Linear antigenic and immunogenic regions of human respiratory syncytial virus N protein

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Three linear antigenic regions on the N protein from human respiratory syncytial virus (RSV) subgroup A (strain A2) were identified by using peptides which reacted in ELISA with sera from humans with recent or previous RSV infection. The determinants were localized within three hydrophilic regions of the protein: Thr1 to Gly30 (N3 peptide), Ser231 to Ala250 (N25 peptide) and Thr331 to Leu391 (N39 peptide). The site represented by the N39 peptide reacted with four subgroup A-specific MAbs. There were minor variations in the amino acid epitope dependencies of each of these MAbs. Two additional antigenic regions Ser1 to Arg150 and Ala231 to Leu290, were represented by peptides that reacted with human convalescent sera, but these peptides did not differentiate between acute and convalescent sera from RSV-infected humans. Rabbit hyperimmune sera raised against selected peptides specifically precipitated different forms of the N protein from a nucleocapsid-containing homogenate derived from extracts of RSV- (subgroup A and/or B)-infected 35S-labelled cells in a radioimmunoprecipitation assay (RIPA); the sera were also used to demonstrate RSV infection in cells by immunofluorescent assay (IFA). Anti-N3 peptide sera precipitated N41, the full-length (Mr 41 000) form of N protein, in a RIPA done on a soluble protein pool. Anti-N39 (C-terminal region) peptide sera precipitated both forms, suggesting that N39 (Mr 38 000) is an N-terminally truncated (probably at position Tyr23 located inside the N3 peptide linear antigenic region) form of N41 protein.

Introduction

Respiratory syncytial virus (RSV) is a negative single-stranded enveloped RNA virus belonging to the genus Pneumovirus in the family Paramyxoviridae. It is the single most important viral agent causing severe lower respiratory tract disease (bronchiolitis and pneumonia) in infants and children (McIntosh & Chanock, 1985). The amino acid sequences of individual RSV proteins have been deduced from the nucleotide sequences of their corresponding genes. This information has allowed a representation of potential T cell and B cell epitopes of the proteins by use of synthetic peptides. Characterization of antigenic and immunogenic properties of such peptides can increase our understanding of RSV immunobiology.

The nucleoprotein (N) of RSV together with phosphoprotein (P), large (L) proteins and viral genomic RNA make up the nucleocapsid core of the virion (Collins, 1991), the site of the transcription–replication complex of the virus (Barik, 1992; Huang et al., 1993). Studies of other negative-stranded viruses (Bishop et al., 1971), for example vesicular stomatitis virus (VSV) (reviewed by Banerjee & Barik, 1992), have defined a multifunctional role for the nucleoprotein in transcription–replication. In order to understand the role of different parts of RSV N protein in these functions, a need exists for more data on the structure and function of the N protein and its antigenic determinants (Ward et al., 1983; Collins, 1991). The aim of our study was to identify antigenic and immunogenic regions on the RSV N protein and to map epitopes within these sites at the level of individual amino acids.

Immunodominant epitopes may be involved in protein–protein interactions within protein complexes, as in the case of the glycoprotein complex of human immunodeficiency virus (HIV) (Neurath et al., 1992). Peptides that mimic VSV P protein sequences or anti-N protein sera that interfere with the viral transcription–replication process have been reported (Yamashita & Kawai, 1990; Hill & Summers, 1982). Thus peptides, anti-peptide animal sera and MAbs can be used for immunochemical characterization of viruses (Norrby et
al., 1987; Åkerlind-Stopner et al., 1990) and may be useful tools for structural–functional studies of the replicative complex of RSV.

Recently we reported similar studies of the RSV P protein (Leonov et al., 1994). In the present study, we identified three immunodominant linear antigenic regions by use of synthetic overlapping peptides representing the RSV N protein. Peptides that reacted with a panel of 20 RSV-positive sera derived from convalescing patients and 14 paired sera from RSV-infected patients were identified. Linear antigenic sites were localized within three hydrophilic regions of the N protein: Thr₁₁ to Gly₂₀ (N3 peptide), Ser₂₃₁ to Ala₄₅₀ (N25) and Thr₂₇₁ to Leu₃₉₅ (N39). The site represented by peptide N39 reacted with four subgroup A-specific MAbs. Within this region the epitope dependence on individual amino acids was characterized. Rabbit hyperimmune sera raised against selected peptides specifically precipitated different forms of the N protein from RSV-infected [³⁵S]labelled cell extracts in a radioimmunoprecipitation assay (RIPA) and indicated RSV infection in cells by immunofluorescent assay (IFA).

