Monoclonal Antibodies to Respiratory Syncytial Virus Proteins: Identification of the Fusion Protein

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Six monoclonal antibodies directed against respiratory syncytial virus proteins were produced. Each was characterized by immunoprecipitation and indirect immunofluorescence. One was directed against the nucleocapsid protein, NP 44, two were directed against a 37,000-dalton protein, two were directed against the major envelope glycoprotein, GP 90, and one was directed against the 70,000-dalton envelope protein, VP 70. Indirect immunofluorescence stain patterns of infected HEp-2 cells defined GP 90 and VP 70 as viral proteins expressed on the cell surface, whereas NP 44 and the 37,000-dalton protein were detected as intracytoplasmic inclusions. One of the anti-GP 90 antibodies neutralized virus only in the presence of complement but did not inhibit cell-cell fusion. The anti-VP 70 antibody neutralized virus without complement and inhibited cell-cell fusion of previously infected HEp-2 cells, thus identifying VP 70 as the fusion protein.

Respiratory syncytial virus (RSV), an RNA virus, is the major cause of lower respiratory tract disease in infants and young children. Electron microscopy shows the virus to be 120 to 200 nm in diameter, with an envelope containing surface projections (2). Attempts to identify and characterize the proteins of RSV have been hampered by several factors. Loss of infectivity during purification procedures and the fact that 80% of sucrose band-purified material is nonviral make the study of purified viral proteins difficult and may account for some of the discrepancies among different laboratories (8). Between 7 and 10 presumed viral polypeptides ranging in molecular weight from 200,000 (200K) to 10K have been identified by polyacrylamide gel electrophoresis of radiolabeled RSV protein (6, 13, 15). A 90K glycoprotein and a 50K glycoprotein have been identified and suggested as surface glycoproteins based upon their removal by trypsin and detergent treatment of purified RSV (12). Recently, the 50K glycoprotein has been shown to be connected to a 20K protein by disulfide bonds, forming a 70K protein (9). The functional designation of the 90K and 70K glycoproteins has not been made. A 44K protein is considered to be the nucleocapsid protein, and a 28K protein is considered to be the matrix protein (12).

Although related to paramyxoviruses, RSV does not possess similar biochemical markers.

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\[ \text{MATERIALS AND METHODS} \]

\textbf{Virus and cells.} Bovine embryonic kidney (BEK), MA 104, and HEp-2 cells were maintained in minimal
essential medium supplemented with 5% heat-inactivated fetal calf serum and 2 mM glutamine. The Long strain of RSV was plaque purified, grown to stock titers in HEp-2 cells (5 × 10⁶ PFU/ml), and frozen at −70°C until use. Purified viral samples were prepared by polyethylene glycol precipitation followed by band purification in a continuous sucrose gradient, as previously described (1).

Hybridoma production. CAF-1 mice (6 weeks old) were immunized in accordance with two schedules. In the first immunization schedule, 0.1 ml of RSV antigen (CF antigen, lot 3-9178; Microbiological Associates) in complete Freund adjuvant was given intraperitoneally (i.p.), followed 2 weeks later by i.p. injection of the same RSV antigen without adjuvant. 3 days after the second injection, spleen cells were used for fusion.

In the second immunization schedule, mice were intranasally infected with 10⁵ PFU of virus of 0.2 ml of medium, and after 6 weeks, 0.1 ml of purified BEK-grown virus (0.1 mg of protein per ml) mixed with complete Freund adjuvant was given i.p. Three weeks later, the same antigen without adjuvant was given i.p., and after another 3 weeks, an injection of unpurified BEK-grown RSV cell pellet was given i.p. Three days later, spleen cells were used for fusion.

The spleen cells from immunized mice were fused with nonsecretor X63/Ag8.653 BALB/c-derived myeloma cells at a 3:1 ratio, in accordance with the method of Fazekas de St. Groth and Scheiddegger (7, 10). Culture fluids were screened for anti-RSV antibody initially by an enzyme-linked immunosorbent assay and later by an indirect fluorescent-antibody (IFA) test on acetone-fixed RSV-infected HEp-2 cells. Uninfected HEp-2 cells were used as controls. Positive hybridomas were cloned twice by limiting dilution, expanded, and injected into pristane-primed mice for ascites production.

