Localization of antigenic sites on the surface glycoprotein of mouse hepatitis virus

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A panel of murine hepatitis virus (MHV) surface (S) glycoprotein-specific monoclonal antibodies (MAbs), which recognize either continuous or discontinuous epitopes, were tested in competitive binding assays. The results indicate that the binding site of MAb 30B amino acids 395 to 406 in the amino-terminal S₁ subunit, is involved in the discontinuous epitope designated antigenic site A. This site is a major determinant for the induction of neutralizing antibodies. These data define, for the first time, the location of a functionally important domain on the MHV S protein.

Introduction

Coronaviruses are associated with diseases of economic importance in animals and humans. Natural infections generally result in enteric or respiratory disease, but the involvement of other organs is not uncommon (Wege et al., 1982; Spaan et al., 1990). In the laboratory, the murine coronaviruses have received much attention owing to their ability to cause a variety of different diseases depending upon the virus isolate, the host animal and the route of infection (Hirano et al., 1981; Sorensen et al., 1982; Barthold & Smith, 1984). For example, infection of rodents with murine hepatitis virus (MHV), which is a model for diseases in humans (Kyuwa & Stohlman, 1990), has been used widely to investigate the mechanisms of virus-induced demyelination. At the moment, interest is focused on the structure and function of coronavirus proteins and their interactions with the immune system. A detailed knowledge of their viral antigens and immunodominant domains which influence pathogenicity and epidemiology is a first step towards developing preventive or therapeutic strategies.

Murine coronaviruses are composed of four major structural proteins: the nucleocapsid protein, the membrane glycoprotein, the haemagglutinin-esterase protein and the surface or spike (S) glycoprotein (Siddell, 1982). The S protein forms large peplomer structures on the surface of the virus particle and plays a central role in the infection process, being involved in the attachment of virus to the cell surface, the induction of neutralizing antibodies and cell-to-cell fusion. By implication it has a role in the fusion of virus to cell membranes (Collins et al., 1982; Vennema et al., 1990).

As might be expected, structural determinants on the S protein strongly influence viral pathogenicity (Dalziel et al., 1986; Fleming et al., 1986; Wege et al., 1988). One approach to the identification of the location and function of these determinants is to define the binding sites of monoclonal antibodies (MAbs) which neutralize virus infectivity or inhibit virus-mediated cell fusion. This approach is particularly suitable for MHV because MAbs can be derived from infected animals. However, it is based, as always, on the assumption that there is a direct spatial relationship between the binding site of the MAb and its ability to interfere with important structural or functional sites.

For this purpose, Wege et al. (1984) have isolated and characterized a set of MAbs specific for the MHV-JHM S protein; competitive binding assays and analysis of escape variants have allowed the delineation of six antigenic sites, A to F, two of which, A and B, strongly bind neutralizing antibodies. Antigenic sites A and B define discontinuous epitopes dependent upon the conformation of the native protein, whereas those defined by antigenic sites C to F are continuous (Wege et al., 1984, 1988; van Regenmortel, 1990).

Recently, Routledge et al. (1991) established a collection of MAbs induced by immunization of mice with a recombinant MHV S protein. These MAbs bind to continuous epitopes on the MHV S protein and it has been possible to determine their precise binding sites by analysing their reactivity with a set of truncated fusion proteins and with synthetic peptides. In this paper we have used competitive binding assays to define the relationship between these continuous and discontinuous epitope-dependent MAbs, and to define the location of
the biologically important antigenic site A on the native MHV S protein.

Methods

Cells and viruses. Sac(-) (Weiland et al., 1978) and DBT cells (Kumanishi, 1967) were grown in monolayers in MEM containing 5% heat-inactivated foetal calf serum (FCS), glutamine and antibiotics. Sac(-) cells were also grown in suspension culture in modified MEM containing 5% FCS and antibiotics. MHV-JHM was propagated in Sac(-) cells at low multiplicity and purified as described by Wege et al. (1979).

Virus neutralization. To neutralize virus infectivity, serial dilutions of MAbs were incubated with 40 p.f.u. MHV-JHM for 1 h at 4 °C. Ascites fluids containing approximately equal Ig concentrations (determined by gel electrophoresis) were inactivated for 30 min at 56 °C before mixing with the virus. After incubation, the amount of infectious virus was measured on monolayers of DBT cells in 24-well Costar plates. Microplaques were stained with May-Grünewald solution 16 to 20 h after infection and the antibody dilution resulting in 50% plaque reduction was calculated.

