Synergistic Interactions of Anti-NS1 Monoclonal Antibodies Protect Passively Immunized Mice from Lethal Challenge with Dengue 2 Virus

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(Accepted 25 March 1988)

SUMMARY

Non-neutralizing, serotype-specific anti-NS1 monoclonal antibodies partially protected passively immunized mice from lethal dengue 2 virus intracerebral challenge. There was no apparent correlation between complement-fixing activity and protective capacity among individual anti-NS1 monoclonal antibodies. Immunization with specific combinations of non-protective or partially protective antibodies resulted in prolonged survival or reduced mortality. Solid protection, equal to that achieved after immunization with neutralizing polyclonal antibody, was achieved only with an antibody pair which individually fixed complement to high titre with homologous virus. Some groups of mice had increased morbidity after immunization with combinations of protective monoclonal antibodies that bind to overlapping epitopes. These results may affect the design of recombinant dengue vaccines which may require the inclusion of serotype-specific antigenic domains.

Previous studies have established the importance of the humoral response for survival after lethal flavivirus challenge (Monath, 1986). However, the functional characteristics of the antibodies required for protection remain unclear. While neutralizing antibodies against the envelope (E) glycoprotein have been regularly associated with immunity to flavivirus infections, a lack of correlation between protection and heterologous neutralizing antibody levels has been reported (Wisseman et al., 1966; Whitehead et al., 1970; Halstead et al., 1973; Price et al., 1973; Porterfield, 1980). Neutralizing and non-neutralizing mouse monoclonal antibodies (MAbs) prepared against flavivirus E glycoproteins have been used to protect mice from lethal challenge (Heinz et al., 1983; Henchal et al., 1984; Mathews & Roehrig, 1984; Brandriss et al., 1986; Gould et al., 1986). Similar results have now been reported for non-neutralizing MAbs directed against the yellow fever virus non-structural glycoprotein NS1 found on the surface of flavivirus-infected cells (Schlesinger et al., 1985; Gould et al., 1986). In one study, protection appeared to correlate with the complement-fixing activity of the MAbs (Schlesinger et al., 1985). The protective capacity of anti-NS1 antibody and the presence of substantial amounts of similar antibody in convalescent sera from dengue fever and dengue haemorrhagic fever patients (Falker et al., 1973; W. E. Brandt, personal communication; E. A. Henchal & J. J. Schlesinger, unpublished data) suggest a role for complement-fixing, anti-NS1 antibodies in the protective immune response to dengue virus infections.

We previously reported the preparation of MAbs against a large serotype-specific antigenic domain (five epitopes) which overlaps with a cross-reactive epitope on the dengue 2 virus (DEN-2) NS1 protein (Henchal et al., 1987). These data were consistent with results presented by others (Russell et al., 1970; Qureshi & Trent, 1973) that suggested the presence of serotype-
specific antigenic determinants on this non-structural antigen. In the present study we report the functional characteristics of these MAbs in mouse passive protection assays and show that a combination of two MAbs exerts a synergistic protective effect equal to that of polyclonal antibody.

The preparation of MAbs against Thai DEN-2 isolates was described previously (Henchal et al., 1987). Thai DEN-2 virus strains, D80-100 and D80-141, were isolated in LLC-MK₂ (monkey kidney) cells from the serum of dengue haemorrhagic fever patients treated at Bangkok Children's Hospital in 1980 (Henchal et al., 1987), and passaged in C6/36 (Aedes albopictus) cells and BALB/c suckling mice (Henchal et al., 1982). Six-week-old female BALB/c mice were immunized with viral antigens in 20% (w/w) suckling mouse brain homogenates in phosphate-buffered saline pH 7.4 (PBS), and immune splenocytes were fused with the P3-X63-Ag8.653 mouse myeloma cell line as described by Gentry et al. (1982). Selected hybridomas producing non-neutralizing MAbs were subcloned to homogeneity using the limiting dilution method (Hengartner et al., 1978). Hybridoma 6A8 was prepared using BALB/c mice immunized with purified DEN-2 NS1 protein (Schlesinger et al., 1987). High titre mouse ascitic fluids were produced in pristane-primed BALB/c mice (Kennett et al., 1978). The protein concentration of MAbs in mouse ascitic fluids was estimated by electrophoretic analysis on cellulose acetate membranes using the method of Brandriss et al. (1986). Control normal mouse ascitic fluids (NMAF) and neutralizing DEN-2 hyperimmune mouse ascitic fluids (DEN-2 HMAF) were prepared using standard methods (Brandt et al., 1967).

