Antigenic and Structural Variation in the Major Nucleocapsid Protein of Respiratory Syncytial Virus

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SUMMARY

Antigenic and structural variation in the major nucleocapsid protein, VPN41, from different strains of respiratory syncytial (RS) virus was observed using a combination of monoclonal antibodies and two-dimensional peptide mapping. Limited trypsin treatment of intact nucleocapsids produced two peptide fragments Mr 27K and Mr 14K. Two monoclonal antibodies, N1 and N2, reactive with primary sequence epitopes located on intact nucleocapsids also reacted with either the 27K fragment (N2) or the 14K fragment (N1). Competitive radioimmunoassay studies using N1 and N2 antibodies revealed two discrete antigenic groups among the seven human strains of RS virus examined. A bovine strain of RS virus, although antigenically similar to the human strain of RS virus, was placed in a separate group. Two-dimensional peptide mapping of 125I-labelled VPN41 purified by SDS-PAGE revealed extensive structural homology between all strains. However, several unique tryptic/chymotryptic peptides supported the grouping obtained with the monoclonal antibodies.

INTRODUCTION

The peak prevalence of severe human respiratory syncytial (RS) virus infection lies between the ages of 6 weeks and 5 months (Gardner, 1973; Parrott et al., 1973) suggesting that maternal IgG persisting in the baby's blood confers little or no protection and perhaps even contributes to disease by interaction with RS virus antigen in the lung (Chanock et al., 1970). The major nucleocapsid polypeptide VPN41 is the most immunogenic RS virus protein in the natural infection and significant levels of maternal antibody to VPN41 are present in the sera of infants during the high risk period for bronchiolitis (Ward et al., 1983). Presumably, such antibodies may form immune complexes in the lung by interaction with nucleoprotein which is expressed on the surface of infected cells and syncytia (Fernie et al., 1981; Walsh & Hruska, 1983).

The major nucleoprotein of RS virus has been identified as a polypeptide of Mr 41K (Wunner & Pringle, 1976) which may be cleaved by cellular proteases to a form detected as VP38 (Cash et al., 1979). Despite its potential importance in the pathogenesis of bronchiolitis few data are available on the structure and function of the RS virus major nucleoprotein. In other negative-stranded RNA viruses the nucleoprotein has been completely sequenced and a multifunctional role defined with each nucleoprotein molecule binding about 20 RNA bases (Choppin & Compans, 1975) while complexes of nucleoprotein with RNA and polymerase are active in transcription (Bishop et al., 1971). Despite these key functions, studies using monoclonal antibodies have detected considerable antigenic variation in the nucleoprotein of influenza virus (van Wyke et al., 1980). In a search for structural and antigenic variation in the major nucleoprotein of selected RS virus isolates we have studied the binding of two monoclonal antibodies designated N1 and N2 which react with primary structure epitopes of VPN41. Differences in monoclonal antibody-binding properties were found to correlate with variations in the tryptic/chymotryptic digest maps of VPN41.
**Methods**

Cells. HEp-2 and Vero cell lines were cultured in Dulbecco's minimal essential medium (DMEM) supplemented with 10% (v/v) or 2% (v/v) foetal calf serum (FCS, Flow Laboratories) respectively. Cells were incubated at 37 °C in an atmosphere of 5% (v/v) CO₂ in air.

Growth of virus and preparation of nucleocapsids. Human strains of RS virus [Long, Edinburgh (Ogilvie et al., 1981), CC79, SR80, HN81, AV82, CP83 (local Southampton isolates from sequential epidemic years 1979, 1980, 1981, 1982, 1983 respectively)] were grown in HEp-2 or Vero cells maintained in DMEM supplemented with 1% (v/v) FCS or 0.5% (v/v) FCS respectively. The Compton 127 strain of bovine RS virus was grown in bovine embryonic kidney cells and was generously provided by Dr E. J. Stott (A.F.R.C. Institute for Research on Animal Diseases, Compton, Berkshire, U.K.). Viral nucleocapsids were purified from infected HEp-2 or Vero cells as previously described (Ward et al., 1983).

