Monoclonal antibody neutralization escape mutants of respiratory syncytial virus with unique alterations in the attachment (G) protein

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Five monoclonal antibody (MAb) neutralization escape mutants of respiratory syncytial virus (RSV) were produced by growing the Long strain RSV (group A virus) in the presence of a neutralizing, group cross-reactive MAb specific for the attachment protein (G). Four viruses (RSV-2, -6, -14 and -15) had amino acid replacements clustered within a highly conserved centrally located 13 amino acid region (position 164–176). Reactivity with group A-specific MAbs and with polyclonal anti-G serum was maintained and growth kinetics were unaffected. An additional virus (RSV-3) had four amino acid substitutions in the cytoplasmic tail and transmembrane region of G, and had restricted growth and formed small syncytia. Immunofluorescent and Western blot analysis indicated that G protein was not membrane associated and had reduced incorporation into the virion, thereby escaping neutralization by L9 and polyclonal anti-G serum. The predominant form of G produced by RSV-3 was found in infected cell supernatants, consistent with the size of secreted G.

Introduction

Respiratory syncytial virus (RSV) is an enveloped, single-stranded negative-sense RNA virus of the genus Pneumovirus in the family Paramyxoviridae, and is the most important cause of serious respiratory illnesses in infants (Collins, 1991). Two major antigenic groups, A and B, are readily distinguished by differences in nucleotide sequence and monoclonal antibody (MAb) reactivity found in several of the 10 RSV proteins (Huang et al., 1985; Sullender et al., 1991; Anderson et al., 1991; Cane & Pringle, 1991; Mufson et al., 1985). One of these is the envelope glycoprotein G, which mediates virus attachment to an undefined cell receptor (Levine et al., 1987). G is a type II glycoprotein with an N-terminal signal/transmembrane region between amino acids 38 and 66, and may exist on the virion surface as a trimer (Langedijk et al., 1996). The G protein of group A RSV is synthesized as a 298 amino acid backbone with a predicted molecular mass of ~33 kDa. Subsequent post-translational modification of the A2 strain G protein by the addition of four N-linked and numerous O-linked carbohydrate side-chains results in a mature protein of ~90 kDa when analysed by SDS–PAGE (Wertz et al., 1989; Fernie et al., 1985). The O-linked carbohydrates are linked to serine and threonine residues which together account for ~30% of the amino acids (Wertz et al., 1985). A high proline content (~10%) is thought to promote a linear, non-globular configuration (Collins & Mottet, 1992).

Despite similarities in overall physical structure, based upon amino acid and carbohydrate composition and hydrophobicity analysis, substantial differences in the primary amino acid sequence and in antigenic relatedness have been noted between the G proteins of the two major RSV groups (Johnson et al., 1987; Walsh et al., 1987). Johnson reported a 53% amino acid sequence identity between G proteins from prototype group A and B viruses (Johnson et al., 1987). Furthermore, 12–20% intragroup variation in amino acid sequence has also been described (Cane & Pringle, 1995; Sullender et al., 1991). Strain differences are most frequent in the extracellular C-terminal region of G, which has only ~43% amino acid identity, in contrast to the relatively well-conserved N-terminal cytoplasmic tail and transmembrane regions (84% identity) (Johnson et al., 1987). The longest stretch of exact identity in the ectodomain is a 13 amino acid region in the mid-portion of
the molecule which is completely conserved among all
naturally occurring RSV strains of either group analysed thus
far (Cane et al., 1991; Garcia et al., 1994; Sullender et al., 1991).
This region, which encompasses amino acids 164–176, includes
the first two of the four conserved cysteine residues proposed
to form a cysteine noose which contributes to the structural
integrity of G (Langedijk et al., 1996; Doreleijers et al., 1996).
Because this region is so highly conserved among RSV isolates
it is speculated that it either possesses cell receptor-binding
activity or provides critically important structural support for
this activity.

