Quantification of Respiratory Syncytial Virus Polypeptides in Nasal Secretions by Monoclonal Antibodies

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SUMMARY

An indirect enzyme-linked immunosorbent assay (ELISA) which uses monoclonal antibody as solid-phase immunosorbent was developed to measure specific polypeptides of respiratory syncytial virus (RSV). The assay was used to examine 43 nasopharyngeal (NP) aspirates from RSV-positive infants that had been examined previously for RSV by culture, direct immunofluorescence, and polyclonal antibody ELISA. Frozen NP aspirates were serially diluted and examined for the 66K mol. wt. fusion glycoprotein (F), the 84K large surface glycoprotein (G) and the 41K nucleoprotein (N) by monoclonal capture ELISA. F protein was detected in all 43 specimens, G protein was detectable in 20 (47%) and N protein in 22 (51%) of 43 NP aspirates. In specimens with detectable G and N proteins, F was detected by endpoint titration at approximately tenfold greater dilutions than either G or N. In 19 sequential NP aspirates from five patients with RSV infection, F was present in higher titre throughout infection. In 20 cases, matching cell culture isolates were examined by immunofluorescence with strain-specific monoclonal antibodies. Three of 20 isolates showed strain-specific differences by their lack of reaction with anti-G monoclonal antibody. Titration of the 20 cell culture isolates by monoclonal antibody capture ELISA showed the relative amount of F and N proteins to be equal in all cases, whereas levels of G protein tended to be slightly lower. Reconstruction experiments with NP aspirates demonstrated that degradation of F and N proteins did not occur in NP aspirates, but that G protein antigenicity appeared to be affected by nasal secretions. When compared with cell culture-grown material, nasal secretions contained abundant F protein but a surprisingly low concentration of N protein.

INTRODUCTION

Respiratory syncytial virus (RSV) is the single most important cause of lower respiratory tract infection in children (Glezen & Denny, 1973). In vitro biochemical and genetic studies have identified ten major RSV gene products (Bernstein & Hruska, 1981; Fernie & Gerin, 1982; Dubovi, 1982; Lambert & Pons, 1983; Collins et al., 1984) and assigned their location within the virion (Peeples & Levine, 1979; Gruber & Levine, 1983; Huang et al., 1984). Both the 84K to 90K mol. wt. (G) and 66K to 70K mol. wt. fusion (F) surface glycoproteins have been shown to be the major targets for antibody-mediated neutralization in cell culture (Cote et al., 1981; Fernie et al., 1982; Walsh & Hruska, 1983; Walsh et al., 1984a) and in animal models (Taylor et al., 1984; Walsh et al., 1984b). A third RSV structural protein, the non-glycosylated 41K mol. wt. nucleoprotein (N) has been identified as a major viral component recognized by human

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Table 1. Characteristics of antibodies used

<table>
<thead>
<tr>
<th>Designation</th>
<th>Immunizing virus strain</th>
<th>Protein specificity*</th>
<th>Isotype/species</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>13-1</td>
<td>RSV Long</td>
<td>F (66K)</td>
<td>IgG1/mouse</td>
<td>Cote et al. (1981)</td>
</tr>
<tr>
<td>6-3</td>
<td>RSV Long</td>
<td>N (41K)</td>
<td>IgG2a/mouse</td>
<td>Cote et al. (1981)</td>
</tr>
<tr>
<td>MOPC-21</td>
<td>-</td>
<td>-</td>
<td>IgG2a/mouse</td>
<td>Hendry et al. (1984)</td>
</tr>
<tr>
<td>M-117</td>
<td>RSV Bovine</td>
<td>Polyclonal</td>
<td>Gnotobiotic calf</td>
<td>Cranage et al. (1981)</td>
</tr>
<tr>
<td>HaRS</td>
<td>RSV Long</td>
<td>Polyclonal</td>
<td>Horse</td>
<td>Hendry &amp; McIntosh (1982)</td>
</tr>
</tbody>
</table>

* As determined by radioimmunoprecipitation.

serum antibodies and is probably present on the surface of RSV-infected cells (Ward et al., 1983).