Monoclonal antibodies. The procedure for the production of hybridomas secreting monoclonal antibodies reactive with RS virus polypeptides has been described previously (Ward et al., 1983). Briefly, BALB/c mice were immunized intramuscularly with 100 μg of nucleocapsids purified from the Edinburgh strain of RS virus grown in HEp-2 cells. Antigen was administrated in complete Freund's adjuvant and mice were boosted at weeks 4 and 8 with the same dose in incomplete Freund's adjuvant and 4 days prior to fusion with 50 μg intravenously. The spleen cells were fused with BALB/c NS1/1 (NS-1) myeloma cells and hybridomas were screened for the production of anti-nucleoprotein antibodies by ELISA and were cloned by limiting dilution. Phenotypically stable hybrids were expanded by growth in culture and approximately 10⁵ cells were injected intraperitoneally into BALB/c mice primed with Pristane (2,6,10,14-tetramethylpentadecane; Sigma). Ascitic fluid was clarified by centrifugation at 15000 g for 10 min and stored at −70 °C.

Purification of monoclonal antibodies. Two monoclonal antibodies specific for VPN41, designated N1 and N2 (both subclass IgG2a) were purified by affinity chromatography on Protein A-Sepharose (Watanabe et al., 1981). Ascitic fluids were applied to the column in 0.14 M-phosphate buffer pH 8.2 and the IgG was eluted with 0.2 M-sodium citrate pH 4 and neutralized by collection into 1 M-Tris-phosphate buffer pH 7.2.

Radioiodination of Protein A and monoclonal antibodies. Purified monoclonal antibodies and staphylococcal Protein A (Sigma) were labelled with Na¹²⁵I using the solid-phase chloramine-T reagent, 'Iodo-beads' (Pierce, Rockford, Ill., U.S.A.). Protein was adjusted to a concentration of 1 mg/ml in phosphate-buffered saline (PBS) and mixed with 500 μCi Na¹²⁵I (15 mCi/μg) in a total volume of 200 μl. A single Iodo-bead was added and the reaction allowed to continue for 5 min. Unreacted iodide was removed by chromatography on Sephadex G-25 packed in a Pasteur pipette.

Radioimmunoassay and competitive inhibition studies. Purified nucleocapsids from the human RS virus isolates and bovine strain were diluted to a concentration of 1 μg protein/ml in coupling buffer (100 mM-Na₂CO₃/NaHCO₃ pH 9.6) and 100 μl volumes added to the wells of 'high-activated' PVC immunoassay plates (Flow Laboratories). In parallel experiments nucleocapsids at a concentration of 100 μg protein/ml were denatured with an equal volume of 2°(w/v) SDS, 1% (w/v) dithiothreitol (DTT) at 100 °C for 10 min prior to dilution in coupling buffer. After 16 h incubation antigen-coated plates were washed four times with PBST [PBS containing 0.05% (v/v) Tween 20] and the antibody was added. For each antigen eight replicate tests using 100 ng of iodinated N1 or N2 (both 1 μCi/μg) per well were performed with a tenfold excess of unlabelled antibody in the competitive inhibition studies. After incubation at room temperature for 2 h the plates were washed as before and the amount of radioactive antibody bound was determined by solubilization with 1% (w/v) SDS in 0.1 M-NaOH and γ-counting. Results were expressed as ng of antibody bound to the well and the percentage inhibition of binding was determined from the formula: 100 × (1 − (ng of labelled antibody bound in the presence of unlabelled antibody + ng of labelled antibody bound in the absence of unlabelled antibody)).