The G protein carries several MAbs-defined neutralizing
epitopes (Walsh et al., 1989; Anderson et al., 1985; Storch &
Park, 1987). The majority of neutralizing MAbs to G are group
specific and even polyclonal antibodies to G demonstrate only
minimal group cross-reactivity, suggesting that this common
region is not immunodominant (Walsh et al., 1987). However,
several MAbs which recognize virus strains from both RSV
groups have been described (Anderson et al., 1985; Walsh et
al., 1989; Mufson et al., 1985). One such antibody, L9, binds to
G protein from both group A and B viruses, neutralizes both
viruses in vitro, and when passively administered to mice
protects against pulmonary challenge with group A and B
virus (Walsh et al., 1989). Results of Western blot analysis
suggest that L9 binds to a linear epitope on G which is not
dependent upon glycosylation since it reacts with the de-
natured 33 kDa protein backbone (Walsh et al., 1989). Thus, we
were interested in exploring the binding site of L9 on G, both
to study possible receptor-binding activity of G and for use in
the design of a synthetic peptide antigen capable of stimulating
cross-protective immunity.

Therefore, the G genes of five L9 neutralization-resistant
mutants of a group A RSV virus were sequenced and their G
proteins characterized. The results from four of the escape
mutants suggest that several amino acids located in the 13
amino acid conserved region are important for L9 binding,
especially a phenylalanine at position 170. The final mutant
virus unexpectedly had four amino acid substitutions in the
cytoplasmic tail and transmembrane region, and appeared to
escape neutralization by markedly reducing the amount of G
protein in the viral envelope.

Methods

**Viruses and cells.** Viruses were grown in HEp-2 cells in minimal
essential medium (MEM) with 5 % foetal calf serum (FCS). The Long
strain of RSV (a group A virus) was plaque-purified twice under agarose,
expanded to a stock titre of \( \approx 10^7 \) p.f.u./ml and stored at \(-70 \) °C.
Growth kinetics were determined by inoculating virus on \( 2 \times 10^5 \) HEp-
2 cells in 24-well plates at an m.o.i. of 0.01 in 200 \( \mu l \) of MEM for 1 h at
room temperature. The media were then aspirated, the cells washed three
times with MEM followed by the addition of 10 ml of MEM, and
incubated at 37 °C at 5 % CO\(_2\). Culture supernatants were collected at
various time-points, cell debris was removed by pelleting, fast frozen and
stored at \(-70 \) °C until titration. Neutralization assays were performed
using a standard plaque reduction neutralization assay using 50–100 p.f.u.
virus and a 1:50 dilution of antibody.

**Antibodies.** Previously described MAbs to G, the RSV fusion
protein (F) and nucleocapsid protein (N) were used for immuno-
fluorescence (IFA), Western blot and neutralization assays. L9 and K6 are
G protein-specific, group cross-reactive MAbs, while K1, K2, K5, 78G,
130-2G, and 232-1F are G protein, group A-specific MAbs (Walsh et al.,
1989). L4 and D14 are MAbs specific for RSV F and N proteins,
respectively (Walsh & Hruska, 1983). A 17DD yellow fever virus MAb
(1A5) was used as a negative control. Previously described polyclonal
rabbit anti-G or anti-F sera were also used (Walsh et al., 1986, 1987).

**Production of L9-resistant virus.** L9 (80 \( \mu g/ml \)), fresh rabbit
complement (1:10 final dilution) and 100 p.f.u. of RSV were incubated in
100 \( \mu l \) MEM in 96-well plates for 30 min at room temperature. Then,
\( 1.5 \times 10^5 \) HEp-2 cells in 100 \( \mu l \) were added to each well and the plates
were incubated for 3 days at 37 °C. Culture supernatants were
sequential passaged twice to new wells containing fresh L9–
complement mixture. All wells with maximal cytopathic effect (CPE)
were screened for reactivity with L9 by indirect IFA. Virus from L9-
negative wells was plaque-purified, passaged once, retested with L9 by
IFA, and frozen. Finally, all viruses were tested for L9 resistance in a
plaque reduction neutralization assay.