Methods

**Virus.** RSV prototype strain A2 (subgroup A) was passaged in Vero cells and stored at −70 °C before use.

**Cells.** Vero cells were grown in Eagle's MEM supplemented with 3% fetal calf serum (FCS) that had been inactivated by heating at 56 °C for 30 min. Virus was passaged as described elsewhere (Åkerlind-Stopner et al., 1988) in nearly confluent monolayer cultures at 10 p.f.u./cell. At the time of inoculation the medium was changed to Eagle's MEM with 0.5% FCS.

**Peptides.** The derived amino acid sequence of RSV strain A2 N protein (Galmiski, 1991) was used for simultaneous multiple solid-phase peptide synthesis according to the method of Houghten (1985). Peptide amides were synthesized using t-Boc amino acids (Bachem) and p-methylbenzhydrylamine resin (Fluka) and cleaved by liquid hydrogen fluoride in a multi-vessel apparatus. The peptides demonstrated 55–97% homogeneity, as tested by HPLC on a Nova-pak C₁₇ column (Waters Associates). Amino acid composition was confirmed by amino acid analysis. A Cys residue was coupled to the C-terminal end of those peptides which lacked this amino acid in their sequence to enable coupling to keyhole limpet haemocyanin (KLH) as described by Sambrook et al. (1989).

**MAbs.** Antibodies specific for the N protein and, as a control, the P protein were used. One group of MAbs was produced using the Long replicative complex of RSV.

**Antibodies.** Antibodies specific for the N protein and, as a control, the P protein were used. One group of MAbs was produced using the Long replicative complex of RSV.

**Animal sera.** Rabbits were injected subcutaneously with 100 μg of KLH-conjugated synthetic peptides in Freund's complete adjuvant and then with two injections of the same peptides in Freund's incomplete adjuvant at 3 week intervals. The animals were bled 10 days after the last injection. Pre-immune and hyperimmune rabbit sera were collected and stored at −20 °C until use.

**ELISA.** ELISA with purified RSV antigen and peptides was performed as previously described (Åkerlind-Stopner et al., 1990; Leonov et al., 1994). The results were expressed either as A₄₉₂ (mean of three determinations) or, for substituted peptide analogues, as the percentage of peptides binding. The titres of immune anti-peptide sera were calculated as the highest dilution that gave a mean A₄₉₂ value that exceeded the 'cut-off' value by more than 0.2 absorbance units, i.e. exceeded the mean of three determinations of A₄₉₂ performed in wells with the same dilution of pre-immune sera plus two standard deviations.

The peptide competition ELISA was performed as described previously (Leonov et al., 1994). Briefly, different concentrations (from 0–20 μg/ml) of the competitive peptide together with a standardized dilution of MAb (giving 50% of the maximum A₄₉₂) in ELISA dilution buffer were mixed, added to the wells pre-adsorbed with either homologous or heterologous peptide and allowed to react for 3 h at 37 °C. Competition ELISA between MAbs and HCS was performed in a similar manner using different dilutions (from 1:50–1:1000) of HCS as the competitive reactant. To analyse the peptide analogues in soluble form a competition ELISA based on a double antibody sandwich (DAS) protocol (Joisson & Van Regenmortel, 1991) was prepared with some modifications. Briefly, different concentrations of competitive peptide analogues together with standardized (giving 50% of the maximum A₄₉₂ value, i.e. without competitor) dilution of purified nucleocapsids were mixed and added to the wells pre-adsorbed with one of the affinity purified MAbs (either B90, C3222, RS102 or RS204C). Rabbit anti-N3 peptide serum was used as the source of the capture antibodies. Results were calculated as percentage inhibition compared to the value obtained with the original full-length N39 peptide (assumed to give 100% inhibition).

**RIPA.** The preparations of RIPA RSV antigen (from subgroup A, strain A2- and subgroup B, strain CH18537-infected Vero cells) and the RIPA procedure were slightly modified from previously described methods (Leonov et al., 1994). Briefly, PBS-washed [³⁵S]methionine-labelled cells were disrupted on ice in hypotonic Tris–HCl buffer pH 8.0

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Table 1. Amino acid sequences of the immunoreactive N protein peptides

