Antigenic structure of the human respiratory syncytial virus G glycoprotein and relevance of hypermutation events for the generation of antigenic variants

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A set of monoclonal antibodies (MAbs) specific for the attachment (G) glycoprotein of a recently isolated strain of human respiratory syncytial virus (HRSV) is described. Antibody reactivity with a series of HRSV isolates belonging to antigenic groups A and B identified three epitope categories: (i) strain-specific or variable epitopes that were present in a limited set of viruses from the same antigenic group, (ii) group-specific epitopes shared by viruses from the same antigenic group and (iii) conserved epitopes present in all HRSV isolates. Sequence analysis of escape mutants was used to map relevant antigenic sites of the G glycoprotein. Strain-specific epitopes were located preferentially in the variable C-terminal third of the G polypeptide, in agreement with previous studies of the Long strain. However, a new strain-specific epitope was mapped into another variable region, N-terminal to the cluster of cysteines in the G protein ectodomain. In contrast, the group-specific and conserved epitopes were located in the central conserved region of the G protein primary structure. These results, together with previous analysis of the Long strain, provide a detailed antigenic map of the HRSV attachment protein. Some mutants selected with group-specific antibodies contain multiple A–G substitutions (hypermutations) and lack one or two of the four cysteines which are conserved in all HRSV isolates. The genetic mechanism implicated in the generation of the hypermutated viruses and its relevance for the natural history of HRSV are discussed.

Introduction

Human respiratory syncytial virus (HRSV) is the leading cause of lower respiratory tract infections in very young children and infants (reviewed in Collins et al., 1996). HRSV is a member of the genus Pneumovirus, family Paramyxoviridae. It has two surface glycoproteins (G and F), which are the main targets of neutralizing and protective antibodies. The G glycoprotein is responsible for virus binding to the cell surface receptor (Levine et al., 1987). The F glycoprotein mediates the fusion of the viral and cell membranes (Walsh et al., 1985), allowing the entrance of the virus nucleocapsid into the cell cytoplasm and the initiation of a new infectious cycle.

The G molecule is a type II glycoprotein with a single hydrophobic domain between residues 38 and 66 which serves as both membrane anchor and signal sequence. The protein precursor is synthesized as a 32 kDa polypeptide which is cotranslationally modified by the addition of high-mannose N-linked carbohydrate side-chains to asparagine residues, forming intermediates of 45–48 kDa (Wertz et al., 1989; Collins & Mottet, 1992). Maturation of the N-linked sugar chains occurs in the Golgi compartment. At later stages of the maturation pathway, O-linked sugars are added to serine and threonine residues of the protein ectodomain yielding a mature molecule of 80–90 kDa, as estimated by SDS–PAGE. In the centre of the G protein ectodomain there is a cluster of four cysteines (residues 173, 176, 182 and 186) and a short amino acid segment (residues 164–176) of identical sequence among all HRSV isolates (Johnson et al., 1987; Cane et al., 1991; Sullender et al., 1991; García et al., 1994). This region is slightly hydrophobic and has been proposed as the putative receptor binding site (Johnson et al., 1987). Flanking the cluster of cysteines, there are two protein segments that have a high content of serine, threonine and proline, resembling the amino acid composition of mucins, a class of proteins secreted by epithelial cells.

Based on the reactivity with certain panels of monoclonal

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antibodies (MAbs), HRSV isolates have been subdivided into two antigenic groups (A and B) (Anderson et al., 1985; Mufson et al., 1985) that correlate with different genotypes (Cristina et al., 1990). The G glycoprotein is the viral gene product with the highest degree of antigenic and genetic diversity among viral isolates (Anderson et al., 1985; Cristina et al., 1990; García-Barreno et al., 1989; Mufson et al., 1985). Sequence differences are clustered preferentially in the two mucin-like regions of the G protein ectodomain (Cane et al., 1991; Sullender et al., 1991). Reinfections by HRSV have been related to changes in the antigenic properties of the virus, particularly the G protein (Mufson et al., 1987; Waris, 1991), suggesting that immune selection of antigenic variants may contribute to the epidemiology of HRSV. This idea is further supported by the correlation of genetic and antigenic changes reported for virus isolates of antigenic group A (García et al., 1994; Cane et al., 1995).

