Analysis of 17D Yellow Fever Virus Envelope Protein Epitopes Using Monoclonal Antibodies

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SUMMARY

Sixteen monoclonal antibodies that reacted with the envelope glycoprotein (E) of 17D vaccine strain yellow fever virus (17D YF), including two antibodies produced against dengue 2 virus, were used in a solid phase competitive binding assay (CBA) to define spatial relationships among antigenic determinants on 17D YF E. The antibodies showed YF strain, type or flavivirus group specificities and nine epitopes were identified on 17D YF E by patterns of neutralization, haemagglutination inhibition and competition of antibody binding. Epitopes defined by neutralizing antibodies with strain and type specificities appeared spatially distant but competition between type-specific neutralizing antibodies and some non-neutralizing antibodies against type and group determinants suggested close proximity among epitopes in these regions. Despite competition between some neutralizing and non-neutralizing monoclonal antibodies in CBA, plaque assays revealed no interference with neutralization by non-neutralizing antibody.

INTRODUCTION

Current knowledge of flavivirus structure indicates that the envelope of each of the 60 members of this genus contains multiple copies of a single glycoprotein, E (53 to 59 kilodaltons), which subserves both haemagglutination and neutralization. Although earlier studies partially defined antigenic relationships among various flaviviruses (Clarke, 1960; Madrid & Porterfield, 1974) and demonstrated the presence of type, complex and group-specific antigenic determinants on E (Trent, 1977), knowledge of the structural basis for the remarkable heterogeneity among these viruses is incomplete. Recently, the availability of monoclonal antibodies has enabled more detailed analysis of flavivirus structure and has helped to define further the antigenic relationships within the genus (Gentry et al., 1982; Peiris et al., 1982; Schlesinger et al., 1983; Kimura-Kiroda & Yasui, 1983; Roehrig et al., 1983; Heinz et al., 1983).

We have reported serological reactivity patterns among monoclonal antibodies against 17D vaccine strain yellow fever virus (17D YF; Schlesinger et al., 1983), a strain unique among flaviviruses for its use as a highly effective vaccine. At least five distinct epitopes were defined on 17D YF E. In the present study we employed a solid phase competition antibody assay to analyse their topographical distribution and to identify additional ones.

METHODS

Virus and cells. Avian leukemia virus-free 17D YF (lot no. 2091, Connaught Laboratories) was passed in Vero cells maintained in MEM supplemented with 5% foetal calf serum. A single virus pool containing $5 \times 10^6$ p.f.u./ml was used for neutralization assays. Virus was purified from infected culture fluids by polyethylene glycol 6000 precipitation followed by sequential centrifugation in discontinuous and linear sucrose gradients (Trent & Qureshi, 1971). Virus protein concentration was determined with a Bio-Rad protein determination kit.

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Table 1. Serological characteristics of monoclonal antibodies reactive with 17D yellow fever virus (17D YF) envelope glycoprotein (E)

<table>
<thead>
<tr>
<th>Inducing virus</th>
<th>Group</th>
<th>Number of hybridomas</th>
<th>Flavivirus specificity</th>
<th>Neutralizes 17D YF</th>
<th>Neutralizes Asibi YF</th>
</tr>
</thead>
<tbody>
<tr>
<td>17D YF</td>
<td>A</td>
<td>1</td>
<td>17D YF strain</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>3</td>
<td>YF type</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>5</td>
<td>YF type</td>
<td>-</td>
<td>+</td>
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<tr>
<td></td>
<td>D</td>
<td>4</td>
<td>Group*</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>1</td>
<td>YF type</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Dengue 2</td>
<td></td>
<td>2</td>
<td>Group†</td>
<td>-</td>
<td>ND‡</td>
</tr>
</tbody>
</table>

* Haemagglutination inhibition against Zika, Banzi and/or dengue 2.
† Haemagglutination inhibition against 17D YF, Asibi YF and Japanese encephalitis virus.
‡ ND, Not done.