Characterization of monoclonal antibodies. Monoclonal immunoglobulin class and subclass were determined by Ouchterlony double gel diffusion analysis of hybridoma tissue culture fluids by using goat antiserum with heavy- and light-chain specificities (Meloy and Litton Bionetics).

Virus neutralization. The ability of the monoclonal antibodies to neutralize RSV was assayed by a 90% plaque reduction neutralization test. Heat-inactivated ascitic fluid dilutions (100 μl) were mixed with 100 μl of media containing 50 to 100 PFU of virus, incubated for 1 h at 37°C, and then inoculated onto HEp-2 cells in 24-well Costar plates. After an adsorption period of 1 h, the virus-antibody mixture was removed and the cell sheet was overlaid with 0.6% LTG agarose (type VII; Sigma Chemical Co.) containing minimal essential medium and 5% fetal calf serum. Typical syncytial plaques were read at 4 to 5 days after fixation and methylene blue staining. Complement-enhanced neutralization was performed in parallel by the inclusion of a 1:24 dilution of fresh rabbit serum in the antibody mixture. Control experiments were done with heat-inactivated rabbit serum.

Radiolabeling of virus, RIP, and polyacrylamide gel electrophoresis. RSV-infected and sham-infected HEp-2 cells were radiolabeled with [35S]methionine and [3H]glucosamine-labeled RSV.

TABLE 1. Monoclonal antibodies to RSV proteins

<table>
<thead>
<tr>
<th>Monoclonal antibody</th>
<th>Immunoglobulin class</th>
<th>RSV protein specificity (mol wt)*</th>
<th>Virus neutralization titer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Without complement*</td>
</tr>
<tr>
<td>D14</td>
<td>IgG2α</td>
<td>NP 44(44K)</td>
<td>&lt;1:4</td>
</tr>
<tr>
<td>G2</td>
<td>IgG1</td>
<td>VP 37(37K)</td>
<td>&lt;1:4</td>
</tr>
<tr>
<td>L2</td>
<td>IgG1</td>
<td>VP 37(37K)</td>
<td>&lt;1:4</td>
</tr>
<tr>
<td>L4</td>
<td>IgG2β</td>
<td>VP 70(70K)</td>
<td>1:512</td>
</tr>
<tr>
<td>L7</td>
<td>IgG2α</td>
<td>GP 90(90K)</td>
<td>&lt;1:4</td>
</tr>
<tr>
<td>L9</td>
<td>IgG2α</td>
<td>GP 90(90K)</td>
<td>&lt;1:4</td>
</tr>
</tbody>
</table>

* Determined by RIP of [35S]methionine- or [3H]glucosamine-labeled RSV.
† Determined by 90% plaque reduction neutralization of ascitic fluid dilution without complement.
‡ Determined by 90% plaque reduction neutralization of ascitic fluid dilution with complement.
infection in HEp-2 cells was determined by incubating infected cell monolayers in media containing monoclonal antibodies. HEp-2 cells were grown on glass cover slips in 12-well plates until nearly confluent. The monolayers were infected with 50 to 100 PFU of virus, allowed to adsorb for 60 min, covered with 2 ml of minimal essential medium, and incubated at 37°C. After 8 h, the medium was aspirated and replaced with medium containing a 1:50 dilution of heat-inactivated monoclonal antibody. Cover slips were removed daily for 4 days, washed five times in phosphate-buffered saline, air dried, and fixed in cold acetone. Detection of virus was assayed by syncytial plaque formation and by an IFA test, using anti-NP 44 monoclonal antibody (D14) to detect viral antigen within cells.

RESULTS

Isolation and characterization of monoclonal antibodies to RSV proteins. Six hybridoma cell lines secreting monoclonal antibodies to RSV were isolated (Table 1). Three antibodies were of subclass IgG2a, two were of subclass IgG1, and one was of subclass IgG2b. RIP of [35S]methionine-labeled RSV-infected cell lysates demonstrated that one monoclonal antibody (D14) was directed against the 44K protein (Fig. 1). This protein is considered to be the nucleocapsid protein (NP 44) (12). Two monoclonal antibodies (L2 and G2) precipitated a 37K viral protein (Fig. 1). Monoclonal antibody L4 precipitated both a 70K and a 50K protein (Fig. 1). The relative intensities of the 70K and 50K proteins detected in [35S]methionine-labeled viral lysates was determined by the duration of the labeling period. As the labeling period increased from 1 to 3 h, the amounts of 70K protein decreased and the amounts of 50K protein increased. This finding is consistent with that reported by Dubovi (6). Two monoclonal antibodies (L7 and L9) immunoprecipitated a 90K glycoprotein (Fig. 2). This glycoprotein is best labeled with [3H]glucosamine and is not labeled with [35S]methionine (6, 13, 15).

