1991

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Monoclonal Antibodies to the Fusion Protein of Bovine Respiratory Syncytial Virus†

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Received 17 December 1990/Accepted 29 May 1991

Five monoclonal antibodies specific for bovine respiratory syncytial virus were characterized by Western immunoblotting, radioimmunoprecipitation, and epitope mapping. The monoclonal antibodies were found to be specific for the fusion protein, and there were at least two antigen binding sites, one of which was neutralizing.

Bovine respiratory syncytial virus (RSV) is a member of the *Paramyxoviridae* family and apparently is closely related to human RSV (2, 3). Bovine RSV now is recognized as a major contributor to bovine respiratory disease (3). Isolation and identification of the virus during outbreaks of bovine respiratory disease have been hampered by the apparent lability of the virus and by the lack of high-quality diagnostic reagents (3). Additionally, only one known study has been directed at the characterization of bovine RSV structural proteins that induce biologically important antibodies (5). Antibody to the 90-kDa protein apparently is capable of neutralizing the virus but does not inhibit syncytia formation (9). Antibody to the fusion protein is capable of both neutralizing the virus and inhibiting syncytia formation (8, 10). Five monoclonal antibodies (MAbs) specific for bovine RSV were produced and partially characterized (Table 1 (6). Three of the MAbs (8G12, 15C7, 16A12) were isotype immunoglobulin G1 (IgG1; κ), and two (14D3 and 14E3) were isotype IgG2a (κ). MAbs 8G12 and 15C7 (ascites) inhibited syncytia formation in vitro and exhibited neutralizing activity, whereas the remaining three MAbs did not. All five MAbs reacted with bovine RSV-infected cells by direct and indirect fluorescent-antibody tests. The objective of the present study was to further characterize these MAbs by radioimmunoprecipitation (RIP), Western immunoblotting, and epitope mapping.

Bovine RSV strain 375 was grown in bovine turbinate cells cultured in HALS medium (GIBCO) supplemented with 50 μg of gentamicin per ml and 10% horse serum in a 37°C humidified incubator with 5% CO₂. Confluent monolayers were adsorbed with bovine RSV at a multiplicity of infection of 1 for 1 h and then refed HALS medium containing gentamicin. Virus culture fluid was frozen after the cells formed syncytia and cell lysis was approximately 80%. The titer of the virus culture fluid ranged from 10⁵.⁵ to 10⁶.² 50% tissue culture infective doses per ml of fluid. The virus culture fluid was clarified of cell debris and concentrated 10 times with an Amicon DC-2 hollow fiber dialyzer-concentrator (10-kDa cutoff) and purified by linear sucrose gradient (20 to 60%) centrifugation in TEN buffer (0.13 M NaCl, 0.001 M EDTA, 0.05 M Tris base [pH 7.7]) at 25,000 rpm (114,000 × g) in a Beckman L8-70M ultracentrifuge with an SW28 rotor for 18 h. A band with a density of 1.23 g/ml contained the bovine RSV; this was confirmed by a characteristic cytopathic effect in tissue culture.

To radiolabel bovine RSV, confluent monolayers were adsorbed with bovine RSV with a multiplicity of infection of 1.0 for 1 h at 37°C in a humidified incubator with 5% CO₂. The inoculum was then removed, and 2 ml of HALS medium plus 2% horse serum containing 50 μCi of d-[1-¹⁴C]glucosamine hydrochloride (Amersham) was added. One milliliter of 2× RIP buffer (150 mM NaCl, 0.1% sodium dodecyl sulfate [SDS], 1% Triton X-100, 1% deoxycholate, 10 mM Tris [pH 7.2]) was added when the cytopathic effect was approximately 80%. Labeled virus lysate was divided into aliquots and stored at −70°C.

The MAbs used in this study were prepared as described by Kluczka and Anderson (6). Polyclonal antibodies were prepared by immunizing a calf with purified bovine RSV mixed with Freund complete and incomplete adjuvants.

Purified bovine RSV and a negative cell control were resolved under reducing and nonreducing conditions by SDS-polyacrylamide gel electrophoresis (PAGE) on 10% gels (7). Prestained molecular weight markers (Deversified Biotech) were used to determine the relative mobilities of the bands. The separated proteins were then transferred to nitrocellulose paper (Schleicher & Schuell) in a transblot cell (Bio-Rad). After transfer, the nitrocellulose was blocked with RIP buffer containing 2% bovine serum albumin (BSA; Sigma) for 30 min at room temperature. The blocked nitrocellulose was then incubated with MAb or polyclonal antiserum diluted in phosphate-buffered saline (PBS) with 0.05% Tween 20 plus 1% BSA for 1 h at room temperature and was then washed three times with PBS–Tween 20. Alkaline phosphatase-conjugated goat anti-mouse IgG or goat anti-bovine IgG (Kirkegaard and Perry) diluted in PBS plus 1% BSA was then added and incubated for 1 h at room temperature. The nitrocellulose was washed again and developed with 5-bromo-4-chloro-3-indolyl phosphate and nortroblue tetrazolium substrate (Kirkegaard and Perry). One hundred microliters of labeled virus lysate was preadsorbed with 50 μl of 10% formalin-fixed Staph A (from *Staphylococcus aureus* Cowen strain) for 1 hour at 4°C, to remove any material that may have nonspecifically bound to Staph A, and then microcentrifuged (Fisher) to remove the Staph A. Forty-five microliters of monoclonal ascitic fluid

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†This work is published as paper 9558 of the Nebraska Agricultural Research Division.