The role of RSV polypeptides expressed during infection in stimulating host immunity remains largely undefined. Re-infection with RSV occurs at all ages in the presence of serum antibody (Henderson et al., 1979; Glezen et al., 1981) and the importance of specific mucosal immunity in recovery and protection is unclear (McIntosh et al., 1978; Kaul et al., 1981). Furthermore, variation amongst RSV isolates has been shown when individual polypeptides are compared (Cash et al., 1977; Ward et al., 1984; Gimenez et al., 1984; Anderson et al., 1985), and differences at this level may be important in the design of diagnostics and vaccines and in understanding the incomplete natural immunity seen following RSV infections.

We have developed a sensitive enzyme-linked immunosorbent assay (ELISA) and have demonstrated its ability to measure specific RSV polypeptides in crude preparations (Hendry et al., 1985). In this report, we have applied this technique to study the production of G, F and N proteins of RSV in infant nasal secretions and describe antigenic variation during a community outbreak.

METHODS

Patients. All study specimens were from respiratory secretions received in the Diagnostic Virology Laboratory of the Children's Hospital, Boston, during the RSV epidemics of 1980 to 1981 and 1981 to 1982. The patients were for the most part hospital inpatients and ranged in age from less than 1 month to about 2 years (mean 5.4 months), with 95% of them under 1 year of age. Specimens were taken at the discretion of the house staff on admission, shortly thereafter, or, in the case of nosocomially acquired infections, at the onset of symptoms. If serial specimens were sought, permission was first obtained from the parents, and all subsequent samples were taken by a specially trained research nurse.

Clinical specimens and viral cultures. Nasopharyngeal (NP) aspirates were obtained by gentle suction through a No. 5 or a No. 8 French catheter into a suction trap. Aspirated secretions were transported to the laboratory undiluted at 4 °C. Secretions were suspended in 0.5 to 1.0 ml of phosphate-buffered saline (PBS) and portions were removed for viral culture and indirect immunofluorescence (IF) as described previously (McIntosh et al., 1982; Gardner & McQuillin, 1980). The remaining specimen was dispersed by probe sonication for 30 s at 4 °C and assayed for total protein by the method of Bradford (1976), modified for microtitre plates. Secretions were assayed for RSV antigen by ELISA as described previously (Hendry & McIntosh, 1982; McIntosh et al., 1982) and stored at −70 °C until needed.

Specimens were inoculated onto HEp-2, rhesus monkey kidney, and human foreskin fibroblasts as described previously (McIntosh et al., 1982). When 2+ to 3+ cytopathic effects were seen, infected cells were scraped into the culture medium and isolates were stored at −70 °C.

Immunofluorescence (IF). Methods for IF of RSV isolates in NP aspirates and HEp-2 cell cultures by use of a polyclonal bovine anti-RSV antisera and mouse monoclonal antibodies have been described previously (Gardner & McQuillin, 1980). The remaining specimen was dispersed by probe sonication for 30 s at 4 °C and assayed for total protein by the method of Bradford (1976), modified for microtitre plates. Secretions were assayed for RSV antigen by ELISA as described previously (Hendry & McIntosh, 1982; McIntosh et al., 1982) and stored at −70 °C until needed.

Immunoglobulin fractions of ascitic fluids or sera were absorbed to polyvinylchloride microtitre plates at −70 °C.

Antibodies. The antibodies used are shown in Table 1. Immunoglobulin fractions were obtained from either mouse ascitic fluids or sera by ammonium sulphate precipitation at one-third saturation (25 °C) followed by dialysis against PBS. Affinity-purified fluoresceinated antibodies to mouse immunoglobulin G (γ chain), bovine immunoglobulins (heavy and light chains), and horseradish peroxidase-conjugated anti-bovine antibodies were obtained from Kirkegaard and Perry Laboratories, Gaithersburg, Md., U.S.A.