The serological and functional characteristics of the MAbs were determined and are summarized in Table 1. The isotype of each MAb preparation was identified using the competitive blocking assay described previously (Gentry et al., 1982) or by Ouchterlony double diffusion assay. Solid-phase radioimmuno assay (RIA) using D80-100 or New Guinea C DEN-2 virus antigen was performed as described previously (Zollinger et al., 1976; Henchal et al., 1987). RIA antigen was prepared by homogenizing infected or uninfected C6/36 cells in 0.5% Triton X-100, 0.05 M-NaCl, 0.02 M-Tris-HCl pH 8.0, 5 µg/ml PMSF. The concentration of clarified antigen preparations was optimized using DEN-2 HMAF (Gentry et al., 1982). The Laboratory Branch complement fixation (CF) test (Centers for Disease Control, 1981) adapted for microtitre plates was performed using sucrose-acetone-extracted suckling mouse brain antigen (Clarke & Casals, 1958). This antigen was used in RIA at a 1:10 dilution. The virus and viral protein specificities of each preparation were identified previously by indirect immunofluorescence and radioimmune precipitation assays (Henchal et al., 1987).

Although most of the antibody preparations belonged to the IgG1 subclass, 40-21/9, 47-10/10 and 6A8 were identified as IgG2a, IgG2b and IgM respectively. The IgG antibody preparations had RIA endpoint titres in the range 6-0 to 7-6 against D80-100 and New Guinea C antigens. However, two preparations, 16-25/3 and 47-10/10, had significantly lower RIA titres (1000-fold decrease) against the challenge neurotropic DEN-2 New Guinea C virus. Monoclonal antibody 47-10/10 also had decreased reactivity with a minor genetic topotype of DEN-2 strains circulating at the same time in Bangkok during 1980 (Walker et al., 1988). Four MAbs had marginal or low CF titres against homologous virus, but one (68-5/16) fixed complement to high titre (>10⁵-⁶). Although most of the preparations lacked a CF titre all were able to react with sucrose-acetone-extracted DEN-2 (New Guinea C) mouse brain antigen by RIA. Consistent with previous results, MAbs 16-25/3 and 47-10/10 lost CF reactivity when tested against New Guinea C virus antigen. Nine of the ten MAbs reacted specifically with DEN-2 viruses, but 27-12/4 cross-reacted with Japanese encephalitis virus antigens.

To evaluate the protective capacity of anti-NS1 MAbs, mouse passive protection assays were conducted using the guidance provided by Smith & Westgarth (1956). This statistical method has the advantage of evaluating the protective capacity of antibody preparations which may not completely protect all challenged mice. A neurotropic strain of the prototype New Guinea C virus was used as the challenge virus in passive protection assays, since low passage Thai DEN-2 strains were not lethal for adult mice. Tenfold dilutions of a reference DEN-2 (New Guinea C) mouse brain stock, passage 34, were titrated by intracerebral (i.c.) inoculation (0.02 ml per mouse) of 20- to 23-day-old BALB/c mice. Mice were observed daily for morbidity for 21 days.
Table 1. Serological characteristics of anti-NS1 monoclonal antibodies