0095-1137/91/092038-03$02.00/0
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TABLE 1. Biological activities of MAbs to bovine RSV*  

<table>
<thead>
<tr>
<th>MAb</th>
<th>Isotype</th>
<th>Serum neutralization</th>
<th>Syncytia inhibition</th>
<th>IFA*</th>
<th>Western blot with proteins of the following size (kDa):</th>
<th>RIP with proteins of the following size (kDa):</th>
</tr>
</thead>
<tbody>
<tr>
<td>8G12</td>
<td>IgG1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>20 46 70 120 20 46</td>
<td>20 46</td>
</tr>
<tr>
<td>15C7</td>
<td>IgG1</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16A12</td>
<td>IgG1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14D3</td>
<td>IgG2a</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14E3</td>
<td>IgG2a</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* +, positive; --, negative.  
* IFA, immunofluorescence assay.

diluted 1:10 in PBS was added to 45 μl of the preadsorbed labeled virus preparation and incubated overnight at 4°C. Fifty microliters of 10% Staph A was added to the antigen-antibody mixture and incubated for 1 h at room temperature. The Staph A-antibody complex was pelleted in a microcentrifuge and washed three times with RIPA buffer. After the final wash, 45 μl of 1× SDS-PAGE sample buffer was added to the pellet. The sample was then heated to 100°C for 5 min and centrifuged to remove the Staph A, and 40 μl was loaded onto a 10% SDS-polyacrylamide gel. After electrophoresis, the gels were fixed (40% methanol:10% acetic acid), soaked in a scintillant (Ampliphy; Amersham), and dried with a Bio-Rad gel drier. The dried gels were exposed to X-ray film (X-Omat; Kodak) and developed by fluorography (4).

Each MAb (8G12, 15C7, 14D3, 14E3, 16A12) was partially purified by ammonium sulfate precipitation and dialyzed against 6 liters of 0.1 M NaHCO₃ (pH 8.4) for 24 h with two changes of buffer. The protein concentration of the purified antibodies was adjusted to 1 mg/ml with 0.1 M NaHCO₃. The MAbs were biotinylated by dissolving 1.0 mg of biotinami-doacproate N-hydroxysuccinimide ester (Sigma) in 1.0 ml of dimethyl sulfoxide and immediately adding 200 μl of the biotin solution to 1 ml of each of the MAb solutions and reacting them at room temperature for 2 h. The biotinylated MAbs were then dialyzed against PBS (pH 7.2) overnight at 4°C with two changes of buffer. The conjugates were diluted 1:2 with glycerol, divided into aliquots, and stored at −20°C.

Competitive interactions among the MAbs were evaluated by solid-phase enzyme-linked immunosorbent assay (ELISA) similar to that described by Anderson et al. (1). Thirty nanograms of purified virus in 100 μl of coating buffer (0.01 M sodium borate [pH 9.2]) per well was adsorbed onto 96-well polystyrene plates (Nunc) at 4°C for 18 h. The wells were emptied, washed three times with PBS–TWEEN 20, and blocked with coating buffer containing 2% BSA for 30 min at 37°C. Competition among the MAbs was assessed by simultaneous incubation at 37°C of biotinylated MAb with various concentrations of unlabeled competitor MAb on the antigen-coated plates. The biotinylated MAb was used at a dilution that gave the optimum signal-to-noise ratio without the competing MAb. Plates were then washed with PBS–TWEEN 20, 100 μl of steptavidin peroxidase (Kirkegaard and Perry) in PBS containing 1% BSA (1:4,000) was added to each well, and the plates incubated for 1 h at 37°C. The plates were washed as described above; this was followed by the addition of 2,2’-azino-di(3-ethyl-benzthiazole sulfonate) substrate (Kirkegaard and Perry). The A₄₀₅ was recorded on a Dynatech MR650 micro-ELISA Reader after 30 min of incubation at 37°C. The percent competition was calculated by the following formula: (1 − A(A₄₀₅)) × 100, where A is the mean of the specific absorbance of wells with the competing MAb, and A₄₀₅ is the mean of the specific absorbance for wells with diluent in place of the competing MAb. Competition was considered significant when the blocking was 50% or greater.