A detailed analysis of the G protein antigenic structure should help to understand both the basis of virus neutralization and the mechanisms used by HRSV to evade a pre-existing immune response. Previous studies with MAbs specific for the G glycoprotein of the HRSV Long strain illustrated both the plasticity of the G molecule in accommodating sequence changes and the relevance of the C-terminal third for its antigenic structure (García-Barreno et al., 1990; Rueda et al., 1991, 1994, 1995). However, the Long strain is the oldest isolate of HRSV (Chanock et al., 1957). It has been passed in tissue culture cells for long periods of time and is distantly related to viruses isolated during recent epidemics (García et al., 1994). Consequently, many of the MAbs specific for the G protein of the Long strain do not react with recently isolated viruses and cannot be used as typing reagents for currently circulating strains (García-Barreno et al., 1989). In addition, sequence changes may influence the location of carbohydrate side-chains which are important determinants of the G protein antigenic structure (Palomo et al., 1991; García-Beato et al., 1996). For all these reasons, we decided to explore the antigenic organization of the G glycoprotein of a recent isolate (Mon3/88, isolated in 1988, Montevideo, Uruguay) with a shorter passage history than the Long strain, and prototype of one of the major lineages of group A viruses (García et al., 1994). In the present report, we describe the preparation and characterization of a new panel of anti-G MAbs, the location of residues important for epitope integrity in the G protein primary structure and the mechanisms involved in selection of antigenic variants.

**Methods**

- **Cells and viruses.** The year and place of isolation of the HRSV strains used in this study have been described (García et al., 1994). All viruses were grown in HEp-2 cells (García-Barreno et al., 1988). Mon3/3/88 virus, used to immunize mice, was purified as described (García-Barreno et al., 1988). Briefly, the virus was precipitated from the supernatant of infected cultures with 6% polyethylene glycol 6000 (Sigma) and spun down in a 20–60% sucrose gradient at 120,000 g for 22 h. A visible virus band was collected and pelleted through a 33% sucrose cushion at 120,000 g for 2 h. The pellet was resuspended in TNE buffer (10 mM Tris–HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA) and kept at −20 °C until used.

- **Cell extracts and G protein purification.** Monolayers of infected cells were scraped off the culture plates with a rubber policeman when cytopathic effect was evident (usually 48 h post-infection). Cells were pelleted by centrifugation at low speed and after washing with PBS they were solubilized in extraction buffer (10 mM Tris–HCl, pH 7.6, 5 mM EDTA, 140 mM NaCl, 1% octylglucoside). Extracts were clarified by centrifugation for 5 min in a minifuge. Partial purification of the G protein was achieved as follows. Protein extracts were resolved by 10% SDS–PAGE with 100 mg/ml Coomasie blue added to the running buffer to visualize protein bands without fixing the gels. A wide band that included proteins with the expected mobility of the G molecule was sliced into pieces and the proteins were eluted by stirring for 16 h at 4 °C in 0.1 M NH₄HCO₃, 0.01% SDS. Gel fragments were eliminated by filtration and the protein solution was lyophilized. Finally, the protein was resuspended in a small volume of distilled water.

- **Preparation of MAbs.** Purified Mon3/3/88 virus was used to immunize BALB/c mice. Two doses of 40 µg were administered intraperitoneally 4 weeks apart. The first dose was mixed with an equal volume of Freund’s complete adjuvant and the second with incomplete adjuvant. Four and two days before sacrificing the mice, they received two doses of virus without adjuvant. Immune splenocytes were fused to Sp2-0 cells as described (Sanchez-Fauquier et al., 1987). Hybridomas were originally grown in 96-well microtitre plates. Identification of antibody-producing cultures was done by ELISA with either infected cell extracts or gel-purified G protein as antigens. Positive cultures were cloned at least twice, by limiting dilution. Specificity of the antibodies for the G protein was confirmed by Western blot and the antibody isotypes were determined by ELISA using anti-murine class and subclass specific antibodies (Southern Biotechnology). Characterization of antibody reactivity in ELISA, Western blot and neutralization tests was done as described (García-Barreno et al., 1989).

- **Isolation and characterization of antibody escape mutants.** Mon3/3/88 virus was plaque-purified twice in soft agar (Rueda et al., 1994). Twelve individual plaques were passed independently in the presence of antibodies. For each passage the virus was mixed with an equal volume of hybridoma supernatant and incubated for 30 min at 37 °C. Then, rabbit anti-mouse Ig serum was added at a final 1/10 dilution and incubation continued for another 30 min at 37 °C. The virus antibody mixtures were used to infect HEp-2 monolayers growing in 96-well microtitre plates and hybridoma supernatant was added to the cultures at a 1/4 dilution. After 3–4 days at 37 °C, cell supernatants were collected and incubated with antibodies as before. The selection cycle was repeated 5–10 more times until cell extracts from individual wells did not react with the antibody used in the selection process. Then, viruses were plaque-purified from the supernatants and one plaque from each well was grown for further characterization.