ELISA. The specificity, sensitivity and methods used are described in detail elsewhere (Hendry et al., 1985). Immunoglobulin fractions of ascitic fluids or sera were absorbed to polyvinylchloride microtitre plates (0-1
ml/well) at a total protein concentration of 2 µg/ml in PBS and stored at 4 °C until needed. On the day of the test, the wells were aspirated and 0·1 ml of PBS plus 0·5% gelatin was added and incubated for 30 min at 37 °C to block unbound sites on the solid phase. The plates were washed three times with PBS plus 0·05% Tween-20 (PBS-T), and 0·1 ml of secretion diluted in PBS plus 0·15% Tween-20, 0·5% gelatin and 3 mM-EDTA (PBS-T-G-E) was added to wells containing monoclonal capture antibodies. After incubation overnight at ambient temperature (25 °C) the contents were aspirated and the wells washed three times with PBS-T. Fifty µl of a bovine antiserum to RSV diluted 1:500 in PBS plus 0·15% Tween-20 and 0·5% gelatin (PBS-T-G) was added and incubated for 1·5 h at 37 °C. The wells were washed three times with PBS-T and 0·1 ml of a 1:1000 (100 ng/ml) dilution of peroxidase-labelled goat anti-bovine antibody in PBS-T-G was added and incubated for 1 h at 37 °C. The wells received a final three washes with PBS-T and 0·125 ml of OPD substrate (0·4 mg/ml orthophenylene diamine in 0·1 M-citrate-phosphate pH 5·5 containing 0·03% H₂O₂) was added and incubated for 30 min in the dark at room temperature. The peroxidase reaction was terminated by the addition of 0·025 ml of 3·5 M-HCl and the absorbance at 490 nm determined with an ELISA spectrophotometer (EL. 307 I.P., Bio-tek Instruments, Burlington, Vt., U.S.A.).

Reconstruction experiments were performed by a modification of the method described above. A HEp-2 cell-grown preparation of RSV (Long strain) diluted 1:100 in PBS-T-G-E was used as the standard viral antigen. Selected nasal secretions were also diluted in PBS-T-G-E to a final concentration of 1:30, and the RSV antigen was incubated with diluted secretion or diluent alone overnight at 25 °C in microtitre wells containing capture antibodies. The percent reduction in absorbance in antigen preparations containing secretions was computed for each monoclonal capture antibody according to the formula \[1 - \left(\frac{A_{490\text{ with secretion}}}{A_{490\text{ without secretion}}}\right)\] × 100.

Data analysis. For ELISA, all data points represent the mean of duplicate wells. The outside wells of each microtitre plate received substrate only and served as blanks for zeroing the spectrophotometer. Specimens were diluted at half-log intervals ranging from 1:3 to 1:1000 for aspirates and from 1:10 to 1:3000 for HEp-2 cell culture isolates. A positive ELISA value was determined by using a cutoff value of 2 times the absorbance of the negative control (MOPC-21 capture antibody) at the lowest dilution used. An interactive statistics program (Minitab) was used for graphics and data analysis. For immunofluorescence, all slides were read under code by at least two readers.

RESULTS

Titration of RSV polypeptides in nasal secretions

NP aspirates previously shown to be positive for RSV by cell culture isolation and/or immunofluorescence were examined by polyclonal ELISA (McIntosh et al., 1982; Hendry & McIntosh, 1982). Forty-three specimens that yielded an absorbance > 0·5 at a 1:1 dilution were examined by monoclonal capture antibody ELISA. Typical results for titration of RSV polypeptides in a secretion are shown in Fig. 1. The titres obtained were 1:1000 with polyclonal capture (HaRS), 1:100 with F protein capture (13-1), and 1:30 with G protein (111-2) and N protein (6-3) capture antibodies. Similar titrations were performed on a total of 43 NP aspirates and endpoint titres were determined as above. The results summarized in Fig. 2 indicate that polyclonal capture antibody is the most sensitive reagent for detecting RSV antigens. Relative to F, both G and N polypeptides were detected at approximately tenfold lower concentrations and were not found at all (< 1:3) in 23 and 21 specimens, respectively. One-way analysis of variance on the distribution of log₁₀ titres indicated that this difference was significant (\(P < 0·05\)).