Results of the analysis by immunoblotting are shown in Fig. 1. Analysis of purified bovine RSV under nonreducing conditions with the bovine polyclonal antiserum showed six proteins (approximately 120, 70, 42, 34, 29, and 22 kDa). Reducing conditions revealed at least six virus-specific proteins (approximately 46, 42, 34, 29, 25, 22, and 20 kDa); the 120- and 70-kDa proteins disappeared and the 46- and 20-kDa protein bands stained much more intensely. Western blot analysis with MAb 8G12 showed activity similar to that of the polyclonal antiserum with regard to the fusion protein. Under nonreducing conditions, the MAb identified two bands (120 and 70 kDa). Under reducing conditions, MAb 8G12 reacted only with one band (46 kDa), suggesting that the epitope recognized by this MAb is on the 46-kDa subunit of the fusion protein. This MAb was a neutralizing antibody (6), indicating that there may be a neutralizing epitope on the 46-kDa subunit of the fusion protein. The MAb 15C7 reacted similarly to MAb 8G12; i.e., under reducing conditions it reacted with a 46-kDa protein and under nonreducing conditions it reacted with 120- and 70-kDa proteins, but with less intensity than MAb 8G12 did. MAb 16A12, 14D3, and 14E3 each reacted with a 120-kDa protein under nonreducing conditions, but they did not react with any protein under reducing conditions. RIP with 14C-glucosamine-labeled virus demonstrated specificity for the fusion protein with all five MAbs (data not shown). Two bands were evident on the fluorograms with each MAb; the bands were 46- and 20-kDa glycoproteins.
TABLE 2. Competition ELISA with biotinylated and nonbiotinylated MAb

<table>
<thead>
<tr>
<th>Biotinylated MAb</th>
<th>% Competition for the following competing MAb*: 8G12</th>
<th>15C7</th>
<th>14E3</th>
<th>14D3</th>
<th>16A12</th>
</tr>
</thead>
<tbody>
<tr>
<td>8G12</td>
<td>60</td>
<td>68</td>
<td>4</td>
<td>-3</td>
<td>0</td>
</tr>
<tr>
<td>16A12</td>
<td>20</td>
<td>15</td>
<td>10</td>
<td>3</td>
<td>42</td>
</tr>
<tr>
<td>15C7</td>
<td>57</td>
<td>82</td>
<td>-2</td>
<td>-5</td>
<td>14</td>
</tr>
<tr>
<td>14D3</td>
<td>0</td>
<td>12</td>
<td>10</td>
<td>16</td>
<td>3</td>
</tr>
<tr>
<td>14E3</td>
<td>-11</td>
<td>3</td>
<td>7</td>
<td>15</td>
<td>-55</td>
</tr>
</tbody>
</table>

* Percent blocking is indicated for 2.5 μg of competing MAb, and blocking of 50% or more was considered significant.

Competition (epitope mapping) data are presented in Table 2. At least two, and possibly three, epitopes were identified by the five MAb s. There was two-way competition between MAb s 8G12 with 15C7. None of the other MAb s competed with MAb 8G12 or 15C7. There was weak competition between MAb s 14D3 and 14E3. These two MAb s did not compete to a high degree with the unbiotinylated homologous MAb, suggesting that biotinylation of these MAb s lowers the affinity for their binding sites or indicating that each MAb had a low affinity for the epitope. Competition between biotinylated MAb s 14E3 with 16A12 seemed to enhance the binding of MAb 14E3, suggesting that the binding of MAb 16A12 alters the protein such that the epitope is more accessible for MAb 14E3 to bind to. It may be hypothesized that MAb s 14D3 and 14E3 are specific for a different epitope than MAb s 8G12 and 15C7 are. MAb 16A12 competed minimally with all other MAb s except MAb 14E3. When MAb 16A12 was used as the competing antibody, it competed or at least interfered with the other three biotinylated MAb s. When biotinylated MAb 16A12 was used with the other nonbiotinylated MAb s, no competition was observed.

A summary of the MAb activities observed in this study is presented in Table 1. Western blotting activity with the 46-kDa subunit was observed only under reducing conditions. Activity with the 70- and 120-kDa proteins occurred only under nonreducing conditions. RIP with the MAb s showed activity only with the 20- and 46-kDa subunits. In reality, the MAb s probably reacted with the 120- and 70-kDa proteins, but when the precipitate was put into SDS-PAGE sample buffer and heated, the protein may have been reduced to the subunits. Western blot analysis demonstrated that the 46-kDa epitope recognized by MAb s 8G12 and 15C7 is stable under denaturing and reducing conditions. However, epitopes recognized by the remaining MAb s (14D3, 14E3, 16A12) apparently were not stable under these conditions.

All the data indicate that the five MAb s used in this study are specific for the fusion protein of bovine RSV and that there are at least two distinct antibody-binding sites on the fusion protein. On the basis of competition ELISA, RIP, and Western blotting, an epitope on the fusion protein (46 kDa) of bovine RSV appears to be responsible for neutralization. In addition, these studies provide evidence that a diagnostic assay (ELISA) may be developed with these MAb s; MAb 8G12 would probably be the antibody of choice, since it appears to be specific for a stable and biologically significant epitope. Additional MAb s will be useful to further characterize the fusion protein and the other proteins of bovine RSV.

This study was supported in part by funds provided by the U.S. Department of Agriculture under the Animal Health Act, Public Law 95-113; the University of Nebraska Research Council; and the U.S. Department of Agriculture Special Grants Program (grant 87-CRSR-2-3156).

REFERENCES