**Nucleotide sequencing of G gene.** Reverse transcription from
sucrose gradient-purified virion RNA (vRNA), or the RNA present in an
infected cell lysate, was followed by PCR amplification of the cDNA as
previously described (Sullender et al., 1991). The resulting DNA was
cloned into a plasmid vector or asymmetric PCR was performed to
produce a single-strand DNA template for direct sequence determination
(Sullender, 1995). The following primers, with additional nucleotides to
provide restriction sites underlined, were used for cDNA synthesis and
amplification.

\[
\begin{align*}
5' \text{ primer RSL1} & : \text{TAGTCATAACAATGAA, mRNA sense, bases} \\
& : 1–17 \text{ in the SH to G gene intergenic region} \\
5' \text{ primer SH246} & : \text{CTTTGAGTATCAAGACGTCAGAG mRNA} \\
& : \text{sense, bases 240–268 in the SH gene mRNA} \\
5' \text{ primer 5222A1} & : \text{GTCGAGCTACTAGGATTCCCGGAAAATGTCGAGCTACCCCGCTTTTTAGAA, mRNA sense, bases 10–30 in the G gene} \\
& : \text{mRNA} \\
3' \text{ primer G914b} & : \text{CGCGATCCAACTAATGGCG mRNA, sense, complementary to bases 905–918 in the G gene mRNA} \\
3' \text{ primer FIR} & : \text{GATTAGCAGTTCAG, vRNA sense, bases 14–28 in the F gene} \\
3' \text{ primer 5222B1} & : \text{AGCAATTTCGAGCTGTTACGGCGGCTTTTTATGACTAC vRNA sense, complementary to the last 10 bases of the G gene mRNA and the first seven bases of the intergenic region} \\
5' \text{ primer F164} & : \text{GTATGACACTGGTATACCAAC, vRNA sense, complementary to bases 164–186 in the strain 18537 F gene mRNA.} \\
\end{align*}
\]

DNA to be cloned was cleaved internally and the ends were modified
to allow cloning into plasmids. All DNA manipulations and cloning steps
were performed using standard techniques (Ausubel et al., 1997).
Nucleotide sequences were determined from the cloned DNA or direct
sequencing of the amplified cDNA or the product of asymmetric
amplification of the cDNA was performed. Dideoxynucleotide chain
termination sequencing was performed using Sequenase version 2.0
DNA Sequencing (United States Biochemical) or Fidelity DNA Se-
sequencing kits (Oncor, Appligene) and [α-32P]dATP (DuPont NEN) or Thermosequenase radiolabelled terminator cycle sequencing kits and 32P-labelled dideoxynucleotides (United States Biochemical).

Sequence data were analysed using GCG software (Program Manual for the Wisconsin Package, version 8).

Western blot analysis. Samples were separated on 8 or 10% polyacrylamide gels under non-reducing, non-denaturing conditions (0.1% SDS and without heating) and transferred to nitrocellulose paper and developed according to published methods (Walsh et al., 1986).

Indirect immunofluorescence. HEp-2 cells, grown on glass cover-slips, were inoculated with virus at an m.o.i. of 0.01 and then incubated for 24 h at 37 °C. Cover slips were acetone-fixed for development with MAbS and rabbit antibodies. For detection of cell surface-expressed proteins, live infected cell monolayers were incubated with antibody on wet ice before fixing with methanol and staining with FITC-labelled conjugates.

**Results**

**L9 neutralization-resistant viruses**

Since L9 only neutralizes 50–60% of the input virus, selection of escape mutants by single-step neutralization was considered unlikely (Walsh et al., 1989). Therefore virus was grown over a prolonged period in the presence of L9 and complement to promote emergence of resistance. After three cycles of growth (~ 9 days) in L9 and complement, most wells of the 96-well plates showed virus growth, although the extent of CPE was variable. Cells in the majority of wells remained reactive with L9 by IFA, although ~ 35% of wells out of 576 were considered L9-negative by IFA and also had extensive CPE. Viruses from these wells were expanded through a single growth cycle in the absence of L9 and frozen. Five viruses were randomly selected for further evaluation and are identified as RSV-2, RSV-3, RSV-6, RSV-14 and RSV-15. Each virus was plaque-purified under agarose at least twice and tested for L9 reactivity. Four of the escape mutants were consistently negative by IFA, but RSV-3 was persistently, but weakly L9-positive. RSV-3 was then plaque-purified three additional times. Infected cells remained weakly positive by IFA, although the virus continued to be neutralization resistant.