Trypsin treatment of purified nucleocapsids. Nucleocapsids prepared from HEp-2 cells were washed twice in TE buffer (10 mM-Tris-HCl pH 7.4, 0.1 mM-EDTA) by centrifugation at 10000 g for 30 min, and resuspended at 1 mg protein/ml in TE buffer. TPCK-trypsin (Sigma, type XIII) was added to a final concentration of 5 μg/ml and the mixture incubated at 37 °C. Aliquots were withdrawn at various time intervals and the cleavage products analysed by electrophoresis and reaction with the N1 and N2 monoclonal antibodies.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and protein electroblotting. Polypeptides separated by gradient SDS-PAGE (Ward et al., 1983) were transferred to nitrocellulose sheets (BA85, 0.45 μm, Schleicher & Schüll) essentially as described by Towbin et al. (1979). Transfer was at 7.5 V/cm for 16 h at 4 °C in 20 mM-Tris, 150 mM-glycine, 20% (v/v) methanol. Free binding sites on the nitrocellulose were blocked by incubation in a solution containing 3% (w/v) bovine serum albumin (BSA; Sigma, fraction V), 0.9% (w/v) NaCl, 10 mM-Tris-HCl pH 7.4 for 1 h at 37 °C. The nitrocellulose sheets were rinsed with distilled water, blotted dry and incubated with ascitic fluid diluted 100-fold in gelatin buffer: 0.15 M-NaCl, 5 mM-EDTA, 0.05% (v/v) NP40, 0.25% (w/v) gelatin (Difco), 50 mM-Tris-HCl pH 7.4. After 1 h at room temperature the nitrocellulose sheets were rinsed with gelatin buffer. Radioiodinated Protein A (7 μCi/μg) was diluted in gelatin buffer to 1.5 μCi/ml and applied to the sheets for 1 h at room temperature. The labelled sheet was then washed in gelatin buffer, dried and radioactive
bands detected by autoradiography at \(-70^\circ\text{C}\) using Kodak XAR-5 film with a DuPont Cronex Lightning Plus intensifying screen.

**Peptide mapping.** Proteolytic digests of carboxymethylated VPN41 radiolabelled with methyl-3,5-di\([^{125}\text{I}]\)iodo-hydroxybenzimidate were compared by two-dimensional peptide mapping as described by Lambden (1982). Freeze-dried nucleocapsid preparations containing 1 mg protein were solubilized in 1 ml modification buffer: 0.5 M-Tris-HCl pH 8.3, 6 M-guanidine hydrochloride, 25 mM-sodium EDTA, 10 mM-2-mercaptoethanol. The solution was flushed with N\(_2\) and incubated at 37\(^\circ\text{C}\) for 2 h. Nucleocapsids were carboxymethylated by the addition of iodoacetic acid (5 mg/ml final concentration) followed by incubation in the dark for 2 h at room temperature. The reaction was stopped by the addition of 50 \(\mu\)l 2-mercaptoethanol and the nucleocapsids were dialysed exhaustively against 0.4\% (w/v) \(\text{NH}_4\text{HCO}_3\). The carboxymethylated nucleocapsids were freeze-dried and each sample was dissolved in 100 \(\mu\)l 4 M-guanidine hydrochloride, 100 mM-N-ethylmorpholine-acetic acid pH 9 and transferred to a tube containing a dried aliquot (225 \(\mu\)Ci) of methyl-3,5-di\([^{125}\text{I}]\)iodohydroxybenzimidate (4000 Ci/mmol; Amersham). The tubes were sealed and incubated at 37\(^\circ\text{C}\) for 48 h. Incorporation of radiolabel was approximately 40\%. The radiolabelled nucleocapsids were then dialysed against 0.4\% (w/v) \(\text{NH}_4\text{HCO}_3\), freeze-dried and the labelled protein was separated by SDS-PAGE. After electrophoresis the VPN41 band was located by staining, excised and washed in 50\% (v/v) methanol. The washed gel slice was cut into small fragments, freeze-dried and rehydrated in 200 \(\mu\)l 0.4\% (w/v) \(\text{NH}_4\text{HCO}_3\) containing trypsin (Sigma, type III) plus chymotrypsin (Sigma, type II) each at a final concentration of 25 \(\mu\)g/ml. The gel slices were incubated with the proteases for 6 h at 37\(^\circ\text{C}\) when 200 \(\mu\)l of fresh protease solution was added and the incubation continued for a further 16 h. The supernatant fraction containing the solubilized peptides released by proteolysis was removed, clarified by centrifugation at 10000 \(\times\) g for 5 min and freeze-dried. The peptides were re-dissolved in 50 mM-\(\text{NH}_4\text{OH}\) and samples (approx. 0.015 \(\mu\)Ci) spotted onto silica gel thin-layer chromatography plates (10 \(\times\) 10 cm; Polygram SilG, Machery-Nagel, Düren, F.R.G.). Peptides were resolved by electrophoresis (250 V for 40 min) at pH 3.5 (pyridine/acetic acid/water; 1:10:189, by vol.) in the first dimension followed by ascending chromatography (n-butanol/pyridine/acetic acid/water; 15:10:3:12, by vol.) in the second dimension. Radioactive peptides were detected by autoradiography.