<table>
<thead>
<tr>
<th>MAb*</th>
<th>Isotype</th>
<th>RIA endpoint titre†</th>
<th>CF titre (log_{10})</th>
<th>Virus specificity‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>16-25/3</td>
<td>IgG1</td>
<td>7.0</td>
<td>4.0</td>
<td>0.9</td>
</tr>
<tr>
<td>20-1/1</td>
<td>IgG1</td>
<td>7.4</td>
<td>6.6</td>
<td>&lt;0.9</td>
</tr>
<tr>
<td>27-12/4</td>
<td>IgG1</td>
<td>6.7</td>
<td>6.7</td>
<td>0.9</td>
</tr>
<tr>
<td>34-23</td>
<td>IgG1</td>
<td>7.0</td>
<td>6.7</td>
<td>&lt;0.9</td>
</tr>
<tr>
<td>40-21/9</td>
<td>IgG2b</td>
<td>6.9</td>
<td>6.7</td>
<td>&lt;0.9</td>
</tr>
<tr>
<td>47-10/10</td>
<td>IgG2a</td>
<td>7.1</td>
<td>3.8</td>
<td>1.2</td>
</tr>
<tr>
<td>63-15</td>
<td>IgG1</td>
<td>6.7</td>
<td>6.0</td>
<td>0.9</td>
</tr>
<tr>
<td>68-5/16</td>
<td>IgG1</td>
<td>6.9</td>
<td>6.0</td>
<td>&gt;3.3</td>
</tr>
<tr>
<td>101-4/7</td>
<td>IgG1</td>
<td>7.6</td>
<td>6.5</td>
<td>&lt;0.9</td>
</tr>
<tr>
<td>6A8</td>
<td>IgM</td>
<td>ND§</td>
<td>ND</td>
<td>&lt;0.9</td>
</tr>
</tbody>
</table>

* All MAbs, except 101-4/7 and 6A8, were produced using D80-100 antigen. D80-141 antigen was used to produce 101-4/7 and New Guinea C antigen was used to produce 6A8.
† The reciprocal of the highest dilution that resulted in binding equal to 1.5 times the c.p.m. bound by a similar dilution of NMAF was taken as the RIA endpoint.
‡ Adapted from Henchal et al. (1987). JEV, Japanese encephalitis virus.
§ ND, Not determined.

Individuals dying before day 4 were disregarded, and mice paralysed on day 21 were regarded as having died on that day. The LD_{50} was calculated using the method described by Reed & Muench (1938). Test animals were passively immunized with 0.5 ml of ascitic fluid by the intraperitoneal route. After 24 h mice were challenged (i.c.) with 100 LD_{50} of the reference virus. Mice were observed as before. The survival time index (STI) was calculated for each group using the following formula:

$$STI = 1000/K \sum_{i=1}^{K} 1/t_i$$

where K is the number of mice in the group and t_i is the number of days survived by the mouse no. i. Survivors were regarded as dying on day 42 so that the minimum STI value (23.8) was obtained only when the whole group survived. Using the statistical methods described by Smith & Westgarth (1956), a MAb preparation was considered protective if the STI value was below 56.

Only the neutralizing, polyvalent antibody preparation, DEN-2 HMAF, conferred solid protection against lethal DEN-2 challenge. However, several MAb preparations provided partial protection. After passive immunization with NMAF, challenged animals had an average (mean ± S.D.) day of death of 12 ± 1.2 and an STI value (which accounts for the day of death of each animal) of 77.8. In comparison, mice inoculated with MAbs 63-15 or 68-5/16 had increased survival and lower STI values (50.8 and 43.0). The best survival rates (93% to 50%) were obtained with MAbs 6A8, 63-15 or 68-5/16. Mice immunized with some MAbs had increased (79 to 87) but not significantly (P > 0.025) different STI values relative to NMAF (78).

To determine whether combinations of anti-NS1 MAbs that bind to the same antigenic domain could be used to provide the protective capacity of polyclonal antisera, MAbs which define six different NS1 epitopes (Henchal et al., 1987) were paired in aliquots and used to immunize mice passively, as described above. Generally, survival rates remained unchanged or STI values increased when MAb preparations were combined (Table 2). However, survival rates significantly improved and STI values decreased when MAb 27-12/4 was combined with either 16-25/3, 20-1/1 or 47-10/10. These were not protective when used individually. Monoclonal antibody 68-5/16 combined with 47-10/10 conferred solid protection similar to that provided by polyclonal mouse ascitic fluids. This was contrary to results obtained with 68-5/16 combined with 63-15, in which case the survival rate decreased and the STI value increased.

It has been suggested that protection by anti-NS1 MAbs correlates with their ability to promote complement-mediated cytolysis of infected target cells (Schlesinger et al., 1985). To determine whether the same was true for the present antibodies, each was examined...
individually for complement-mediated cytolytic activity as measured by $^{51}$Cr release from labelled DEN-2-infected mouse neuroblastoma (Neuro 2A) cells. Complement-dependent, antibody-mediated cytotoxicity assays using New Guinea C and D80-100 DEN-2 virus-infected Neuro 2A target cells were performed as described previously (Schlesinger et al., 1985). The preparation of control rabbit antiserum to DEN-2 NS1 protein has been described before (Schlesinger et al., 1987). Heat-inactivated antisera and MAbs at 1/20 and 1/100 final dilutions, respectively, were tested in quadruplicate using rabbit complement. While control DEN-2 HMAF and monospecific rabbit anti-dengue NS1 antisera were cytolytic (specific $^{51}$Cr release was 23% and 24% respectively), none of the MAbs demonstrated specific cytolysis.