Neutralization of virus. Ascites fluid containing anti-VP 70 antibody (L4) neutralized virus to a titer of 1:512. The addition of complement to the antibody-virus mixture enhanced neutralization by L4 at least fourfold to a titer of >1:2,048. Neither of the anti-GP 90 antibodies (L7 and L9) alone neutralized virus. However, in the presence of complement, one of the antibodies, L7, neutralized virus to a titer of 1:2,048 (Table 1). The anti-VP 37 antibodies and the anti-NP 44 antibody did not neutralize virus.

Immunofluorescence patterns. Different IFA staining patterns of RSV-infected HEp-2 cells were noted for the various antibodies. The anti-NP 44 antibody (D14) gave intracytoplasmic staining with large inclusion bodies (Fig. 3A). Non-acetone-fixed infected cells demonstrated markedly reduced fluorescence, indicating that the majority of nucleoprotein antigens are within the infected cell. Although not detected on the
surfaces of isolated infected cells. NP 44 antigen was detected on the surfaces of syncytia when cytopathic effect was extensive. IFA staining with anti-VP 70 antibodies (L2 and G2) also showed intracytoplasmic inclusions (data not shown). Both anti-GP 90 and anti-VP 70 antibodies produced a characteristic fluorescent pattern on the peripheries of both acetone-fixed and non-acetone-fixed infected cells (Fig. 3B and C). Filamentous extensions protruding from the surfaces of infected cells were often evident with anti-GP 90 antibodies, whereas anti-VP 70 antibody produced a speckled pattern.

**Inhibition of the spread of infection by monoclonal antibodies.** Antibodies were tested for their ability to inhibit fusion by observing the development of syncytia in RSV-infected HEp-2 cells incubated in the presence of monoclonal antibodies. Infected cultures incubated with standard media or with media containing monoclonal antibodies to the nucleocapsid protein (D14) or to VP 37 (L2) progressed such that by day 3, the entire cell sheet was infected and giant syncytia were noted by light microscopy and IFA staining (Fig. 4A and B). Similarly, incubation of infected cells with anti-GP 90 antibodies (L7 and L9) did not inhibit the progression of syncytium formation, as compared with controls.

In contrast, incubation of infected cells with anti-VP 70 antibody (L4) markedly inhibited cell fusion and ultimate syncytium formation (Fig. 4C and D). On days 2, 3, and 4, only single cells were infected, as determined by IFA staining, and syncytium formation did not occur. The number of infected cells did not appreciably increase over the 4-day period. Because L4 was used at a neutralizing dilution (1:50), any virus released from infected cells would be expected to be neutralized.

**DISCUSSION**

Of the 7 to 10 RSV-specifed polypeptides previously demonstrated, only the 44K nucleocapsid protein has been given a definite structural designation (12, 15). The immunofluorescence patterns we obtained with monoclonal antibodies to GP 90 and VP 70 indicate that these glycoproteins are expressed on the cell surface by 24 h postinfection, a finding consistent with the concept that RSV is released from infected cells by budding. Monoclonal antibodies to VP 37 gave intracytoplasmic IFA staining. The monoclonal antibody to NP 44 localized the nucleocapsid protein in an intracellular location early in the infectious cycle, as determined by IFA staining, but demonstrated its presence on the surfaces of syncytia late in the course of infection. This may represent staining of nucleocapsid protein underlying a damaged cell membrane. As expected, monoclonal antibodies to VP 37 or to NP 44 did not neutralize virus or exert any effect on the growth pattern of RSV in tissue cultures.