**RESULTS**

**Monoclonal antibodies to VPN41**

Analysis of the immunoreactivity of hybridoma ascitic fluids towards RS virus nucleocapsid protein separated by SDS-PAGE and electroblotted onto nitrocellulose revealed two independent monoclonal antibodies, N1 and N2, reactive with the major nucleoprotein VPN41. These two monoclonal antibodies were presumed to be specific for primary structure epitopes because of their reactivity towards protein denatured with SDS and 2-mercaptoethanol. The N1 and N2 epitopes were shown by limited proteolytic hydrolysis of intact nucleocapsids to lie on separate tryptic fragments of VPN41. A 30 min incubation with trypsin yielded two polypeptide fragments \(M_1\), 14K and 27K reactive with N1 and N2 respectively (Fig. 1). The \(M_1\), 14K polypeptide accumulated during the incubation whereas the 27K fragment increased in amount over a 3 min period and then declined until there was none detectable at 30 min, suggesting that further proteolysis of the 27K fragment had occurred.

**Binding of monoclonal antibodies to VPN41**

The binding of monoclonal antibodies N1 and N2 to either intact, native nucleocapsids or SDS/DTT-denatured nucleocapsids was measured by radioimmunoassay. The results presented in Table 1 showed that equal amounts of antigen prepared from different RS virus isolates varied two- to threefold in binding capacity for the N1 monoclonal antibody. SDS/DTT-denatured nucleocapsids from all strains bound a greater amount of N1 than N2. For each strain the binding capacity of native VPN41 for N1 was approximately half that of the denatured protein. However, a comparison of the binding properties of N2 suggested that nucleocapsid proteins from the seven human isolates fell into two groups. The Long, Edinburgh, SR80 and CP83 strains showed approximately equal binding of N1 and N2 to the native capsid and the binding of both N1 and N2 was increased by SDS/DTT treatment of the nucleocapsids. In contrast, native nucleocapsids from the CC79, HN81 and AV82 strains bound less N2 than N1 and there was little or no increase in N2 binding on SDS/DTT denaturation. These differences in antibody binding were best illustrated by comparison of the binding ratio N1/N2 (Table 1). The bovine viral nucleocapsid shared the N1 and N2 epitopes with the human isolates.
Table 1. Binding of monoclonal antibodies N1 and N2 to the major nucleocapsid protein from different RS virus strains

<table>
<thead>
<tr>
<th>RS virus strain</th>
<th>Native nucleocapsids</th>
<th>Denatured nucleocapsids*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgG bound (ng)†</td>
<td>N1/N2</td>
</tr>
<tr>
<td></td>
<td>N1</td>
<td>N2</td>
</tr>
<tr>
<td>Long</td>
<td>17-0</td>
<td>14-8</td>
</tr>
<tr>
<td>SR80</td>
<td>10-5</td>
<td>10-2</td>
</tr>
<tr>
<td>Edinburgh</td>
<td>9-1</td>
<td>9-2</td>
</tr>
<tr>
<td>CP83</td>
<td>12-5</td>
<td>10-4</td>
</tr>
<tr>
<td>AV82</td>
<td>9-5</td>
<td>5-8</td>
</tr>
<tr>
<td>HN81</td>
<td>7-5</td>
<td>3-5</td>
</tr>
<tr>
<td>CC79</td>
<td>5-7</td>
<td>3-1</td>
</tr>
<tr>
<td>Bovine</td>
<td>7-3</td>
<td>8-8</td>
</tr>
</tbody>
</table>

* Nucleocapsids were denatured with 1% SDS/0-5% DTT.
† Based on a specific activity of 1 μCi/μg protein for both N1 and N2 antibodies.