Monoclonal antibodies. The production and characterization of mouse monoclonal antibodies to 17D YF and to dengue 2 virus (DEN 2) have been described previously (Schlesinger et al., 1983; Gentry et al., 1982). Serological properties of 13 IgG and one IgM monoclonal antibodies against 17D YF E and two DEN 2 monoclonal antibodies against DEN 2 and 17D YF E are summarized in Table 1. An IgG2a monoclonal antibody, 1A5, directed against the non-structural yellow fever virus-specified protein NV3 (Schlesinger et al., 1983) and an IgG2a monoclonal antibody against respiratory syncytial virus were used as controls. All IgG antibodies against 17D YF E were of IgG2a isotype except 5E6 (IgG2b) and 3A3 (IgG1). Monoclonal antibody-rich ascitic fluids (MIAF) were prepared by intraperitoneal injection of pristane-primed syngeneic BALB/c mice with cloned hybridoma cells and a single pool of each ascitic fluid was used. The concentration of monoclonal antibodies in the ascitic fluids was determined by electrophoresis on cellulose acetate membranes.

Purification and radiolabelling of monoclonal antibodies. IgG monoclonal antibodies were purified by Protein A-Sepharose CL-4B (Pharmacia) affinity chromatography (Ey et al., 1978). Fractions were examined by Ouchterlony double gel diffusion using goat antisera (Meloy & Litton Bionetics) with heavy chain specificities. With this method the fractions used contained no detectable immunoglobulin of another subclass. IgM monoclonal antibody 8A3 was purified either by gel filtration on Sephadex G200 or by affinity chromatography using heavy chain-specific goat IgG anti-mouse IgM linked to Sepharose 4B. Purified monoclonal antibody (100 to 300 μg) was radiolabelled by the IODO-GEN technique (Fraker & Speck, 1978). Radiolabelled antibody was separated from unreacted 125I on a Sephadex G50 column. At least 90% of the radioactivity in these preparations was precipitable by TCA and purity of each of the radiolabelled monoclonal antibodies was established by SDS-PAGE (Laemmli, 1970). Monoclonal antibodies were iodinated to a specific activity of 2 to 5 μCi/μg protein.

Antibody binding and competition solid phase radioimmunoassays. Relative binding avidities and competition among the monoclonal antibodies were determined by modification of previously described methods (Frankel & Gerhard, 1979; Stone & Nowinski, 1980). Purified virus (0.02 μg) in 50 μl of bicarbonate coating buffer pH 9.9 was adsorbed (4°C, 18 h) onto the wells of a single lot of polyvinyl Costar micro-test plates. Empty wells were refilled with 200 μl 5% bovine serum albumin in phosphate-buffered saline (PBS-BSA) and incubated for 1 h at 37°C to block further non-specific protein adsorption. For the antibody binding assay, 50 μl MIAF serially diluted in PBS-BSA-Tw were added to duplicate virus-adsorbed wells and incubated for 4 h at 37°C. The amount of antibody was determined from the known concentration in the MIAF. Wells were washed five times with cold PBS-BSA-Tw to remove unbound immunoglobulin and 125I-labelled Protein A (IPA, New England Nuclear, sp. act. 8-68 μCi/μg), 2 × 10⁵ c.p.m. in 50 μl PBS-BSA-Tw was added to each well for 1 h at 37°C. Unbound IPA was removed by washing five times with cold PBS-BSA-Tw and radioactivity bound to separated wells was measured in a gamma counter. To determine binding of IgM antibody, 125I-labelled goat IgG anti-mouse IgM with heavy chain specificity (Cappel Laboratories, Cochranville, Pa., U.S.A.) was used instead of IPA. Competition among monoclonal antibodies for binding to virus attached to the solid phase was determined by modifying the antibody binding assay. 125I-labelled monoclonal antibody, 2 × 10⁵ c.p.m., was added to dilutions of competitor MIAF in PBS-BSA-Tw and 50 μl of the mixtures added to duplicate BSA-blocked wells containing attached virus. Sealed wells were incubated for 4 h at 37°C, washed five times with cold PBS-BSA-Tw, and bound radioactivity measured. The percentage competition was determined from the ratio of 125I-labelled monoclonal antibody c.p.m. bound in the presence and absence of unlabelled anti-E antibody. Monoclonal antibody 1A5 was used as a negative control. The unlabelled homologous antibody was included as a positive control.