Sequence analysis of the escape mutants was done as follows. Nucleotides 1–620 of G mRNA were sequenced by the dideoxy method with reverse transcriptase and specific primers, using poly(A) RNA from infected cells as template (García-Barreno et al., 1990). Nucleotides 620–918 were sequenced from cloned DNA obtained by RT–PCR, using total RNA as template and oligonucleotides spanning positions 295–315 (positive sense) of the G gene and 119–139 (negative sense) of the F gene. The amplified DNA, which crossed the G–F boundary of the viral genome, was digested with the restriction enzyme PstI (cutting after
nucleotides 535 of the G gene and 64 of the F gene) and cloned in pGEM-4 vector (Promega) digested with the same enzyme. Sequenase 2.0 (USB) and primers complementary to the Sp6 and T7 promoters were used for the sequencing reactions.

Results and Discussion

Isolation and characterization of MAbs directed against the G glycoprotein of Mon/3/88 virus

Nineteen hybridomas were obtained that produced antibodies specific for the G protein of Mon/3/88 virus. All these antibodies reacted in Western blots with an 80–90 kDa band corresponding to the mature G protein (Gm) present in extracts of HEp-2 infected cells. Representative examples are presented in Fig. 1. Some antibodies also recognized heterogeneous bands of 40–60 kDa that represent maturation intermediates (Gi) of the G glycoprotein (Wertz et al., 1989; Collins & Mottet, 1992). Only certain antibodies reacted with the unglycosylated precursor (Gp) that accumulates in infected cells treated with tunicamycin and monensin (Fig. 1), indicating that carbohydrate side-chains are essential for integrity of certain epitopes (e.g. antibody 021/10G) (Palomo et al., 1991).

Fig. 2 summarizes the properties of the new set of MAbs. The majority of the antibodies have an IgG1 isotype except antibodies 021/1G (IgG2a) and 021/9G (IgM). Similarly to previously described anti-G MAbs (Anderson et al., 1985; García-Barreno et al., 1989), none of the antibodies raised against the G protein of Mon/3/88 neutralized the virus to significant levels. However, addition of a rabbit anti-mouse Ig serum to virus–MAb mixtures reduced virus titre between one and two log10 units in some cases. It is worth mentioning that all the anti-G antibodies obtained up to date in different laboratories have only low neutralizing capacity (i.e. a fraction of the virus is not neutralized independent of the antibody titre); however, it has been reported that mixtures of anti-G antibodies have a synergistic effect in the neutralization test (Anderson et al., 1988; García-Barreno et al., 1989; Walsh et al., 1989).

Reactivity of the new set of anti-G MAbs with HRSV isolates generated three different patterns (Fig. 2): (i) two antibodies reacted with viruses from antigenic groups A and B, identifying conserved epitopes (C) of the G glycoprotein; (ii) seven antibodies reacted with viruses from antigenic group A but not with viruses from antigenic group B, identifying group A-specific epitopes (GS) and (iii) ten antibodies reacted with some viruses from antigenic group A but not with others, identifying strain-specific or variable epitopes (SS). Whereas all the antibodies that recognized either conserved or group-specific epitopes reacted with the unglycosylated G protein precursor, only two of the antibodies that recognized strain-specific epitopes recognized the precursor polypeptide. The reactivity profile of group A viruses with the strain-specific antibodies has been related previously to the phylogenetic classification of viruses deduced from G protein gene sequences (García et al., 1994; Cane & Pringle, 1995).

MAbs obtained in our laboratory against the G protein of the Long strain recognized exclusively strain-specific epitopes and some of them reacted only with Long virus (García-Barreno et al., 1989). In contrast, almost half of the anti-G MAbs of Fig. 2 recognized either conserved or strain-specific epitopes. Since the methodology for isolation of hybridomas was essentially the same in our original study (García-Barreno et al., 1989) and in the present work, it is unclear why antibodies with restricted reactivity were preferentially isolated when Long virus was used as immunogen. The proportion of anti-G antibodies recognizing either of the three epitope categories is also different among those reported by other laboratories (Anderson et al., 1985; Stoch & Park, 1987). It is possible that the immunizing strain determines, at least in part, the predominant epitope category recognized by the MAbs but this possibility remains to be tested.