Based on the molecular weight and surface location of GP 90 of RSV, Fernie and Gerin (9) have suggested that it may be analogous to the hemagglutinin of the paramyxoviruses which functions as the viral glycoprotein responsible for adsorption of virus to cells (14). Although GP 90 of RSV does not possess hemagglutinating or neuraminidase activity, it is reasonable to suspect that it also might function in cell attachment. The inability to neutralize RSV with our two anti-GP 90 monoclonal antibodies does not negate this possibility. These monoclonal antibodies may be directed toward an epitope on GP 90 distinct from the one responsible for virus-host cell adsorption. Multiple monoclonal antibodies or polyclonal antibodies to GP 90 may be needed to clarify the potential role of this protein in cell attachment. That one of the anti-GP 90 antibodies was capable of neutralizing virus in the presence of complement suggests that neutralization is mediated by viral membrane lysis and that antibodies to GP 90 might be important in immunity to infection. Although able to neu-
FIG. 3. Immunofluorescence staining of RSV-infected HEp-2 cells by monoclonal antibodies. Infected cells were grown on cover slips, air dried, and fixed in cold acetone. Slides were incubated with 1:100 dilutions of monoclonal antibodies followed by a 1:25 dilution of fluorescein isothiocyanate-labeled sheep anti-mouse IgG antibody. Photographs were taken at a magnification of ×225 in black and white with ASA 400 film and 4-min exposures. (A) Intracytoplasmic fluorescence with inclusion bodies produced by the anti-NP 44 antibody (D14). A similar pattern was produced by a anti-VP 37 antibody (L2). (B) Speckled peripheral pattern produced by the anti-VP 70 antibody (L4). (C) Peripheral filamentous extensions produced by anti-GP 90 antibodies (L7 and L9).
neutralize RSV in the presence of complement, this anti-GP 90 antibody was unable to prevent the spread of infection by cell-cell fusion.

Fernie and Gerin, by analysis of iodinated purified virus under reducing and nonreducing conditions, demonstrated that VP 70 is composed of a 20K and a 50K protein bound by disulfide bonds (9). Because of the structural similarity to the fusion proteins of other enveloped viruses, they suggested that VP 70 might be the fusion protein of RSV. In contrast to the anti-GP 90 antibodies, our anti-VP 70 antibody neutralized virus in the absence of complement and in addition prevented fusion of RSV-infected cells, thus identifying VP 70 as the fusion protein.

The fusion protein may be involved in the cell penetration step of some enveloped viruses by fusing the viral envelope to the host cell membrane, resulting in delivery of the ribonucleoprotein into the cell cytoplasm (3). Neutralization of RSV by anti-VP 70 antibody might be mediated by inhibition of virus-cell fusion. The anti-VP 70 monoclonal antibody described by Cote et al. differs from ours in that it was able to neutralize virus but did not inhibit cell-cell fusion (5). This suggests that either the fusion protein facilitates cell adsorption or that neutralization of virus occurs by a mechanism other than inhibition of cell attachment. It is also possible that virus-cell fusion and cell-cell fusion may be mediated by different regions of the fusion protein.

Reinfecction with RSV in the presence of circulating neutralizing antibodies is common in both infants and adults. However, the protein specificity of these neutralizing antibodies has not been fully explored. Because of the cell-cell spread of RSV, neutralizing antibody to GP 90

FIG. 4. Immunofluorescence staining of previously infected cells incubated in the presence of monoclonal antibodies. HEp-2 cells were infected with 50 to 100 PFU of virus and incubated 8 h later with media containing monoclonal antibodies, as described in the text. RSV-infected cells were detected by IFA staining with anti-NP 44 antibody followed by fluorescein isothiocyanate-labeled sheep anti-mouse IgG antibody. (A) Extensive syncytium formation and viral spread on day 3 in untreated control cells (magnification, ×50). Similar syncytium formation was after incubation with anti-NP 44, anti-VP 37, or anti-GP 90 antibody. (B) High-power view (×225) of single syncytium at day 3. (C) Absence of syncytium on day 3 when infected cells were incubated with anti-VP 70 antibody (magnification, ×50). Only single infected cells were seen on day 3, demonstrating the inhibition of fusion by monoclonal antibody L4. (D) High-power view (×225) of single infected cell incubated with anti-VP 70 antibody, demonstrating typical swollen appearance. Cells typically ruptured by day 4 without fusion occurring.
may not be as effective in preventing infection as neutralizing antibody that is directed to the fusion protein and can also limit cell-cell spread of virus.

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LITERATURE CITED