Neutralization assays. Neutralization titres were determined by a previously described plaque reduction method in Vero cells (Shope & Sather, 1979).
RESULTS

Solid phase radioimmunoassays

The binding characteristics of 13 monoclonal antibodies against 17D YF envelope protein are presented in Fig. 1. Binding avidities of the three IgG antibodies that neutralized 17D YF (group B; Fig. 1a) were similar to each other and were considerably lower than several antibodies in groups C and D that did not neutralize 17D YF so that no correlation could be made between neutralizing capacity and avidity. The apparent absence of binding of 3A3, the only IgG1 antibody (Fig. 1b), probably reflects low affinity of staphylococcal Protein A for IgG1 antibodies. At least five antibodies exhibited a prozone effect at the highest antibody concentrations. Because the DEN 2 monoclonal antibodies were prepared using a myeloma protein-secreting cell line (Gentry et al., 1982) accurate determinations of monoclonal antibody concentration in MIAF were not feasible with the method used and their avidities were not determined. Binding studies with the only IgM antibody, 8A3, were attempted using hybridoma supernatants, ascitic fluids and antibody purified by gel filtration and affinity chromatography.
Fig. 2. Enhancement of binding of $^{125}$I-labelled 17D YF-neutralizing monoclonal antibodies. $^{125}$I-labelled 17D YF-neutralizing monoclonal antibody (2 × 10^5 c.p.m.) in the presence of increasing dilutions of non-neutralizing monoclonal antibody 5H3 was added to 17D YF virions attached to the solid phase. $^{125}$I-labelled antibodies used were: 2E10 (○); 2C9 (▲); 4E8 (■). Results of a representative experiment performed in duplicate are shown.

Table 2. Competitive binding assay: 17D YF neutralizing hybridomas*

<table>
<thead>
<tr>
<th>Unlabelled competitor monoclonal antibody and group</th>
<th>Dengue 2</th>
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<tbody>
<tr>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>125I-labelled group B monoclonal antibodies</td>
<td></td>
</tr>
<tr>
<td>4E8</td>
<td>2E10</td>
</tr>
<tr>
<td>2C9</td>
<td>2B8</td>
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<tr>
<td>2E10</td>
<td></td>
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<td>2C9</td>
<td></td>
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<tr>
<td>2E10</td>
<td></td>
</tr>
</tbody>
</table>

* Competitive binding is defined as follows: (−) < 10% competition; (+) >75% competition; (↑) >50% increased binding; (↑↑) >100% increased binding.

All these preparations demonstrated non-specific adsorption under the assay conditions so that relative avidity could not be measured. Attempts to block non-specific adsorption by high concentrations of horse or calf sera or by MOPC 104E, a mouse IgM myeloma protein (which itself gave relatively little non-specific adsorption), were unsuccessful.

To examine the topographical relationship between epitopes that subserve neutralization of 17D YF and those which do not, competitive interference with the binding of the three labelled 17D YF-neutralizing IgG antibodies was measured. Unlabelled competitor MIAF were used at a dilution of 1:100 which corresponded to a concentration on the plateaus of their avidity curves (Fig. 1). Results are presented in Table 2. Homologous and cross-competition of at least 90% was seen among the three 17D YF-neutralizing monoclonal antibodies. Of the other 13 monoclonal antibodies tested, six competed with the three 17D YF-neutralizing antibodies (78 to 98%
Table 3. Competitive binding assay: flavivirus group-specific hybridomas*