Isolation and antigenic characterization of antibody escape mutants

The antibody-resistant mutants listed in Fig. 3 were selected from Mon/3/88 virus, using the same procedure previously described for isolation of escape mutants of the Long strain (García-Barreno et al., 1989; Rueda et al., 1994). Antibodies representing the three epitope categories described in the previous section were included in the selection process. Twelve individual plaques of Mon/3/88 were passed independently in the presence of each antibody to ensure that mutants resistant to the same antibody were generated by independent events. Each mutant virus was tested for reactivity with the set of MAbs described in Fig. 2. Certain escape mutants (e.g. 5G/2B/1) lost reactivity only with the antibody that was used in their selection (Fig. 3). In other cases, however, the mutants also lost reactivity with antibodies of the same category to the one used in their selection. For instance, mutant 16G/2B/6 did not react with either antibody 021/16G (used in the selection process) or antibodies 021/4G and 021/9G, all of which...
recognize strain-specific epitopes (Fig. 2). Finally, some escape mutants lost epitopes recognized by antibodies of a different category, in addition to that recognized by the antibody used in their selection. For instance, mutant 18G/9A/4 did not react with antibody 021/18G (specific for group A viruses) and neither did it react with any of the antibodies that recognized conserved or group A-specific epitopes or with the strain-specific antibody 021/7G. Comparison of the results presented in Figs 2 and 3 indicates similarities of the reactivity profiles of antibodies with virus isolates and escape mutants. For instance, antibodies 021/8G, 021/10G, 021/11G and 021/12G, which lost reactivity with three escape mutants, reacted with the same set of virus isolates.

Identification of genetic changes selected in escape mutants

The location in the G protein primary structure of residues needed for integrity of the different epitopes was determined by sequencing the G protein gene of each mutant presented in Fig. 3. Since all the antibodies reacted in Western blots with the mature G molecule, their epitopes are presumably non-conformational. This seems to be a general characteristic of anti-G MAbs prepared in different laboratories and some previously characterized antibodies indeed reacted with synthetic peptides (Norrby et al., 1987; García-Barreno et al., 1992; Cane, 1997) or G protein segments expressed in bacteria (Cane et al., 1996). However, as seen in Fig. 1, certain epitopes depend on sugar chains for recognition by their antibodies. In these cases, synthetic peptides cannot be used for epitope mapping. This limitation, however, does not influence the mapping of G protein epitopes by sequencing of escape mutants.

The majority of the mutants listed in Fig. 3 have single nucleotide differences in the G protein gene with respect to Mon/3/88 virus; however, three mutants have multiple nucleotide changes and they are discussed below in a separate section. Fig. 4 shows the changes selected in mutants resistant to antibodies reacting with either of the three epitope categories described before.

Mutants selected with strain-specific antibodies have sequence changes in two different positions of the G protein gene. Two mutants resistant to antibody 021/5G have nucleotide substitutions in the hypervariable region of the G protein gene that precedes the cluster of cysteines (Gln<sup>142</sup>Lys and Lys<sup>145</sup>Arg). One of the mutants (5G/9A/1) has in addition a second change (Glu<sup>262</sup>Gly). Since the first two changes are closely located in the G protein primary structure it is likely

![Table](image)

**Fig. 2.** Characteristics of MAbs raised against the G glycoprotein of Mon/3/88 virus. Isotypes were determined with murine Ig class and subclass specific reagents. Serial fivefold dilutions of Mon/3/88 virus were tested in a microneutralization test with (+ RAM) or without (− RAM) addition of a rabbit anti-mouse Ig serum. (+) indicates reduction of a log<sub>5</sub> unit of virus titre. Reactivity of antibodies with G protein precursor was tested by Western blot as in Fig. 1. Reactivity of antibodies with virus isolates was tested by ELISA using extracts of HEp-2 cells infected with the indicated viral strains as antigens. (SS) strain-specific, (GS) group-specific and (C) conserved epitopes. (■) indicates reactivity as with Mon/3/88 virus, (□) indicates partial reactivity and (□□) no reactivity. (k) light kappa chain of immunoglobulins.
that the change at residue 263 is an accompanying mutation, not involved in the loss of antibody reactivity. In agreement with this hypothesis, antibody 021/5G reacts with a fragment of the G protein, expressed as a fusion protein in bacteria, that corresponds to the hypervariable region preceding the cluster of cysteines (Cane et al., 1996).