Analysis of paired RSV cell culture isolates

To test whether antigenic or other phenotypic differences among individual strains of RSV could account for the results in Fig. 2, and to compare the quantity of antigens produced in tissue culture with that found in secretions, we obtained the cell culture isolates of these secretions and examined them in several ways. HEp-2 cell culture isolates were available from 20 of the 43 aspirates shown in Fig. 2. All 20 of these originated in the 1981 to 1982 epidemic.

First, monoclonal capture antibody ELISA was used to examine the 20 isolates for differences in their capacity to produce detectable F, G and N polypeptides in HEp-2 cell cultures. Twenty frozen cell culture isolates (cells plus medium) were titrated as above and the results are shown in Fig. 3(a). All 20 cell culture isolates reacted with monoclonal antibodies 13-1 and 6-3. Three isolates (open circles) were unreactive by ELISA with capture antibody 111-2. With the exception of these three isolates, no significant differences were seen in these tissue culture-grown preparations between the log₁₀ titres for F, G and N polypeptides. Fig. 3(b) represents
Fig. 1. Titration of RSV antigens in nasal secretion. The y axis represents the $A_{490}$ of the indicated capture antibody minus the $A_{490}$ of the control capture antibody, MOPC-21. The cutoff value (broken line) was determined from the $A_{490}$ of MOPC-21 at a 1:3 specimen dilution. Capture antibodies: ●, 13-1 (F protein); ○, 111-2 (G protein); △, 6-3 (N protein); ▽, HaRS.

Fig. 2. Titration of RSV antigens in 43 nasal secretions by monoclonal capture antibody ELISA.
the results of titrations of the matching subset of 20 NP aspirates with results similar to those in the larger sample shown in Fig. 2. These results indicate that, with the exception of G protein in the three isolates indicated in Fig. 3, the relative concentrations of F, N and G polypeptides detected in nasal secretions were probably not due to antigenic heterogeneity or to differences in the phenotypic expression of the three polypeptides produced in HEp-2 cell cultures.

Second, the 20 isolates were examined by IF after staining with polyclonal antiserum M-117 and monoclonal antibody 111-2. The same three isolates which were unreactive by ELISA (Fig. 3(a) also failed to stain with anti-G monoclonal antibody 111-2. Thus, 15% of these 20 isolates showed an absence of G protein in the monoclonal antibody ELISA for reasons of antigenic heterogeneity, and it is likely that some other secretions shown in Fig. 2 would have failed to react for the same reason. In other studies of RSV-containing secretions examined by IF (Kao et al., 1984; Kim et al., 1983) monoclonal antibodies 13-1 and 6-3 were shown to react with virtually all samples positive with polyclonal antiserum.

The differences in RSV antigen content shown in Fig. 2 and 3(b) might have been due to different avidities of the monoclonal capture antibodies or the polyclonal detector antibody used. To investigate this problem further, five NP aspirates were titrated with the use of an additional panel of two G, two N and one F monoclonal antibodies. In all cases, similar endpoint titres were obtained for monoclonal antibodies with similar specificities (data not shown). Experiments using HaRS in the detector position gave endpoints identical to those obtained with M-117 (data not shown). These experiments suggest that the observed RSV antigen concentrations in secretions were probably not due to differences in avidity between the particular antibodies used for ELISA.