<table>
<thead>
<tr>
<th>Unlabelled competitor monoclonal antibody and group</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>Dengue 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>125I-labelled flavivirus group-specific monoclonal antibody</td>
<td>8A3</td>
<td>4E8</td>
<td>2C9</td>
<td>2E10</td>
<td>2B8</td>
<td>3A3</td>
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<tr>
<td>4E11</td>
<td>-</td>
<td>±</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>5H3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4G2</td>
<td>-</td>
<td>±</td>
<td>±</td>
<td>-</td>
<td>-</td>
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</tr>
</tbody>
</table>

* Competitive binding is defined as follows: (-) < 10% competition; (+) 35 to 60% competition; (++) > 60% competition; (T) 50 to 100% increased binding; (T+) 110 to 265% increased binding.

inhibition of binding). These included three of the six YF type-specific antibodies that did not neutralize 17D YF (groups C and E), both DEN 2 flavivirus group-reactive antibodies and one of the four YF flavivirus group-reactive antibodies (group D). The group-reactive monoclonal antibody 5H3 augmented binding of two of the three 17D YF-neutralizing antibodies by 50 to 120% (Fig. 2). Group A 17D YF strain-specific antibody 8A3 did not compete with any group B antibody but reciprocal assays could not be evaluated because of high background non-specific adsorption of radiolabelled antibody 8A3.

Competitive binding assays among the group-reactive antibodies were limited because iodination of two of them (5E5 and 5E6) resulted in loss of specific binding activity. The results using the other two YF group-reactive and one of the DEN 2 group-reactive antibodies (4G2) which were successfully labelled are presented in Table 3. All of the group-reactive antibodies competed with the three labelled ones, with the exception of 5E5, which competed with none of them. The three 17D YF-neutralizing antibodies gave different patterns, which were similar to the results of the reciprocal experiments reported in Table 1. There was no competition with labelled 4E11, there was augmentation of binding of 5H3 (50 to 65%) and there was competition with binding of 4G2 (38 to 57%), although it was not as pronounced as the reciprocal 4G2 competition with the neutralizing antibodies (93 to 98%). The two YF type-specific antibodies that did not neutralize 17D YF but did compete with the neutralizing antibodies in the solid phase assay (2B8 and 3A3; group C) augmented the binding of the labelled group-reactive antibodies in five of six combinations (Table 3). Iodination of group C monoclonal antibodies resulted in loss of specific binding activity of each of the five members of this group.

Neutralization assays

The capacity of heat-inactivated yellow fever strain and type-specific MIAF to neutralize 17D YF was measured in the presence and absence of low dilutions of each of the non-neutralizing MIAF directed at 17D YF E. No interference with neutralization by non-neutralizing antibody was observed.

DISCUSSION

Previous studies utilizing monoclonal antibodies have identified strain, type, complex and flavivirus group-specific serological activities associated with up to eight distinct antigenic determinants or epitopes on the flavivirus envelope protein. In general, most of these epitopes have been partially linked and have either clustered into antigenic domains on Japanese encephalitis (JEV), tick-borne encephalitis (TBE) and DEN 2 viruses (Kimura-Kirotada & Yasui, 1983; Heinz et al., 1983) or have formed a continuum of overlapping domains on St. Louis encephalitis virus (SLE) (Roehrig et al., 1983) as defined by solid phase competitive binding assays (CBA). In some instances the biological activities of the monoclonal antibodies, such as neutralization and haemagglutination inhibition capacities, have segregated and been directed to distinct antigenic sites on E (JEV, DEN 2) whereas in others (SLE, TBE) no such relationship
has been apparent. Single flavivirus group-reactive sites have been identified for JEV, TBE and DEN 2 and two overlapping group-reactive sites for SLE.

