalitis (TBE) (Heinz et al., 1984) and yellow fever (Schlesinger et al., 1984) viruses. For JE virus, synergism between MAbs was shown in the NT test (Kimura-Kuroda & Yasui, 1983).

Domains assigned to the physical map could also be distinguished on the basis of antigenic specificity, as was previously found for TBE and yellow fever viruses (Heinz et al., 1983; Cammack & Gould, 1986).

All JE strain-specific MAbs (Hs-1 to -4), showed pronounced HI, NT (in vitro and in vivo) and protection activity, but none showed ADPE even at sub-neutralizing concentrations. Thus, binding of antibodies to domain B epitopes could render the virus non-infectious and therefore incapable of showing ADPE. On the other hand, dissociation of HI, NT and protection was seen with domain A epitopes and this has previously been reported by Kimura-Kuroda and Yasui (1983).

A distinction was also observed between the NT activity in vivo and in vitro. The MAbs that showed NT activity exclusively in vitro possibly formed infectious complexes which could be internalized in vivo as suggested by McCullough (1986). This was a characteristic of the non-HI MAbs. Also, in the passive protection test significant protection could be seen mostly with the MAbs that were NT-positive in vitro. Marginal protection seen with the MAbs that were NT-positive only in vitro could have been mediated by complement or antibody-dependent cell cytotoxicity. Passive protection by non-neutralizing MAbs, as shown for yellow fever and dengue 2 viruses (Gould et al., 1986; Brandriss et al., 1986) was not observed in this study.

The NHs MAbs representing domain C showed NT activity only in vitro and conferred protection in the passive protection test only against a very low challenge dose of virus. The D and E regions did not prove to have much functional significance in the tests carried out. The results therefore support the view that, from the biological standpoint, non-HA epitopes occupy less significant positions in the E polypeptide than HA epitopes.

On the whole, domain B has the most significant role in inducing a protective and hence beneficial response in the host. It is perhaps of most importance that these epitopes did not function in ADPE. If such domains could be identified with other flaviviruses, particularly dengue viruses, it might be possible to develop vaccines that do not run the risk of inducing immune enhancement, as seen with dengue and yellow fever viruses (Halstead et al., 1973; Scott et al., 1983; Barrett & Gould, 1986; Gould et al., 1987).

We have recently observed in HI tests that domain B MAbs failed to react with six out of 24 JE strains taken from different geographical locations, suggesting that the domain is either masked or absent in these strains (data not shown). It is noteworthy that the B domain was absent from the JE Nakayama-Biken vaccine strain but present in another Nakayama strain obtained from the Rockefeller Foundation. Differences in the immunological characteristics of various Nakayama substrains have been reported (Kobayashi et al., 1984) and we are currently looking into the significance of our observations to see if protection is affected by these changes.

In conclusion, there does appear to be an antigenic domain within the viral envelope protein of JE virus that has all the attributes required of a vaccine.
REFERENCES


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