**Stability of RSV polypeptides in nasal secretions**

A series of reconstruction experiments was performed to examine whether the relatively low levels of G and N polypeptides in secretions were due to specific degradation by factors in human nasal secretions. A standard preparation of HEp-2-grown RSV (Long strain) was diluted in PBS-T-G-E buffer either with or without selected nasal secretions, and then assayed by...
Table 2. Effect of nasal secretions on RSV polypeptides as determined by monoclonal capture antibody ELISA

<table>
<thead>
<tr>
<th>Specimen in diluent</th>
<th>RSV†</th>
<th>Benzamidine‡</th>
<th>Percent inhibition of absorbance with indicated capture antibody*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>13-1</td>
</tr>
<tr>
<td>82-298</td>
<td>+</td>
<td>-</td>
<td>20</td>
</tr>
<tr>
<td>82-416</td>
<td>+</td>
<td>-</td>
<td>-14</td>
</tr>
<tr>
<td>82-331</td>
<td>+</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>15</td>
</tr>
<tr>
<td>82-554</td>
<td>-</td>
<td>-</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>-3</td>
</tr>
</tbody>
</table>

* A HEp-2 cell-grown isolate (Long strain) was used at a 10⁻² dilution in PBS-T-G-E, or in the same buffer containing a 1:30 dilution of the indicated nasal secretion. †% Inhibition of control = [1 - A₄₉₀ with PBS-T-G-E + 1:30 secretion/A₄₉₀ with PBS-T-G-E] × 100. ‡+, Specimen positive for RSV antigen by ELISA; -, specimen negative for RSV antigen by ELISA.

ELISA (Table 2). The results indicate that both F and N polypeptides were unaffected by the presence of nasal secretions; in contrast, the specific ELISA signal for G was reduced by 32 to 84% with such treatment, thus indicating that the low titres of G in secretions could be due to specific degradation of this protein, specific antibody, or both. The addition of benzamidine hydrochloride, an inhibitor of proteolytic enzymes, abolished this effect for a RSV antigen-negative secretion, but failed to do so for a secretion containing detectable RSV antigen. None of the four secretions shown in Table 2 showed detectable anti-RSV antibody by live cell immunofluorescence. In addition, repeated freeze-thaw cycles did not affect the relative concentrations of RSV antigens in either secretions or cell culture isolates (data not shown).

Thus, although the mechanism(s) for inhibition of G protein remains unknown, it is likely that, for the F and N proteins, the differences in relative antigen concentrations in secretions were due to expression in vivo, rather than differences in antigenic stability or subsequent degradation by factors contained in nasal secretions. For the G protein, differences might have been due to a number of mechanisms.

Titration of RSV polypeptides in sequential specimens

For five patients, 19 sequential NP aspirates were obtained during the course of their RSV infections. Results from a typical patient shown in Fig. 4 indicate that F protein was detected throughout infection, and was the only antigen detectable by 2 days after the initial specimen, at a time when no virus was isolated. The cell culture isolate from this patient reacted with all three monoclonal capture antibodies. Similar patterns were seen with the other four patients (data not shown).

DISCUSSION

We have used a sensitive monoclonal antibody ELISA to measure the relative concentrations of three RSV structural proteins in infant secretions. The antigen specificity of the assay has been demonstrated previously (Hendry et al., 1985). To our knowledge, specific viral proteins in respiratory secretions during acute infection have never been measured before. By our assays, F protein is present in higher concentrations than either N or G proteins when compared to concentrations present in the homologous cell culture isolates (Fig. 1, 2 and 3).

When tissue culture-grown patient isolates were examined by IF, we observed no staining with the anti-G monoclonal antibody in three of the 20 tested. These three isolates probably represent Group 2 strains as described by Anderson et al. (1984), and this was confirmed by their reaction with a Group 2-specific monoclonal antibody (data not shown). Similar antigenic heterogeneity may have been responsible for some of the G-negative nasopharyngeal secretions in Fig. 2.
RSV polypeptides in secretions

Fig. 4. Titration of RSV antigens in sequential specimens from a patient with RSV infection. Capture antibodies: ○, 13-1; △, 111-2; ▲, 6-3; △, HaRS.