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<tr>
<th>Peptide</th>
<th>Amino acid sequence*</th>
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<tr>
<td>N3</td>
<td>T<del>1</del> TmLDQGSSSSKYz1TIQRSTGz2C</td>
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<tr>
<td>N15</td>
<td>S<del>13</del> RKSYYKMLKEMGEVAPEYRz15C</td>
</tr>
<tr>
<td>N20</td>
<td>A<del>18</del> VRRANNVLKMKRMKLYKGLz20C</td>
</tr>
<tr>
<td>N25</td>
<td>S<del>23</del> STRGGSRVEGIFAGLFMNz25C</td>
</tr>
<tr>
<td>N39</td>
<td>T<del>37</del> AAELAEKHQILPKDNDVz39C</td>
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* The amino acids (in bold) represent identified antigenic determinants containing epitopes (underlined). The amino acids positions in the original RSV strain A2 N protein sequence (Galinski, 1991) are indicated.

by Dounce homogenization and clarified samples were overlaid onto a two-step 20% (w/w)-60% (w/w) sucrose gradient. After centrifugation (90 min, 12 °C, 260 000 g) the ‘free’ (FF) protein pool fraction from the top of the 20% sucrose and the ‘nucleocapsid-associated’ (NCF) protein pool fraction from the 20-60% sucrose interface were sequentially collected by gentle aspiration and dissolved in lysis buffer (Leonov et al., 1994); after final centrifugation (13000 g) for 5 min the supernatant was used as RIPA antigen. A mixture of protease inhibitors (Protease inhibitors set), prepared according to the manufacturer (Boehringer Mannheim) was added to the samples at each step of RIPA antigen preparation. RIPA of these fractions was done as described previously (Leonov et al., 1994).

IFA. IFA of acetone-fixed (-20 °C, 20 min) and formaldehyde-fixed (0.5% in PBS, room temperature, 20 min) RSV (A2)-infected cells was done as previously described (Norrby et al., 1982).

Computer analysis. Analysis of the secondary structure of RSV P protein was performed by use of the DNA Strider 1.1 computer program based on Kyte-Doolittle and Chou-Fasman algorithms.

Results

ELISA reactivity of late convalescent and paired human sera with peptides representing the RSV N protein

Thirty-nine 20-amino-acid-long peptides (with a 10 residue overlap) representing the complete amino acid sequence of the N protein of RSV (strain A2) were synthesized. Each of 20 HCS (from persons of different ages) was tested in ELISA with each of these N protein peptides (designated as Nn, where n is the peptide number). Peptides N3, N15, N20 and N25 (Table 1) reacted with all 20 HCS ("cut-off" A492 value 0.2) and peptide N39 reacted with 17 HCS (Fig. 1). The other N protein peptides demonstrated little reactivity (data not shown). The ELISA reactivity values of each of 16 paired human sera (expressed as A492 convalescent sera:A492 acute sera at one given dilution = C/A ratio) with peptides N3, N15, N20, N25 and N39 (Fig. 2) confirmed their antigenic reactivity. Peptides N3, N25 and N39 exhibited more pronounced differences between convalescent and acute sera than did peptides N15 or N20 (Fig. 2).

Reactivity of MAb's with peptides representing the RSV N protein

The complete collection of 39 peptides was tested in ELISA with anti-N protein MAb's, including four that were subgroup A-specific and 11 MAb's specific to both
subgroup A and B strains. The four subgroup A-specific MAbs (B90, C3222, RS102 and RS204C) demonstrated strong reactivity (A_499 > 1.0) (data not shown) with only one peptide, N39. None of the other peptides reacted with any of the other MAbs. The binding competition ELISA and RIPA showed that N39 can block the interaction between these MAbs and the N protein from RSV (A2)-infected cells (data not shown). In addition, each of 17 HCS blocked the interaction between these four MAbs and the N39 peptide (data not shown).

**Characterization of amino acid dependencies of the epitopes with peptide analogues**

To define the dependence on individual amino acids of epitopes recognized by different MAbs we synthesized substituted analogues of N39 peptide in which each amino acid (starting from the C-terminal Cys) of the original peptide sequence was substituted in turn by Gly. These N39 Gly-substituted analogues were tested in ELISA with RSV subgroup A-specific MAbs B90, C3222, RS102 and RS204C. All the amino acids in the stretch of residues Leu_382 to Glu_390 were crucial (reduced binding of 50% and more) for MAb B90 to show reactivity with the corresponding peptide analogues (Fig. 3a). The reactivity of MAb C3222 showed dependence on this antigenic site and also somewhat on Lys_379 (reduced binding of 20%) (Fig. 3b). A pronounced dependence on Lys_379 (reduced binding of more than 50%) and slight dependence on Gln_381 (reduced binding of 20%) was found with MAb RS102 (Fig. 3c). A few amino acids, Leu_382 to Val_391, were important for interaction of MAb RS204C with these analogues (Fig. 3d). Thus, the antigenic site represented by N39 contained at least four, and possibly more, distinct epitopes.

