Monoclonal Antibodies to the E1 and E2 Glycoproteins of Sindbis Virus: Definition of Epitopes and Efficiency of Protection from Fatal Encephalitis

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SUMMARY

Protection of mice from fatal neuroadapted Sindbis virus encephalitis can be accomplished by passive transfer of monoclonal antibodies (MAbs) to either the E1 or E2 glycoprotein of Sindbis virus. Both neutralizing and non-neutralizing MAbs can be protective. To define further the characteristics of MAbs that provide protection from fatal disease, antigenic epitopes on the E1 and E2 glycoproteins were identified using a competitive binding enzyme immunoassay. Four distinct epitopes on E1 and three on E2 were defined. MAbs to all E1 epitopes, both neutralizing (three) and non-neutralizing (one) protected mice from fatal encephalitis. MAbs to the E2 neutralizing epitopes (two) protected mice from fatal encephalitis while those to the non-neutralizing epitope did not. The efficiency of protection from fatal Sindbis virus encephalitis of four neutralizing and non-neutralizing protective anti-E1 and anti-E2 MAbs representing different epitopes was compared. The neutralizing MAbs (against epitopes E2-ab, E2-c and E1-c) gave 50% protection at lower doses (2 to 20 μg) than the non-neutralizing MAb representing epitope E1-e (150 μg) when given before virus challenge. When given after virus challenge, MAbs to E2-ab and E2-c protected at lower doses (0.03 to 0.3 μg) than did either MAbs to E1-c (> 100 μg) or E1-e (10 μg). The MAbs to E1-e, E2-ab and E2-c were required in larger amounts to afford protection before than after challenge, while the opposite was true for MAb to E1-c.

INTRODUCTION

Recovery from experimental alphavirus infections correlates best with the development of antiviral antibody, rather than virus-specific cellular immunity (Griffin, 1986). The availability of monoclonal antibodies (MAbs) to a number of alphaviruses has made possible a more detailed analysis of the specificities, biological activities and efficiencies of protective antibodies. Previous studies have demonstrated that MAbs to either the E1 or E2 glycoprotein of Sindbis virus (SV) can provide passive protection from fatal encephalitis. Furthermore, both neutralizing and non-neutralizing MAbs to E1 are protective (Schmaljohn et al., 1982; Stanley et al., 1986). Studies of MAb-mediated protection from other fatal alphavirus encephalitides caused by Venezuelan equine encephalitis virus (VEEV) and Semliki Forest virus (SFV), have also shown that MAbs to both the E1 and E2 glycoproteins, and non-neutralizing as well as neutralizing MAbs, can provide passive protection from fatal infection (Mathews & Roehrig, 1982; Boere et al., 1983, 1984, 1985; Mathews et al., 1985; Roehrig & Mathews, 1985). In SFV and VEEV infections MAbs with different biological properties show substantial differences in the efficiency with which they provide protection. When given before virus challenge protective doses of MAbs varied from 0.1 μg to 100 μg of immunoglobulin, and neutralizing anti-E2 antibodies have generally been found to provide the most efficient protection (Boere et al., 1983, 1984; Mathews & Roehrig, 1982; Mathews et al., 1985). In the present study we have identified
epitopes on SV using a previously reported panel of MAbs (Stanley et al., 1985) and have used known amounts of four neutralizing and non-neutralizing IgG MAbs, representing different E1 and E2 glycoprotein epitopes, to determine the efficiency of MAb protection when given either 24 h before (prophylaxis) or 24 h after (recovery) challenge with a lethal dose of neuroadapted SV (NSV).

METHODS

Virus. Two strains of SV were used: AR339 (American Type Culture Collection) and NSV isolated after six intracerebral passages of AR339 in mice (Griffin & Johnson, 1977). SV AR339 is avirulent for weaning mice, while NSV is virulent with an intracerebral LD₅₀ of 2 to 20 p.f.u. Stock viruses were grown and assayed in BHK-21 cells.