**Growth kinetics**

Growth kinetics from a typical experiment are shown (Fig. 1). RSV-2, RSV-6, RSV-14 and RSV-15 had growth kinetics comparable to the parent virus (RSV-1). In contrast, RSV-3 consistently grew at a slower rate and reached a 100–1000-fold lower titre. RSV-3 was also characterized by slower development of CPE and generally smaller syncytia than the parent virus or the other mutants. In repeated experiments, RSV-3 never exceeded 10⁶ p.f.u./ml, whereas each of the other mutants achieved titres of ~ 10⁸ p.f.u./ml.

**Nucleotide sequence and predicted amino acid sequence of G**

The parent virus (RSV-1) contained four nucleotide changes from the published sequence of the Long strain G protein (Johnson et al., 1987). Two of the nucleotide changes resulted in amino acid substitutions at position 215 (histidine to leucine) and at 244 (isoleucine to threonine), similar to those also reported by Rueda et al. (1994). All of the neutralization escape mutants contained six to eight nucleotide substitutions resulting in three or four amino acid substitutions when compared to the parent virus (Table 1 and Fig. 2). All of the nucleotide substitutions in the escape mutants were U to C changes and spanned the entire genome from nucleotide position 85 to 836. RSV-2, RSV-6, RSV-14 and RSV-15 contained several nucleotide and amino acid substitutions in common, generally clustered within or adjacent to the central common region between amino acids 164–176. All four escape mutants had a substitution at amino acid position 170 from phenylalanine to leucine (RSV-2) or proline (RSV-6, RSV-14, RSV-15). RSV-2 and RSV-6 also had an arginine substitution at the fourth conserved cysteine residue (position 186). In addition, RSV-2 and RSV-15 had a leucine for phenylalanine substitution at position 165, and RSV-6 and RSV-14 had a serine substitution at position 168. Finally, RSV-15 had a unique substitution at position 171.

In contrast, RSV-3 had all but one nucleotide change and all four amino acid substitutions were confined to the cytoplasmic tail and transmembrane region of G. The amino acid substitutions, which were all prolines, were at positions 33 and 35 (cytoplasmic tail) and 51 and 54 (transmembrane region).
Table 1. Nucleotide and amino acid changes in escape mutants

Nucleotide and amino acid changes in the G mRNA and protein are shown relative to the parent strain of virus, RSV-1. The nucleotide number and the substitution which occurred are shown in the first column. The nucleotide substitution shown in the first column occurred in the virus indicated by the asterisk (*). Amino acid substitutions are as shown with the parent G protein amino acid followed by the amino acid found in the escape mutant protein. *S, silent nucleotide substitution.