The possibility that all of the epitopes defined by anti-NS1 MAbs might not be expressed on the surface of infected cells was tested by reacting antibody preparations with unfixed, infected LLC-MK₂ cells in indirect immunofluorescence assays. LLC-MK₂ cells infected for 7 days with D80-100 virus were reacted in suspension ($1 \times 10^6$ cells per 130 μl) for 1 h on ice with each MAb diluted 1/80 in Hanks' balanced salt solution (HBSS). Cells were washed extensively with HBSS and reacted for 1 h on ice with a 1/50 dilution of fluorescein isothiocyanate–goat anti-mouse IgG. After extensive washing with HBSS, cells were fixed with 1% paraformaldehyde in PBS and evaluated visually with a u.v. microscope. Acetone-fixed slide preparations were used as controls. All of the MAb preparations were able to react with NS1 antigen expressed on the surface of infected cells. No differences could be detected in the immunofluorescence reactions between unfixed and acetone-fixed cell preparations.

We have shown that mice passively immunized with selected serotype-specific anti-NS1 monoclonal antibodies were protected from lethal DEN-2 i.c. challenge. Moreover, specific combinations of antibodies interacted cooperatively to increase survival. The protective capacity of one pair of MAbs (68-5/16 and 47-10/10) equalled that of neutralizing polyclonal DEN-2 HMAF. Although the ability of individual anti-NS1 antibodies to protect did not appear to correlate with their isotype or CF activity, it is of interest that complete protection was achieved only with a pair of MAbs that individually fixed complement to high titre with homologous virus.

<table>
<thead>
<tr>
<th>Antibody preparation</th>
<th>Dose (mg/mouse)</th>
<th>Mortality (survivors/total)</th>
<th>STI*</th>
<th>Protection</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMAF‡</td>
<td>4.3</td>
<td>2/21</td>
<td>77.8</td>
<td>—</td>
</tr>
<tr>
<td>DEN-2 HMAF</td>
<td>ND‡</td>
<td>12/12</td>
<td>23.8</td>
<td>+</td>
</tr>
<tr>
<td>16-25/3</td>
<td>10.3</td>
<td>1/12</td>
<td>82.8</td>
<td>—</td>
</tr>
<tr>
<td>20-1/1</td>
<td>13.4</td>
<td>0/10</td>
<td>84.6</td>
<td>—</td>
</tr>
<tr>
<td>27-12/4</td>
<td>4.8</td>
<td>2/11</td>
<td>67.2</td>
<td>—</td>
</tr>
<tr>
<td>34-23</td>
<td>2.9</td>
<td>0/12</td>
<td>86.7</td>
<td>—</td>
</tr>
<tr>
<td>40-21/9</td>
<td>1.9</td>
<td>0/10</td>
<td>78.7</td>
<td>—</td>
</tr>
<tr>
<td>47-10/10</td>
<td>5.3</td>
<td>0/12</td>
<td>82.5</td>
<td>—</td>
</tr>
<tr>
<td>63-15</td>
<td>6.4</td>
<td>5/10</td>
<td>50.8</td>
<td>+</td>
</tr>
<tr>
<td>68-5/16</td>
<td>16.8</td>
<td>6/11</td>
<td>43.0</td>
<td>+</td>
</tr>
<tr>
<td>101-4/7</td>
<td>4.6</td>
<td>0/11</td>
<td>83.5</td>
<td>—</td>
</tr>
<tr>
<td>6A8</td>
<td>1.5</td>
<td>14/15</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>27-12/4, 16-25/3</td>
<td>7.6§</td>
<td>4/6</td>
<td>38.9</td>
<td>+</td>
</tr>
<tr>
<td>27-12/4, 20-1/1</td>
<td>9.1</td>
<td>5/6</td>
<td>27.8</td>
<td>+</td>
</tr>
<tr>
<td>27-12, 47-10/10</td>
<td>5.1</td>
<td>2/6</td>
<td>52.4</td>
<td>+</td>
</tr>
<tr>
<td>68-5/16, 47-10/10</td>
<td>11.1</td>
<td>15/15</td>
<td>23.8</td>
<td>+</td>
</tr>
<tr>
<td>68-5/16, 63-15</td>
<td>11.6</td>
<td>2/11</td>
<td>68.9</td>
<td>—</td>
</tr>
<tr>
<td>63-15, 47-10/10</td>
<td>5.9</td>
<td>4/12</td>
<td>53.8</td>
<td>+</td>
</tr>
</tbody>
</table>