Competitive binding assays. The production and biological characterization of the anti-SV MAbs used have been described (Stanley et al., 1985). MAbs were purified from ascitic fluid either by binding to staphylococcal Protein A-Sepharose (Pharmacia) (Oi & Herzenberg, 1980) or by precipitation with 40% ammonium sulphate. Purified MAbs were biotinylated using 200 μg biotinyl-e-aminocaproic acid N-hydroxysuccinimide ester (Calbiochem) per mg protein (Leary et al., 1983). Half-area polystyrene enzyme immunoassay plates (Costar) were coated with SV or NSV. Virus for coating was precipitated from tissue culture supernatant fluid with 10% polyethylene glycol 8000 in the presence of 0.5 M-NaCl (Bell et al., 1979) and diluted to 3 μg/ml in phosphate-buffered saline pH 7.4 (PBS). Fifty μl of this antigen solution was added to each well and allowed to adsorb overnight at 4 °C. Excess antigen was removed and 25 μl of unlabelled (competing) MAb (1:100 and 1:300) and 25 μl biotinylated MAb optimally diluted in PBS containing 0.2% Tween 20 and 5% chick serum were added to each well and incubated for 1.5 to 2 h at 37 °C, or overnight at 4 °C. Plates were washed and 50 μl of a 1:10 dilution of avidin--biotinylated horseradish peroxidase complex (ABC, Vector Laboratories, Burlingame, Ca., U.S.A.) in PBS was added, incubated at room temperature for 45 to 60 min and the plates were washed. Colour was developed with 100 μl of o-phenylenediamine in citrate-phosphate buffer as substrate (Stanley et al., 1985). A MAb was considered to compete if it consistently inhibited binding of the biotinylated MAb by > 50%. MAbs to SV developed and characterized in the laboratories of Drs Alan Schmaljohn (Schmaljohn et al., 1983) and Robert Johnston (Davis et al., 1987) were kindly provided by these investigators for comparative purposes.

Protection. MAbs were purified from ascitic fluid by precipitation with 33% ammonium sulphate. Immunoglobulin concentration was determined by isotype-specific radial immunodiffusion (Miles Laboratories). Four IgG MAbs representative of four different epitopes were studied for efficiency of protection: 101 (E1-c, non-neutralizing), 106 (E1-c, neutralizing), 202 (E2-ab, neutralizes NSV, but not SV) and 209 (E2-c, neutralizes SV and NSV equally). Three- to four-week-old BALB/c An NrlBR mice (Charles River Breeding Laboratories, Wilmington, Mass., U.S.A.) were inoculated intracerebrally with 10 LD₅₀ (20 p.f.u.) NSV (Griffin & Johnson, 1977; Stanley et al., 1986) in 0.03 ml of Hanks' balanced salt solution (HBSS) and observed for paralysis and death for 21 days. To test for protection, groups of six to 12 mice were injected intravenously with 0.1 to 1000 μg MAb immunoglobulin in 1 volume of 0.2 ml HBSS either 24 h before (prophylaxis) or 24 h after (recovery) inoculation of NSV. HBSS served as a negative control. Curves of protection from death and paralysis were drawn for each MAb and a 50% MAb protective dose was determined from the mortality curves.

RESULTS

E1 and E2 epitopes

Ten MAbs were used for identification of the epitopes on the E1 glycoprotein (Fig. 1). Four topographically independent sites were identified. The majority of these anti-E1 MAbs mapped to two sites which appeared to contain two or three overlapping epitopes within each. All of these MAbs are IgG isotypes and are protective against fatal NSV encephalitis when 0.2 ml of ascitic fluid is given prophylactically. Most are also protective when given 24 h after challenge with NSV (Stanley et al., 1986).