With the exception of the three Group 2 RSV isolates mentioned above, HEp-2 cell culture-grown RSV isolates produced F, G and N antigens which were detectable at the same level by ELISA (Fig. 3a). This finding contrasts with the apparent relative concentrations of the three proteins found in NP aspirates (Fig. 2 and 3b) and suggests that the expression of these RSV polypeptides in vivo differs from that seen in vitro. Further evidence for this hypothesis can be seen in reconstruction experiments designed to measure the inhibitory effect of nasal secretions on the monoclonal capture antibody ELISA (Table 2). No specific inhibition of the amount of F and N proteins measured was seen, although the amount of antigenically active G protein was somewhat reduced. In the case of G protein, these findings may reflect the stability of the specific epitope defined by monoclonal antibody 111-2 or they may be due to specific degradation of, or antibody binding to G. Our failure to detect free anti-RSV antibody in these secretions does not exclude the possibility of immune complexes in these secretions, although it seems likely that in some cases we would have detected excess free antibody had it been present. Further experiments examining a larger number of secretions will be needed to distinguish these possibilities. In the case of F and N antigens, however, lack of inhibition of the ELISA signal in these reconstruction experiments indicates that the results in Fig. 2 probably reflect viral antigen expression in the respiratory tract of infants.

A test such as this which depends on the affinity of several serological reagents (in this case monoclonal antibodies) to measure distinct antigens cannot be assumed to be measuring quantities of these antigens relative to each other. On the other hand, an analysis of the quantity of each of the three antigens in secretions in relation to the same antigens in cell culture isolates from these secretions (Fig. 3) is not affinity-dependent. Moreover, insertion of five other monoclonal species in the capture position gave identical results, suggesting that affinity may not have played an important role in our findings.

The high levels of F found in nasal secretions with this assay suggest that F may be the predominant RSV antigen released into nasal secretions from infected respiratory epithelia. This may represent tissue-specific modulation of the expression of viral proteins (Minor et al., 1979; Kristensson et al., 1983). Examples of differential expression of specific viral gene
products have been described at the transcriptional (Sixbey et al., 1984), translational (Carter et al., 1983), and post-translational levels (Schiel et al., 1983; Roux et al., 1984), and alterations in the antigenicity of post-translationally modified viral glycoproteins have been shown (Alexander & Elder 1984; Skehel et al., 1984). Furthermore, factors such as defective interfering particles (Roux & Waldvogel, 1983) and specific antibody (Fujinami et al., 1984) have been shown to affect the expression of specific viral proteins in vitro. Whatever mechanisms play a role in RSV antigen expression, it is important to understand the nature of the antigenic stimulus that occurs during viral replication in the respiratory epithelium. Such information is crucial to the understanding of both local and systemic immune responses to RSV infection.

The low level or absence of detectable N antigen in secretions in relation to the high level in matched cell culture isolates was somewhat surprising. Ward et al. (1983) found high levels of anti-N and anti-F antibodies in sera of infants and adults, and lower or undetectable amounts of anti-G activity. Differences in the antigenicity of RSV polypeptides, antibody isotype, virus strain or local versus systemic antibody response may account for their findings. Further experiments will be needed to distinguish these factors and to confirm our results. We (Kao et al., 1984) and others (Bell et al., 1983; Kim et al., 1983) have used monoclonal antibody to examine by IF epithelial cells shed from the respiratory tract of infants infected with RSV. Both F protein and N protein were seen in all but a few specimens examined. It is interesting that in our own work cells containing the N protein appeared to be less numerous than those with F protein and were at times difficult to find (Kao et al., 1984). In any case, these IF studies examined only intracellular antigens and were not quantitative. The presence and gradual disappearance of detectable RSV antigens in sequential specimens also are consistent with findings in a previous study (McIntosh et al., 1982).

Immunoprecipitation of secretions labelled in situ (Anderson et al., 1984) or Western blotting (Gimenez et al., 1984; Walsh et al., 1984a; Ward et al., 1984) may allow us to examine further the nature of RSV polypeptides produced in nasal secretions and to confirm these results. Successful immunoprophylaxis for RSV infection will require an understanding of antigen expression in vivo, the importance of antigenic variation, and the nature of the protective host response to RSV infections.

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