To characterize the minimal epitope composition which reacted with each of these four MAbs, we tested a set of N39 truncated peptides (Fig. 4). These shortened peptides revealed the need for the neighbouring amino acids Gln_381 and Leu_391 in order to maintain 100% binding of RS204C and B90, 60% binding of C3222 and 48% binding of RS102 compared with the original full-length N39 peptide. The peptide representing amino acid sequence Gln_381 to Leu_391 (N39T) was selected for evaluation of its immunogenic activity in rabbits (sera raised against N39T are referred to subsequently as R210 sera). Interestingly, removal of amino acid Gln_381 allowed the truncated peptide (Leu_382 to Leu_391) to be recognized only by RS204C and B90 (reactivity levels 95% and 65%, respectively). The reaction patterns of peptides (data not shown) were similar when they were studied in soluble form by use of a DAS assay.

**Immunogenic properties of N protein peptides**

Rabbit hyperimmune sera against peptides N3 (R205), N15 (R207), N20 (R208), N25 (R206), N39 (R201) and against the truncated analogue of the N39 peptide N39T (R210) were tested in an ELISA with homologous synthetic peptides and purified RSV subgroup A strain A2 (Table 2) and subgroup B strain CH18537 (data not shown). These sera demonstrated relatively high anti-peptide antibody titres. However, the capacity of these sera to react with whole virus was variable: (i) the highest immunoreactivity was found with R205 antisera; (ii) relatively moderate reactivities were observed for R201, R210 and R206 sera; (iii) borderline antibody activities were found with R207 and R208 sera. The reactivity of the sera in IFA with subgroup A (strain A2) and subgroup B (strain CH18537)-infected Vero cells (data not shown) correlated with ELISA results employing whole virions. The reactivities of sera R207 and R208 were weak, but specific.
Table 2. *ELISA of rabbit anti-peptide sera immunoreactivity with different antigens*

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<td>6400</td>
<td>12500</td>
<td>12500</td>
<td>25600</td>
<td>32000</td>
</tr>
<tr>
<td>RSV (A2) virions</td>
<td>25600</td>
<td>100</td>
<td>100</td>
<td>400</td>
<td>1600</td>
<td>1600</td>
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* Calculated as described (see Methods).
† Short analogue of N39 peptide, i.e. Gln3s to Leua91 (the amino acids positions in the original RSV strain A2 N protein sequence are as referred to in Table 1).

The capacity of the different anti-peptide sera to react with different conformational states of the nucleocapsid protein assumed to be represented in the FF and NCF fractions (see Methods) was assessed in a RIPA using as antigen protein fractions from 35S-labelled cells infected with subgroup A (strain A2) and subgroup B (strain CH18537) (data not shown). These assays (Fig. 5) demonstrated that antisera R205 (lane 1), R206 (lane 6), R201 (lane 5) and R210 (lane 4) precipitated 'N₄₁' (the full-length form of the N protein, apparent Mr 41000) together with 'N₃₈' (the truncated form of the N protein, apparent Mr 38000). R207 (Fig. 5, lane 2) and R208 (data not shown) weakly precipitated the same protein doublet from the NCF fraction and R205 (Fig. 5, lane 7) precipitated only N₄₁ from the FF fraction. All other anti-peptide sera precipitated both N protein forms (as shown for R201, Fig. 5, lane 8) from the FF fraction. In addition, R201 and R210 co-precipitated from the same protein fraction protein bands of apparent Mr 36000, which corresponds to the Mr of mature P protein (Cash et al., 1979). The bands of apparent Mr 30000 were observed in precipitated immune complexes between anti-peptide sera or MAbs and the NCF fraction of RSV subgroup A strain A2-infected cells. All anti-peptide sera precipitated N₄₁ together with N₃₈ forms from the NCF fraction of CH18537-infected cells (data not shown).