<table>
<thead>
<tr>
<th>Nucleotide no. (change)</th>
<th>RSV-2</th>
<th>RSV-3</th>
<th>RSV-6</th>
<th>RSV-14</th>
<th>RSV-15</th>
</tr>
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<tbody>
<tr>
<td>85 (U → C)</td>
<td>*S</td>
<td></td>
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<tr>
<td>108 (U → C)</td>
<td>*S</td>
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<tr>
<td>112 (U → C)</td>
<td>*33 (L → P)</td>
<td></td>
<td></td>
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<tr>
<td>113 (U → C)</td>
<td></td>
<td></td>
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<tr>
<td>119 (U → C)</td>
<td>*35 (L → P)</td>
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<tr>
<td>167 (U → C)</td>
<td>*51 (S → P)</td>
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<tr>
<td>177 (U → C)</td>
<td>*54 (L → P)</td>
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<tr>
<td>414 (U → C)</td>
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<tr>
<td>456 (U → C)</td>
<td>*S</td>
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<tr>
<td>495 (U → C)</td>
<td>*S</td>
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<tr>
<td>508 (U → C)</td>
<td>*165 (F → L)</td>
<td></td>
<td></td>
<td>*165 (F → L)</td>
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<tr>
<td>518 (U → C)</td>
<td>*S</td>
<td>*168 (F → S)</td>
<td>*168 (F → S)</td>
<td></td>
<td></td>
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<tr>
<td>519 (U → C)</td>
<td></td>
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</tr>
<tr>
<td>523 (U → C)</td>
<td>*170 (F → L)</td>
<td></td>
<td></td>
<td>*170 (F → P)</td>
<td>*170 (F → P)</td>
</tr>
<tr>
<td>524 (U → C)</td>
<td>*170 (F → P)</td>
<td></td>
<td></td>
<td>*170 (F → P)</td>
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<tr>
<td>525 (U → C)</td>
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<td></td>
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<tr>
<td>527 (U → C)</td>
<td></td>
<td>*171 (V → A)</td>
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<tr>
<td>539 (U → C)</td>
<td>*175 (I → T)</td>
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<tr>
<td>571 (U → C)</td>
<td>*186 (C → R)</td>
<td>*186 (C → R)</td>
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<tr>
<td>657 (U → C)</td>
<td>*S</td>
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<td>675 (U → C)</td>
<td>*S</td>
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<tr>
<td>689 (U → C)</td>
<td>*225 (V → A)</td>
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<tr>
<td>750 (U → C)</td>
<td>*S</td>
<td>*S</td>
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</tr>
<tr>
<td>836 (U → C)</td>
<td></td>
<td></td>
<td>*274 (L → P)</td>
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</table>

Fig. 2. Schematic of the RSV G protein. The structure of G is displayed from the N-terminal to the C-terminal end. The dark bar indicates the transmembrane region and the lighter bar represents the 13 amino acid common region. The cysteine residues are indicated by the black circles. The amino acid substitutions found in the expanded sections are shown for each of the escape mutants.

Western blot analysis

Cell lysates, purified virus and cell culture supernatants of each of the viruses were separated under non-reducing, non-denaturing conditions on 8 or 10% SDS–PAGE, transferred to nitrocellulose paper, and probed with G- and F-specific rabbit antiserum or MAbs. When infected cell lysates were probed with L9, none of the mutant G proteins were reactive with the exception of RSV-3 which showed faint bands at 90, 45 and 33 kDa (data not shown). However, rabbit anti-G serum reacted strongly with G from each of the mutants except RSV-3, which was barely detectable (Fig. 3 a). The molecular mass of the mutant G proteins, including RSV-3, was similar to that of the parental virus, suggesting that there was no significant alteration in the glycosylation pattern of the G proteins.
RSV neutralization escape mutants

Fig. 3. Western blot analysis of parental (RSV-1) and five L9 neutralization-resistant escape mutants (RSV-2, RSV-3, RSV-6, RSV-14 and RSV-15). SDS–PAGE was carried out under non-reducing, non-denaturing conditions. Molecular masses (noted at the left) are based upon migration of prestained standards. (a) Cell lysates of each virus developed with rabbit anti-G serum; (b) sucrose gradient-purified RSV-1 and RSV-3 developed with rabbit anti-F and anti-G serum; (c) infected cell supernatants of each virus developed with rabbit anti-G serum; and (d) RSV-1 and RSV-3 cell lysates and supernatants developed with MAbs L9 and K2.