The proximity of the N1 and N2 epitopes on both native and SDS/DTT-denatured nucleocapsids was investigated by competitive binding studies (Table 2). In each case the competition by the homologous unlabelled antibody was close to the expected value of 90% using a 10:1 ratio of unlabelled to labelled antibody. In native nucleocapsids from all strains the binding of N1 was inhibited by N2 (11 to 25%), whereas in denatured nucleocapsids inhibition of N1 binding by N2 did not exceed 6%. However, the inhibition of N2 binding by N1 was greater in the CC79, HN81 and AV82 isolates for both native and denatured antigen, which further supported the grouping of the human RS virus strains on the basis of the N1/N2 binding ratio.

Peptide mapping of the major nucleocapsid protein

Evidence that the grouping of strains by their antigenic behaviour towards two primary sequence monoclonal antibodies reflected structural variations was sought by peptide mapping.
Table 2. Competitive binding of monoclonal antibodies N1 and N2 to the major nucleocapsid protein from different strains of RS virus

<table>
<thead>
<tr>
<th>RS virus strain</th>
<th>Native nucleocapsids</th>
<th>Denatured† nucleocapsids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N1 by N1</td>
<td>N1 by N2</td>
</tr>
<tr>
<td>Long</td>
<td>88 ($P &lt; 0.01$)</td>
<td>16 ($P &lt; 0.01$)</td>
</tr>
<tr>
<td>SR80</td>
<td>86 ($P &lt; 0.01$)</td>
<td>15 ($P &lt; 0.01$)</td>
</tr>
<tr>
<td>Edinburgh</td>
<td>87 ($P &lt; 0.01$)</td>
<td>16 ($P &lt; 0.01$)</td>
</tr>
<tr>
<td>CP83</td>
<td>87 ($P &lt; 0.01$)</td>
<td>18 ($P &lt; 0.01$)</td>
</tr>
<tr>
<td>AV82</td>
<td>87 ($P &lt; 0.01$)</td>
<td>25 ($P &lt; 0.01$)</td>
</tr>
<tr>
<td>HN81</td>
<td>87 ($P &lt; 0.01$)</td>
<td>13 ($P &lt; 0.01$)</td>
</tr>
<tr>
<td>CC79</td>
<td>87 ($P &lt; 0.01$)</td>
<td>16 ($P &lt; 0.01$)</td>
</tr>
<tr>
<td>Bovine</td>
<td>81 ($P &lt; 0.01$)</td>
<td>11 ($P &lt; 0.01$)</td>
</tr>
</tbody>
</table>

* The competing antibody was present in tenfold excess.
† Nucleocapsids were denatured with 1% SDS/0.5% DTT.
Purified nucleocapsids prepared from the seven human strains and the bovine strain of RS virus were each carboxymethylated and radioiodinated with the primary amine labelling reagent methyl-3,5-di[¹²⁵I]iodohydroxybenzimidate. Nucleocapsids were separated into their component polypeptides by SDS-PAGE. The band corresponding to VPN41 was excised from the gel.
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Fig. 3. Diagrammatic representation of the peptide map autoradiographs. Peptide maps were grouped into four distinguishable patterns: (a) SR80, Long; (b) CP83, Edinburgh; (c) CC79, HN81, AV82; (d) Bovine. Spots with solid boundaries represent major peptides and spots with dotted boundaries represent minor peptides. Cross-hatching denotes unique peptides with the major unique peptides arrowed. Acidic peptides within the broken line were poorly resolved.