**Discussion**

Five antigenic regions of the N protein (Table 1) were identified by use of 20 RSV HCS in an assay of the antigenicity of N-specific peptides. Tests with paired human sera (Fig. 2) confirmed the preferential immunoreactivity of three of the five sites, i.e. Thr₁₁ to Gly₃₀ (N3 peptide), Ser₂₃ to Ala₂₈₀ (N25 peptide) and Thr₃₇₂ to Leu₃₉₁ (N39 peptide). One of the five sites, the C-terminal part represented by the N39 peptide was defined by the four subgroup A-specific MAbs (data not shown) in peptide screening by using a large panel of N protein-specific MAbs (Mufson et al., 1985; Örvell et al., 1987;
protein. The high ELISA titre of anti-N3 sera (R205) suggested high availability of the corresponding N protein site in virions of RSV. Moderate ELISA reactivities of human convalescent and paired sera with peptides N25 and N39 correlated well with the capacity of anti-N39 (R201), anti-N39 truncated analogue (R210) and anti-N25 (R206) sera to identify the linear immunogenic site in the folded protein of whole virions (Table 2). ELISA based on N3 (Fig. 2) as well as on a combination of N3 and N39 reacted more often with acute and convalescent phase RSV infection sera than N39, alone or in combination with N25. These findings should be further confirmed by using a larger panel of paired human sera. The shortest (Leu38 to Leu39) analogue of the N39 peptide elicited RSV subgroup A-specific immune sera in animals as detected by IFA and RIPA (data not shown).

The site-specific reagents generated in this study can be employed for analysis of precursor–product relationships and/or protein–protein interactions (Yamashita & Kawai, 1990; Neurath et al., 1992; Barge et al., 1993; Ryan et al., 1993; Gombart et al., 1993). Two different forms of N protein, i.e. N41 (Mr 41000) and N38 (Mr 38000), were detected by RIPA with the nucleocapsid-associated protein fraction of RSV-infected cells (Cash et al., 1979). One of these forms, N38, was thought to be the truncated (by a chymotrypsin-like protease) form of mature full-length N41 protein. Our anti-peptide sera raised against the N-terminal end only precipitated the N41 polypeptide from the ‘free’ protein fraction, whereas the corresponding anti-peptide serum against the C-terminal end precipitated both N41 and N38. This finding suggests that the latter protein is generated by N-terminal truncation of the N41 protein at one of the potential sites of action of the chymotrypsin-like protease(s) (e.g. Tyr23, producing a truncated polypeptide with an expected Mr of 2800). Precipitation of both N41 and N38 from nucleocapsid-associated protein fractions by either anti-N or anti-C terminal peptide sera suggested strong interactions between N41 and N38 inside nucleocapsids in contrast to the ‘free’ protein pool. The exact role of this N38 form in RSV infection is unclear. The sensitivity of RSV N41 (Cash et al., 1979) and influenza virus nuclear protein (NP) (Zhirnov & Bukrinskaya, 1984) to proteolysis suggests that this event is associated with structural or environmental changes to these polypeptides, one such change being incorporation into nucleocapsids or virions. Nucleocapsid dissociation–reassociation experiments (our unpublished data) have indicated that N41 and N38 without P protein can form complexes (or aggregates) with RNA which have buoyant densities corresponding to that of the mature functional nucleocapsids containing N, P, L and M proteins and viral RNA. Self-assembly of NP protein

![Fig. 5. RIPA reactivities of N protein-specific MAbs and rabbit anti-N peptide sera. RSV antigen from NCF (lanes 1, 2, 4, 5 and 6) and FF (lanes 7 and 8) protein pools (see Methods) were precipitated by each rabbit anti-peptide serum: R201 (a-N39, lanes 5 and 8); R205 (a-N3, lanes 1 and 7); R206 (a-N25, lane 6); R207 (a-N15, lane 2); R210 (anti-N39 truncated analogue, lane 4); RSV protein bands identified are indicated on the right. The Mr values of Rainbow markers (Amersham) (lane 3) are indicated on the left. Polypeptides were resolved by 10% SDS-PAGE and the gel was processed as described in Sambrook et al. (1989).](image-url)
of Sendai virus into nucleocapsid-like particles was recently reported (Buchholz et al., 1993). Although N38 protein incorporation by RSV nucleocapsids was low (Cash et al., 1979), we have observed preferential co-precipitation of the N41 form together with P protein by the anti-P MAb (unpublished). The M protein was always found in P protein-containing nucleocapsids (unpublished). These findings prompt the following questions which are now being investigated: (i) does nascent N41 (after some maturation event, possibly in its C-terminal part) associate with the N38 polypeptide and RNA to produce nucleocapsid-like particles (which are probably not functional at this state) in the absence or presence of low concentrations of P protein; (ii) does a newly synthesized P protein associate with mature N41 or compete out part of N38 from N41-N38-RNA complexes; (iii) do these P-N protein complexes incorporate M and L polypeptides and viral RNA to form active transcription-replication complexes appearing as inclusion bodies (Norrby et al., 1970; Garcia et al., 1993) inside RSV-infected cells.

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References


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