Inhibition of cell-to-cell fusion. Monolayer cultures of DBT cells in 48-well cluster plates (Costar) were infected with MHV-JHM (40 p.f.u./well) and dilutions of ascites fluid (1:10 to 1:1000), prepared as described above, were added 3 h later. The number and size of syncytia were evaluated microscopically and the antibody dilution resulting in a 50% reduction in plaque size was calculated.

Purification and biotinylation of hybridoma antibodies. The hybridoma antibodies used in this study have been described in detail by Wege et al. (1984) and Routledge et al. (1991). MAbs were purified from ascites fluids by affinity chromatography with Protein A from Staphylococcus aureus (Pharmacia). After binding, the IgG fraction was eluted with 0.1 M-sodium citrate buffer pH 3.5 and adjusted to a protein concentration of 1 mg/ml. The globulins were biotinylated with biotinyl-N-hydroxysuccinimide ester (BHSE; EY Laboratories, Medac) as described by Wege et al. (1984); the BHSE/IgG concentration that yielded biotinylated antibodies displaying optimal self-competition in the competitive binding assay was 100 µg BHSE/mg protein. The reaction mixture was incubated for 3 h at room temperature and biotinylation was terminated by extensive dialysis against PBS. The Ig isotypes were determined using a commercial kit (Bio-Rad).

ELISA for competitive binding assay. MAbs were used in reciprocal competitive binding assays using density gradient-purified MHV-JHM coated onto wells of flat-bottomed microtitre plates (Dynatech). The individual competitor antibodies (as purified Igs) were applied in 10-fold dilutions to wells coated with excess antigen and incubated for 1 h at 37 °C. The microplates were washed and biotinylated hybridoma antibody was added at a dilution resulting in a net absorbance (A492 of wells with antigen - A492 of wells without antigen) of 1.3 to 1.9 in a non-competitive assay. Avidin coupled to horseradish peroxidase (Dianova) was used to measure bound biotinylated mouse globulin, using 0.25 mg/ml o-phenylenediamine in 0.1 M-citrate buffer pH 5.0 as the substrate.

To define overlapping antigen-binding sites, competition was considered as positive if it occurred reciprocally and over a range of several log10 dilutions; each antibody was used both as competitor and biotinylated probe with the complete set of MAbs. To summarize the data, the degree of competition was evaluated relative to the self-competition curve and rated as strong (+ + + +), significant (+ +) or negative (−). The result was rated as strong if the first two 10-fold dilutions of competitor antibody caused greater than 75% reduction of the net absorbance compared to the self-competition of the biotinylated probe alone; the result was rated as significant if the first two 10-fold dilutions caused a 40 to 60% reduction, and negative if the reduction of the net absorbance was not more than 40% and occurred over fewer than three 10-fold dilutions.

Results

In this study, we compared nine MAbs which bind to the S protein of MHV-JHM. As has been shown previously (Wege et al., 1984; Routledge et al., 1991), the binding of MAbs A1, A4, B35 and B7 depends upon the three-dimensional structure of the protein, whereas MAbs 30B, C8, D12, E16 and F18 bind to continuous epitopes on the polypeptide; all MAbs bind to MHV-JHM virions when assayed by ELISA (data not shown). As is summarized in Table 1, MAbs A1, A4, B35, B7 and 30B neutralized virus infectivity, and MAb A1 was clearly able to block virus-induced cell-to-cell fusion. These data confirm our earlier results and provide a direct comparison of the properties of these MAbs under identical experimental conditions.

The results of the competitive immunoassays are shown in Fig. 1. The competition curves show the net A492 values at various dilutions of competing antibody; these data are summarized in Table 2. It is evident that the MAbs which define antigenic site A all compete with the continuous epitope-dependent MAb 30B (Fig. 1 a, b and c); competition was strong and reciprocal. MAb 30B did not compete with MAbs representing antigenic sites C, D, E and F (Fig. 1 f). MAb B7 (antigenic site B) showed partial, one-way competition with MAb 30B, and MAb B35 displayed a one-way reaction with the