The E2 glycoprotein epitopes were identified using seven E2-specific MAbs previously described to be E2-specific, plus one MAb (303) previously reported to precipitate both E1 and E2 (Fig. 1). By competitive binding assays these MAbs identified three topographically distinct epitopes on E2. Two of these (E2-ab and E2-c) were neutralizing epitopes and one (E2-d) was not. Most of the MAbs to the neutralizing sites were at least partially protective against NSV encephalitis (Stanley et al., 1986). Non-protective MAbs were all Ig subclass A.
**SV MAbs: protection from encephalitis**

Fig. 1. Epitope map obtained by competitive binding enzyme immunoassay of MAbs to the E1 (a) and E2 (b) glycoproteins of SV. Inhibition of binding > 80% (■) or 50 to 80% (□). N, neutralization; HI, haemagglutination inhibition; P, protection. A positive reaction is indicated by +, a negative by −. E, epitope; I, Ig isotype.

**Recovery from lethal encephalitis**

Four protective MAbs, 101, 106, 202 and 209, representing epitopes E1-e, E1-c, E2-ab, and E2-c respectively were chosen for more detailed study of the efficiency of protection (Fig. 2 and Table 1). Promotion of recovery by MAbs was tested by giving each MAb 24 h after intracerebral inoculation of NSV. At this time infection of the nervous system is well established, with the presence of approximately 10^7 p.f.u. virus/g brain tissue (Griffin & Johnson, 1977; Stanley et al., 1986). MAbs 209 and 202, each specific for E2, protected 50% of the mice at lower doses (0.3 and 0.03 μg IgG) than anti-E1 MAbs 101 and 106 (10 and > 100 μg Ig). MAb 202 was the most efficient as it promoted significant protection at < 0.1 μg and full recovery at 10 μg. Ability to neutralize virus infectivity did not necessarily make the MAb a more efficient protector, since neutralizing MAb 106 required the transfer of more antibody for 50% protection (100 μg) than did the non-neutralizing MAb 101 (10 μg).
Fig. 2. Protection of mice from death (○) and paralysis (●) due to encephalitis, by various doses of four representative MAbs to SV [a, e, E1 non-neutralizing (MAb 101); b, f, E1-neutralizing (MAb 106); c, g, E2-neutralizing (MAb 209); d, h, E2-neutralizing (MAb 202)] given 24 h before (prophylaxis) (a to d) or 24 h after (recovery) (e to h) challenge with a 10 LD50 dose of NSV.

**Prophylaxis of lethal encephalitis**

To determine whether there were differences in protective doses when MAbs were used for prophylaxis, mice were given the same four MAbs 24 h before inoculation of NSV (Fig. 2 and Table 1). Again, MAb 202 required the smallest amount of antibody for 50% protection. Neutralizing MAbs (106, 202 and 209) gave 50% protection at lower doses (2 to 20 μg) than did non-neutralizing MAb 101 (150 μg). In contrast to the results obtained for MAbs given after infection, the neutralizing MAb 106 required less antibody than the non-neutralizing MAb 101.
Table 1. Efficiency of MAb protection against lethal encephalitis when given 24 h before (prophylaxis) or 24 h after (recovery) intracerebral challenge with NSV at a dose of 10 LD$_{50}$

<table>
<thead>
<tr>
<th>MAb</th>
<th>Epitope</th>
<th>Isotype</th>
<th>Neutralization</th>
<th>Amount of MAb required (µg) for 50% protection</th>
</tr>
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<tr>
<td></td>
<td></td>
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<td></td>
<td>Prophylaxis</td>
</tr>
<tr>
<td>101</td>
<td>E1-e</td>
<td>IgG2a</td>
<td>-</td>
<td>150</td>
</tr>
<tr>
<td>106</td>
<td>E1-c</td>
<td>IgG2b</td>
<td>+ (NSV = SV)</td>
<td>20</td>
</tr>
<tr>
<td>209</td>
<td>E2-c</td>
<td>IgG3</td>
<td>+ (NSV = SV)</td>
<td>20</td>
</tr>
<tr>
<td>202</td>
<td>E2-ab</td>
<td>IgG3</td>
<td>+ (NSV &gt; SV)</td>
<td>2</td>
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</table>

when given before virus challenge. The E1 or E2 specificity of the neutralizing MAb did not correlate with the 50% protective dose (20 µg) since anti-E1 neutralizing MAb 106 protected at the same dose as anti-E2 neutralizing MAb 209.