In the present study, we were able to define eight antigenic sites on the envelope protein of 17D YF by a combination of serological specificities, biological activities, and competitive binding assays using monoclonal antibodies produced to the virus. An additional site was identified by two monoclonal antibodies produced to DEN 2 and four different group-reactive sites were identified in all. We assume that competition between antibodies in the solid phase assay reflects steric relationships between epitopes although we realize that this may not be the only explanation for the effect. We were not able to perform bidirectional competitive binding assays with all combinations of antibodies because some could not be iodinated without loss of specific activity so that our knowledge of the spatial arrangement of all these epitopes, although fairly extensive, is not complete. However, the only three IgG antibodies that neutralized 17D YF could be labelled. These antibodies were all type-specific and showed complete reciprocal competition with each other in CBA and so appear to be directed toward a single antigenic site. By CBA this site overlapped with sites defined by two of five YF type-specific (group C), one of four YF group-reactive antibodies (group D), and the two DEN 2 group-reactive antibodies. That the latter two did not compete with high titted type-specific neutralizing monoclonal antibodies to DEN 2 in CBA using DEN 2 antigen (Dr E. Henchal, personal communication) may illustrate some of the complexity of the antigenic relationships among flaviviruses. A second neutralization site which appeared to be spatially separate from all other sites was defined by the single 17D YF strain-specific IgM antibody obtained. This conclusion remains provisional, however, since high non-specific background binding of the antibody, a problem commonly encountered with solid phase immunoassays involving IgM antibodies (Meurman, 1983), could not be eliminated.

Although more efficient neutralization of virus strains other than the immunizing one has been reported with monoclonal antibodies to Newcastle disease virus (Nishikawa et al., 1983) and SLE (Roehrig et al., 1983), our finding that nine of 12 IgG antibodies had no neutralizing activity for 17D YF but neutralized Asibi YF efficiently and often in high titre is unusual if not unique. These nine antibodies defined six different antigenic sites, only one of which was associated with neutralization of 17D YF itself. This may indicate an unusual relationship between 17D YF and Asibi YF and might possibly be related to the effectiveness of this vaccine. Four of the nine antibodies that neutralized Asibi YF were flavivirus group-specific, another unusual observation since high-titred neutralizing activity has been a property of type- or strain-specific antibodies in previously reported studies and flavivirus group-reactive antibodies have had little or no neutralizing activity (Gentry et al., 1982; Peiris et al., 1982; Kimura-Kiroda & Yasui, 1983; Roehrig et al., 1983; Heinz et al., 1983). Two of the four group-reactive antibodies also neutralized DEN 2 virus and one of them neutralized Banzi also (Schlesinger et al., 1983). This is a novel observation to our knowledge; with the exception of strain differences, we are not aware of any other examples of neutralization of heterologous flaviviruses by monoclonal antibodies. These findings might bear some relation to the observations that the neutralizing antibody response to dengue vaccine was more predictable and more prolonged in individuals who had previously been vaccinated with 17D YF than those who had not (Scott et al., 1983) and also that 17D YF was particularly effective as the initial immunizing virus when a series of immunizations with different flaviviruses were given to monkeys to confer broadened protection against challenge with related flaviviruses (Price et al., 1973).

Blocking of neutralization by a monoclonal antibody that competed with a neutralizing one in CBA has been described (Massey & Schochetman, 1981) and the observation has been cited as a possible problem in vaccine efficacy or design (Massey & Schochetman, 1981; Heinz et al., 1983). We did not observe blocking by our non-neutralizing antibodies whether or not they interfered with binding of the neutralizing antibodies in CBA. It is possible that this apparent discrepancy reflects different assay conditions. For example, it has been shown that some monoclonal antibodies that bind to mildly denatured Sindbis virus in a solid phase assay appear not to bind at all to the intact virion in suspension (Schmaljohn et al., 1983). Differences in binding characteristics of monoclonal antibodies to 'dried' versus 'wet' influenza virions has also been
described (Gerhard et al., 1980) as have differences in binding to human chorionic gonadotropin in solid versus liquid phase assay (Ehrlich et al., 1983). Other explanations are possible but all are highly speculative. In any case, the observation of competition between antibodies in a solid phase assay should not necessarily lead to the conclusion that similar results would be observed when biological function is evaluated.

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