<p>| Table 1. Characterization of MHV-JHM S protein-specific MAbs |
|--------------------------|---------------------|----------------------|-------------------|</p>
<table>
<thead>
<tr>
<th>MAb</th>
<th>Antigenic site*</th>
<th>Isotype</th>
<th>Virus neutralization†</th>
<th>Fusion inhibition‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>Aa</td>
<td>IgG2a</td>
<td>6.9 × 10⁴</td>
<td>750</td>
</tr>
<tr>
<td>A4</td>
<td>Ab</td>
<td>IgG2a</td>
<td>2.5 × 10⁴</td>
<td>100</td>
</tr>
<tr>
<td>B35</td>
<td>Ba</td>
<td>IgG2a</td>
<td>1.7 × 10⁴</td>
<td>&lt;80</td>
</tr>
<tr>
<td>B7</td>
<td>Bb</td>
<td>IgG2a</td>
<td>3.0 × 10⁷</td>
<td>&lt;100</td>
</tr>
<tr>
<td>C8</td>
<td>C</td>
<td>IgG1</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>D12</td>
<td>D</td>
<td>IgG2a</td>
<td>&lt;10</td>
<td>&lt;10</td>
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<tr>
<td>E16</td>
<td>E</td>
<td>IgG2a</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>F18</td>
<td>F</td>
<td>IgG2a</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>30B</td>
<td>Amino acids 395-406</td>
<td>IgG1</td>
<td>7.8 × 10³</td>
<td>&lt;50</td>
</tr>
</tbody>
</table>

* Data from Wege et al. (1984, 1988) and Routledge et al. (1991).
† Values are the reciprocal antibody dilution causing a 50% reduction in plaque number.
‡ Values are the reciprocal antibody dilution causing a 50% reduction in plaque size.
antigenic site A MAbs and MAb 30B (Fig. 1d and e). The reciprocal assays in which biotinylated C8, D12, E16 and F18 antibodies were used were all negative (data not shown). We interpret these results to indicate that the discontinuous epitope-dependent antigenic site A MAbs and the continuous epitope-dependent MAb 30B bind to the same domain of the MHV S protein. The results also suggest that the function of this domain is essential for virus infectivity.

**Discussion**

The results presented in this paper show that the MAb 30B binding site, amino acids 395 to 406 in the amino-terminal $S_1$ subunit of the S protein, is involved in the discontinuous epitope defined by MAbs A1 and A4. This is important because antigenic site A appears to be a major determinant for the induction of neutralizing antibodies during MHV infection. The conclusion that this area of the MHV S protein represents the immunodominant B cell determinant is also supported by the analysis of several MAb-selected, neutralization-resistant mutants of MHV, which have extensive deletions within a region of the $S_1$ subunit adjacent to, but not encompassing, the MAb 30B binding site (Banner et al., 1990; Gallagher et al., 1990; Parker et al., 1989).

Moreover, data from several laboratories indicate that neutralizing MAbs bind to the amino-terminal region of a variety of coronavirus S proteins (Cavanagh et al., 1986; Correa et al., 1990; Weismiller et al., 1990).

Although the location of MHV antigenic site A has been determined, the data presented here do not allow any direct determination of the functions associated with it. All the MAbs which bind to antigenic site A effectively neutralize virus, but only one of them, MAb A1, convincingly inhibits S protein-mediated cell fusion. Clearly further experiments are needed to establish the relationship, if any, between the neutralization and cell-to-cell fusion-inhibiting activities of the MAbs we have tested. It seems likely that a full interpretation of this type of data will only be possible when a three-dimensional image of the S protein and its interactions with bound MAbs becomes available.

The results obtained have not only defined the location of a functionally important domain of the MHV S protein, but also demonstrate that it is possible to define and analyse discontinuous epitopes without depending upon alterations to the native structure, as is necessary for example when MAb-selected, neutralization-resistant variants are generated. This approach and the reagents we have developed should allow a more detailed analysis of the MHV S protein and, in particular, the functions associated with antigenic site A.

**Table 2. Summary of competitive binding assay results**

<table>
<thead>
<tr>
<th>Competitor MAb</th>
<th>Biotinylated MAb</th>
<th>A1</th>
<th>A4</th>
<th>30B</th>
<th>B35</th>
<th>B7</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>~</td>
<td>~</td>
</tr>
<tr>
<td>A4</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>~</td>
<td>~</td>
</tr>
<tr>
<td>30B</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>~</td>
<td>~</td>
</tr>
<tr>
<td>B35</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>~</td>
<td>~</td>
</tr>
<tr>
<td>B7</td>
<td>~</td>
<td>~</td>
<td>~</td>
<td>+</td>
<td>~</td>
<td>~</td>
</tr>
<tr>
<td>C8</td>
<td>~</td>
<td>~</td>
<td>~</td>
<td>~</td>
<td>~</td>
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</tr>
<tr>
<td>D12</td>
<td>~</td>
<td>~</td>
<td>~</td>
<td>~</td>
<td>~</td>
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<td>E16</td>
<td>~</td>
<td>~</td>
<td>~</td>
<td>~</td>
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<tr>
<td>F18</td>
<td>~</td>
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<td>~</td>
<td>~</td>
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</tr>
</tbody>
</table>

* + + + +, Strong competition; + +, significant competition; ~, no competition.
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References


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