**DISCUSSION**

The epitopes defined on E1 and E2 glycoproteins are similar in number and function to those described for other alphaviruses. The E1 protein has traditionally been associated with haemagglutination of erythrocytes (Dalrymple et al., 1976), is relatively conserved, and probably associated with cross-protection among alphaviruses that are not cross-neutralizing (Griffin, 1986). Identification of epitopes on the E1 glycoproteins of VEEV (Roehrig et al., 1982), SFV (Boere et al., 1984) and Western equine encephalitis virus (Hunt & Roehrig, 1985) have demonstrated five or six epitopes with a mixture of neutralizing and haemagglutination-inhibiting MAb activities. SV epitopes (Fig. 1) are consistent with this pattern. Selected MAbs were tested for competition using representative MAbs from the anti-E1 MAbs previously described by Schmaljohn et al. (1983). These authors identified three non-overlapping sites (abc, d and e). Based on competitive binding of their MAb 33 with our MAb 106 it can be determined that MAb 106 identifies epitope E1-c within a larger complex site encompassing a, b and c (Schmaljohn et al., 1983). Our four MAbs to this site each had distinct patterns of competition, suggesting that the site may be even more complex than previously described. Site abc contains epitopes which elicit neutralizing and haemagglutination-inhibiting antibody. Site E1-d was apparently not represented in our panel. MAb K-42 from Schmaljohn et al. (1983) defining the E1-e epitope competes specifically with MAb 104. E1-e elicits antibodies with primarily haemagglutination inhibition activity. Our four MAbs gave three different patterns of competition suggesting some overlapping epitopes in this site as well. MAb 105 and 107 competed only with themselves, neutralized infectivity and inhibited haemagglutination, and are therefore designated as new sites E1-f and E1-g.

The alphavirus E2 protein has traditionally been associated with elicitation of neutralizing antibodies (Pederson & Eddy, 1974; Dalrymple et al., 1976), is less conserved, and accounts for many of the differences between alphaviruses (Bell et al., 1984). Epitope identification on the E2 glycoproteins of VEEV (Roehrig et al., 1982; Roehrig & Mathews, 1985) and SFV (Boere et al., 1983, 1984) as well as SV have been reported. Four to eight epitopes have been identified which elicit MAbs with a mixture of neutralizing and haemagglutination-inhibiting functions. The SV E2 MAbs reported originally by Schmaljohn et al. (1983) did not identify distinct epitopes, although functional analysis of anti-E2 MAbs by other investigators suggested that distinct epitopes were likely to be present (Roehrig et al., 1980; Stanley et al., 1985, 1986). Further studies of reactivity of MAbs with different strains of SV (Olmsted et al., 1986; David et al., 1987) and analysis of neutralization escape mutants (Stec et al., 1986) has led to identification of three different regions of antibody reactivity on E2. E2-a is defined by MAbs 49 and 50 (Stec et al., 1986). These MAbs compete not only with MAbs to this site but also with MAbs (R8/10) defining site E2-b (Olmsted et al., 1986), which is independently mutable, suggesting that E2-a and E2-b overlap. MAbs 49 and R10 both compete strongly (>90%) with our MAb 202, suggesting that the latter also identifies the E2-ab site.
E2-c is a second neutralization site topographically distinct from E2-ab (Omsted et al., 1986; Stec et al., 1986; Davis et al., 1987). Our four MAbs to E2-c had two different competition patterns suggesting additional complexity within this site. E2-d is a previously unidentified E2 epitope, although a non-neutralizing anti-E2 MAb was described by Roehrig et al. (1980). E2-d elicits little if any neutralizing, haemagglutination-inhibiting or protective MAb activity. This functional pattern may or may not be associated with the fact that these MAbs are both IgA.