* STI, survival time index.
† Average day of death (mean ± S.D.) for NMAF-treated animals was 12.1 ± 1.2.
‡ ND, Not determined.
§ Combined dose of anti-dengue virus antibodies.
Since the antibody preparations were used in passive protection assays at the highest concentration available and not standardized to a single quantitative amount of antibody, it is conceivable that some preparations were not at a concentration sufficient to increase the survival of lethally challenged mice. While the Smith & Westgarth (1956) method provides for the evaluation of antibody preparations which do not completely protect challenged mice, it cannot be used quantitatively to compare the protective capacity of the individual antibody preparations used in this study.

Other investigators have suggested that some non-neutralizing antibodies afford protection by sensitizing infected cells to complement-mediated cytolysis (Schmaljohn et al., 1982; Boere et al., 1985). Results obtained with antibodies to the yellow fever virus NS1 glycoprotein, which is expressed on the surface of infected cells, were consistent with this model (Schlesinger et al., 1985). Failure of the present protective anti-DEN-2 NS1 MAbs individually to sensitize infected target cells to complement-mediated cytolysis suggests that other mechanisms may be involved in immune recognition of NS1 on the surface of flavivirus-infected cells.

The failure of MAbs 16-25/5 and 47-10/10 individually to protect challenged mice may have been related to their decreased reactivity with heterologous mouse neurotropic New Guinea C virus. However, these MAbs were able to increase the survival of challenged mice when combined with another non-protective antibody, 27-12/4. While it is possible that, when combined, these MAbs may have enhanced binding as has been found with some anti-E MAbs (Henchal et al., 1983), these effects were not demonstrated in competitive binding assays using solubilized antigen (Henchal et al., 1987). The paradoxical increase in morbidity when protective MAbs 68-5/16 and 63-15 were combined may be related to their mutual competition for overlapping binding sites (Henchal et al., 1987) and unknown dynamic changes in antigen conformation.

The ability of the combination of 68-5/16 and 47-10/10 to provide the protective effects of neutralizing polyclonal HMAF suggests that a unique configuration of antigen and antibody may be required for protection. Although their antibody-binding sites appear to be contiguous, the MAbs were not mutually competitive, and enhanced binding was not observed with solubilized antigen (Henchal et al., 1987). It is remarkable that even though the reactivity of 47-10/10 is 1000-fold less for the neurotropic challenge virus strain than for the immunizing strain (Table 1), combination with 68-5/16 conferred solid protection. Similar effects were not observed when MAb 47-10/10 was combined with 63-15, a protective antibody that binds to a non-contiguous binding site (Henchal et al., 1987).

Since the NS1 protein has not been implicated in the dengue immune enhancement phenomenon, protective determinants on this antigen might be important additions to the recombinant dengue vaccines currently being developed. In fact, mice were partially protected from lethal DEN-2 challenge in the absence of detectable neutralizing antibody after immunization with purified DEN-2 NS1 containing antigenic sites common to all four dengue virus serotypes (Schlesinger et al., 1987). However, cross-protection against heterologous virus was not observed, and immunized mice developed little or no CF antibody against other dengue serotypes. These data suggest that protective NS1 epitopes from all four serotypes may have to be included in a possible future recombinant dengue vaccine. The MAbs presented in this study may be useful probes for the definition of NS1 antigenic determinants that induce a protective response.

The authors wish to thank Ms Julia Charles and Mr Ming Choohong for their assistance with the animal studies, and Dr Bruce L. Innis for his generous review of the draft manuscript. J. J. S. was funded in part by a grant from the Rockefeller Foundation, GAHS8646, and the WHO, V22/181/8.

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(Received 16 December 1987)