When sucrose gradient-purified RSV-1 and RSV-3 were electrophoresed and blotted under non-denaturing conditions, anti-F polyclonal rabbit serum indicated that the quantity of F was similar for both viruses (Fig. 3b, left). However, G protein was markedly reduced in purified RSV-3 relative to RSV-1 (Fig. 3b, right). When the supernatant of infected cells was analysed with rabbit anti-G serum a dark band was noted for RSV-3 and lighter bands noted for the other viruses (Fig. 3c). This G protein from RSV-3 was also reactive with MAbs L9 and K2 (Fig. 3d). The molecular mass of the supernatant G was consistent with the predicted size (84 kDa) of secreted G (Gs) as originally described by Hendricks et al. (1987, 1988).

Reactivity of antibodies with mutant viruses by IFA

Acetone-fixed, virus-infected cells were probed by IFA with a panel of G protein-specific MAbs and control antibodies. All virus-infected cells were positive with anti-F and anti-N MAbs and were negative with 1A5 control MAb. L9 did not react with RSV-2, RSV-6, RSV-14, or RSV-15. As noted, RSV-3 appeared faintly positive by IFA with L9, and also with the other G-specific MAbs. K6, a MAb with characteristics identical to L9 and which competes with L9 in competitive bindings studies, retained reactivity with RSV-2 but not with the other mutants. All of the group A-specific MAbs (K1, K2, K5, 78G, 130-2G and 232-1F) reacted strongly with each of the mutants except for RSV-3, in which weak reactivity was noted. The markedly reduced reactivity for all of the MAbs with RSV-3 was unexpected since the amino acid substitutions were limited to the cytoplasmic tail and transmembrane region of G. Since the extramembranous domains of RSV-3 G were unaltered we reasoned that polyclonal rabbit anti-G serum should detect RSV-3 G, as noted by Western blot. Therefore, G- and F-specific rabbit polyclonal antisera were used in an IFA on acetone-fixed and unfixed live infected cells. Membrane-associated G and F were readily detected in live RSV-1 (parent virus)-infected cells (Fig. 4a, b). RSV-3-infected cells expressed F protein on the surface in normal fashion (Fig. 4d). However, G protein could not be detected on the surface of live RSV-3-infected cells (Fig. 4c). Acetone-fixed RSV-3-infected cells did
Fig. 4. Indirect immunofluorescent assay of live infected HEp-2 cells with rabbit anti-G and anti-F serum. All incubations were at 4°C. Photographs made at ×200 enlargement. (a) RSV-1 (parental virus)-infected live cells stained with anti-G serum; (b) RSV-1-infected live cells stained with anti-F serum; (c) RSV-3-infected live cells stained with anti-G serum; and (d) RSV-3-infected live cells stained with anti-F serum.
stain positive for G, although much less intensely than RSV-1 (not shown).

Together with the Western blot results, the data suggest that the amount of membrane-bound G produced by RSV-3 is markedly diminished, or that it is not stably associated with the membrane and fails to accumulate on the cell surface, while a secreted form of G is increased. It also indicates that the RSV-3 virus particles have reduced G on their surface, and may thus avoid neutralization by L9 and complement. Consistent with this conclusion, RSV-3 but not RSV-1 or the other mutants, was resistant to neutralization with rabbit polyclonal anti-G serum (data not shown). All viruses, including RSV-3, were neutralized by polyclonal anti-F serum.

Discussion

We report the isolation and characterization of five unique MAb-resistant escape mutants of RSV with alterations in the G protein. Each of the mutants was antigenically and phenotypically stable through at least five passages in cell culture without antibody pressure. This is similar to two RSV escape mutants induced with a group cross-reactive G protein MAb described by Rueda et al. (1994). All of the nucleotide substitutions in our escape mutants were U to C changes, a result also similar to Rueda’s findings, and the mechanism is thought to involve a cellular unwindase enzyme (Bass et al., 1989).