Extensive sequence information is available on strains of SV (Rice & Strauss, 1981; Strauss et al., 1984; Davis et al., 1987; Lustig et al., 1988). Little if any of these data have yet been applied successfully to assigning specific regions for MAb reactivity within E1, but information on E2 is gradually emerging. The glycosylation site at amino acid 196 of the E2 glycoprotein bears a complex carbohydrate and lies in a region of sequence variability (Strauss et al., 1987). The E2-ab site appears to reside in this area since neutralization escape mutants of these MAbs have changes in amino acids 190 and 216 (Strauss et al., 1987). Laboratory strains of SV AR339 are polymorphic at amino acid position 216 and this is reflected in the patterns of anti-E2-ab MAbs (Davis et al., 1987). In addition, SV and NSV differ at amino acid position 209 (Lustig et al., 1988) and show distinct reactivities to MAbs 202 (Stanley et al., 1985), which recognizes the E2-ab epitope, suggesting that this amino acid is also involved in this epitope.

Both neutralizing and non-neutralizing MAbs have been shown to protect mice against lethal encephalitis caused by NSV (Schmaljohn et al., 1982; Stanley et al., 1986). However, the amounts of MAbs immunoglobulin given by passive transfer, and therefore the efficiency of protection, have not been determined. In the present study, we demonstrate that anti-E2 MAbs administered after infection are required in smaller doses than anti-E1 MAbs in order to provide 50% protection. However, when given before infection, neutralizing anti-E1 and anti-E2 MAbs are required in approximately the same amounts, while much more of the non-neutralizing anti-E1 MAbs is required. Similar findings have been reported for prophylactic protection by MAbs to SFV and VEEV (Boere, 1983, 1984; Mathews et al., 1985). In both of these alphavirus infections neutralizing MAbs were more efficient than non-neutralizing MAbs at protecting mice from fatal infection. Neutralization of viral infectivity has been postulated as a mechanism of protection in vivo (Mathews & Roehrig, 1982; Zichis & Shaughnessy, 1940) and may be a particularly important mechanism when antibody is given before virus challenge, since a portion of the input virus may be neutralized prior to infection. The mechanism of protection, however, may or may not be by neutralization of virus infectivity, and the efficiency of neutralization does not necessarily correlate with the efficiency of protection. Neutralizing antibodies may also bind to the surface of virus-infected cells and act by other mechanisms as well.

It is clear that neutralization of viral infectivity is not necessarily required for a MAbs to be protective. Non-neutralizing MAbs toward SFV (Boere et al., 1983, 1984, 1985), VEEV (Mathews et al., 1985) and Western equine encephalitis virus (Hunt & Roehrig, 1985) as well as SV (Schmaljohn et al., 1982; Stanley et al., 1986) and viruses from other groups such as mouse hepatitis virus (Nakanaga et al., 1986) and vesicular stomatitis virus (Lefrancois, 1984) are protective. Our data would suggest that non-neutralizing MAbs may be most efficient when given after virus challenge when virus-infected cells are present. The mechanism by which the non-neutralizing MAbs protect is not known. Antibody-dependent complement-mediated cytosis of virus-infected cells has been proposed (Boere et al., 1985; Schmaljohn et al., 1982; Sissons & Oldstone, 1980). However, several protective MAbs, including MAb 106, are negative in this assay (Stanley et al., 1986). Furthermore, studies of NSV (Hirsch et al., 1979), VEEV (Mathews et al., 1985) and SFV (Boere et al., 1986) infections have shown that antibodies are protective even in complement-deficient mice, suggesting that complement is not necessary for protection, although some MAbs may be more efficient in the presence of adequate levels of complement (Boere et al., 1986). Removal of Fc fragments abolishes protective capacity, suggesting that Fc effector functions other than complement-binding are necessary for protection (Boere et al., 1985; Hirsch et al., 1979; Mathews et al., 1985). Other suggested mechanisms for protection are antibody-dependent cell-mediated cytoxicity (Hirsch et al., 1979; MacFarlan et al., 1977; Stanley et al., 1986), inhibition of virion maturation (Boere et al., 1986).
Our data would suggest that mechanisms of antibody-mediated protection may differ with the specificity and biological activity of the antibody and with the time of administration relative to virus challenge.

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