and subjected to a combined tryptic and chymotryptic cleavage. The resultant radiolabelled peptides were resolved by two-dimensional thin-layer peptide mapping and detected by autoradiography (Fig. 2). Digests from different strains were mixed and the peptide maps compared with individual maps in order to identify common peptides. Consensus peptide map patterns (Fig. 3) were obtained by comparing five mapping experiments from each of three independent tryptic/chymotryptic digests. The major nucleocapsid protein from all strains of RS virus, including the bovine strain, showed a high degree of structural homology, with several major and minor peptides common to all strains. However, some of the major peptides (arrowed in Fig. 3) showed different mobilities in a number of the strains and allowed a classification of the strains into discrete groups (Fig. 3). One group comprising the CC79, HN81 and AV82 strains was characterized by two major unique peptides (Fig. 3c), whereas the other strains showed only one major unique peptide in this area of the map. This peptide was resolved as a discrete spot in maps of the Long and SR80 strains but as a streak in maps of the Edinburgh and CP83 strains. Although it is possible that this streak represents two or more peptides, repeat analyses failed to
confirm this, in contrast to the well-separated peptides on the CC79, HN81 and AV82 maps. Peptide maps of the bovine strain revealed a pattern containing a single major peptide which was shown to be distinct from other strains by mixed digest mapping experiments.

DISCUSSION

RS virus major nucleoprotein, VPN41, was cleaved by trypsin treatment of intact nucleocapsids into two fragments, Mr 14K and Mr 27K. The monoclonal antibodies N1 and N2 reactive with VPN41 in both the native and SDS/DTT-denatured states bound to epitopes located on the cleavage fragments Mr 14K and Mr 27K respectively. Although occurring on separate proteolytic fragments, the N1 and N2 epitopes were presumably in proximity since the binding of antibody to the native N1 site was inhibited significantly by N2 antibodies in all eight strains tested. When nucleocapsids were denatured by treatment with SDS and DTT, the binding of the N1 antibody was doubled and inhibition of N1 by N2 antibodies was lost. These findings suggest that the N1 epitope is partially obscured by the N2 site in the native molecule.

The presence of a trypsin-susceptible cleavage point on the intact nucleoprotein may indicate the existence of an exposed region on VPN41 linking two domains, since in their native conformation such sites are particularly sensitive to proteolysis (Konigsberg & Steinman, 1977). The complete sequence of the 467 amino acids comprising the nucleocapsid protein of RS virus strain A2, has been established (Elango & Venkatesan, 1983). Although the protein shows a relative excess of basic amino acids, their clustering within specific secondary structural domains was not observed when the sequence was subjected to computer analysis (Elango & Venkatesan, 1983). Thus, identification of lysine or arginine residues at the trypsin-sensitive cleavage site will require analysis of N-terminal sequences from the two cleavage fragments.

Peptide maps of VPN41 showed that although the seven human isolates and the bovine strain had the majority of the hydrophobic and most basic peptides in common there was significant variation among the more hydrophilic peptides. In digests from isolates CC79, HN81 and AV82 two major hydrophilic peptides were clearly resolved compared to the single peptide or streak in the other isolates. This division by peptide mapping of VPN41 into structural groups paralleled variation in the binding properties of the monoclonal antibodies N1 and N2. The major nucleoproteins of the CC79, HN81 and AV82 group were characterized by a relatively low binding capacity for N2 compared to N1, and significant inhibition of N2 binding by N1 antibodies was confined to native nucleocapsids of this group. Nucleoproteins from the remaining human strains were characterized by a higher binding capacity for N2 and a lower N1/N2 binding ratio. These findings are compatible with a variable hydrophilic portion of VPN41 exposed at the surface of intact nucleocapsids forming the binding site for the N1 and N2 monoclonal antibodies. The nucleocapsid proteins of the orthomyxoviruses also show significant antigenic variation (van Wyke et al., 1980), and are exposed to immune systems at the cell surface functioning as target antigens for antibody-mediated cytotoxic mechanisms (Townsend & Skehel, 1982). By analogy, strain variation in the antigenic structure of the nucleoproteins of RS virus may have survival value. Clearly, the role of nucleoprotein in the pathogenesis of RS virus infection warrants further attention.

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