It was not surprising that four of the escape mutants (RSV-2, RSV-6, RSV-14 and RSV-15) had amino acid substitutions in or near the centrally conserved 13 amino acid region (164–176), since this is the longest fully conserved segment of the G protein, and was a likely candidate for the L9 binding site. Since none of the mutants had only a single amino acid substitution, it is not possible to ascribe the loss of L9 reactivity to a single amino acid alteration. However, all four mutants had a substitution of the phenylalanine at position 170, suggesting that it may be important for L9 binding. The results are consistent with the findings reported by Sullender (1995), in which L9 binding was eliminated when G protein was truncated beyond position 173 and re-established when the protein was extended to amino acid 213. Two of the mutants (RSV-2 and RSV-6) also had a loss of the fourth conserved cysteine residue at position 186. Presumably, this would result in disruption of the proposed cysteine noose in which the four cysteines participate in 173–186 and 176–182 linkages (Langedijk et al., 1996). Loss of a single cysteine at positions 182 and 186 was also noted by Rueda et al. (1994) in two mutants.

The data suggest that the strictly conserved central region plays a role in the L9 binding site. These data may be useful in designing a synthetic peptide capable of inducing a group cross-reactive neutralizing and protective immune response. To date, only RSV group-specific protective synthetic peptides which mainly represent sequences adjacent to the centrally conserved region have been described (Akerlund-Stopner et al., 1990; Garcia-Barreno et al., 1992; Trudel et al., 1991; Simard et al., 1997).

The presence of a universally conserved 13 amino acid stretch in the G protein surrounded by variable regions suggests that this segment is critically important for the function of G. Doreleijers et al. (1996) describe this region as two short antiparallel helices connected on either end by two disulfide bridges of the cysteine noose structure described by Langedijk et al. (1996). Despite extensive amino acid changes in this region, four mutants had normal growth kinetics which argues against this region functioning as a specific cell-receptor binding site. It is also possible that conservation of this area is not due to structure–function constraints, but because it is not immunodominant and thus not under antibody pressure to mutate.

The final escape mutant, RSV-3, was perhaps the most interesting as all four amino acid substitutions occurred in the cytoplasmic tail and transmembrane region of G. Most intriguing was the finding of reduced amounts of full-length G protein in cell lysates, the absence of G on the cell surface, increased Gs in cell supernatants and reduced incorporation of G into the virion. It is possible that the amino acid substitutions substantially alter the antigenicity of G over its entire length, thus resulting in markedly diminished antibody binding. However, this seems unlikely since L9 has been shown to react with G proteins which lack large segments of the transmembrane region (Lichtenstein et al., 1996). Another possible explanation is that the RSV-3 G transmembrane region is altered such that G is not stable in cell membranes and is thus poorly incorporated into the virion, similar to findings with influenza virus neuraminidase and Sindbis virus E2 protein mutants (Kundu et al., 1996, Mitnaul et al., 1996, Levine et al., 1996).

It is also conceivable that the increased secretion of Gs by infected cells could act to prevent virus neutralization by binding to G protein antibodies, essentially competing with the G protein present in the virion. The G protein mRNA contains two open reading frames, the first resulting in full-length G and the second starting at the methionine at position 48. Subsequent enzymatic cleavage of this second translation product at amino acid 66 results in the final product which is secreted from the cell since it lacks the entire transmembrane region of G and is not membrane associated (Roberts et al., 1994). It is possible that the substitutions in RSV-3 G makes it susceptible to enzymatic cleavage resulting in Gs. Using vaccinia virus recombinants expressing G, Lichtenstein et al. (1996) found that G protein which lacked the proximal or distal portion of the transmembrane region (amino acids 38–50 and 51–63, respectively) was not expressed on the cell surface, and a cleaved G protein was secreted into the cell supernatant. The data reported here do not allow firm conclusions about the mechanisms of intracellular synthesis, processing and trafficking of RSV-3 G and will require further study. Whatever
the mechanisms involved, these results are compatible with RSV-3 escaping neutralization by incorporating less of the G protein into viral envelopes, similar to a mechanism noted for a neutralization-resistant cytomegalovirus phenotype with decreased expression of the protein gH (Li et al., 1995).

In summary, five G protein neutralization escape mutants of RSV are described. Four virus mutants had changes concentrated in the centrally located conserved 13 amino acid region of G. An additional mutant appears to escape neutralization by reducing incorporation of G protein in viral membranes.

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References


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