The other mutants selected with the strain-specific antibodies 021/6G, 021/9G, 021/16G and 021/7G have single nucleotide changes in the C-terminal hypervariable region of the G protein gene, except mutant 9G/9A/4 which has a second change (Asn→Thr). This change, located in the cytoplasmic tail of the G protein, is unlikely to be involved in antibody recognition and probably represents an adventitious accompanying mutation. In addition, mutant 16G/2B/6, which also lost reactivity with antibody 021/9G (Fig. 3), has a single change at residue 244 (Arg→Ile). One of the mutants selected with antibody 021/7G (7G/20B/4) has a nucleotide substitution that introduced a stop codon after amino acid 276, shortening the G polypeptide by 21 amino acids. Consequently, this protein has lost residues 282 and 286 that were changed in the other three mutants selected with antibody 021/7G. The G protein of mutant 7G/20B/4 migrates faster than the wild-type protein in SDS–PAGE (not shown).

Two mutants selected with the group-specific antibodies 021/19G and 021/18G (Fig. 4) have single nucleotide substitutions that changed amino acids 181 (Thr→Ala) and 188 (Arg→Gly), respectively. The last mutant lost reactivity with the majority of antibodies recognizing group-specific epitopes (Fig. 3). Amino acid 181 is included in the cysteine noose motif proposed by Doreleijers et al. (1996) for the three-dimensional structure of a central segment of the G protein ectodomain whereas amino acid 188 is outside that motif. Moreover, the reactivity of antibodies 021/18G and 021/19G with the escape mutants of Fig. 3 indicates that they recognize non-overlapping epitopes. Thus, amino acid residues essential for at least two distinct group-specific epitopes were mapped in the G protein ectodomain segment that is conserved in all group A isolates but that has sequence differences with group B viruses (García et al., 1994). Group-specific epitopes have also been reproduced with synthetic peptides corresponding to the central segment of the G protein of human (Akerlind-Stopner et al., 1990) and bovine (Langedijk et al., 1996) isolates.

Four mutants were selected with antibody 021/1G, which recognizes a conserved G protein epitope (Fig. 4). These four viruses had lost reactivity with antibody 021/1G but reacted with all other antibodies of Fig. 3, including 021/21G, which recognizes a different conserved epitope of the G glycoprotein. One of the mutants (1G/2B/4) has two closely spaced nucleotide substitutions (Phe→Leu and Phe→Leu). Two other mutants (1G/9A/1 and 1G/9A/5) have a single point...
mutation (Phe\textsuperscript{165}Ser). This change was also found in a fourth mutant (1G/10B/1) that has, in addition, a silent nucleotide substitution. These results locate residues essential for the integrity of a conserved epitope (021/1G) in the central region of the G protein ectodomain, within a segment of identical sequences for all group A and group B HRSV isolates (Johnson et al., 1987; Sullender et al., 1991; García et al., 1994; Cane & Pringle, 1995).

In summary, the results of Fig. 4 indicate a clear correlation between the epitope category of antibodies used in the selection of escape mutants and the location of genetic changes in the G protein primary structure. In agreement with previous findings (Rueda et al., 1991, 1995), the strain-specific epitopes were preferentially located in the variable C-terminal third of the G protein sequence but epitope 021/1G was located in the other variable region, previously not considered to be antigenically relevant. In contrast, the group-specific and conserved epitopes were mapped in the central region of the G protein ectodomain. The group-specific epitopes were mapped within the G protein segment conserved among group A viruses. The conserved epitopes were located in a protein segment of identical sequence in all HRSV isolates. These results, together with previously described escape mutants of the Long strain, provide a detailed map of the G protein antigenic structure.

### Escape mutants with multiple A–G substitutions

One mutant resistant to antibody 021/18G and two mutants resistant to antibody 021/19G have multiple nucleotide substitutions that involved almost exclusively A–G transitions (Fig. 5). These two antibodies recognized group A-specific epitopes (Fig. 2) and also selected escape mutants with single nucleotide substitutions (Fig. 4).

Mutant 18G/9A/4 had nine A–G transitions leading to six amino acid changes in the G protein (Fig. 5). One of these changes involved cysteine 176 (Cys→Arg), which is conserved in all HRSV isolates sequenced to date. Another change involved the termination codon (Stop→Gln). Consequently, the mutant protein has an extra amino acid since a second stop codon in the G protein mRNA follows the one lost in 18G/9A/4.

Mutant 19G/8B/1 has 21 nucleotide substitutions, 20 of them representing A–G transitions, which are translated into

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**Fig. 4.** Sequence changes selected in the G protein of escape mutants of the Mon/3/88 virus. A diagram of the G protein primary structure is represented, indicating the transmembrane region (●) and the cysteine residues (●). The gene segments of identical sequence in all HRSV isolates of antigenic groups A and B (amino acids 164–176, continuous line) or in all group A isolates (amino acids 163–189, dotted line) are indicated below the protein diagram. Sequence changes selected in each mutant are listed at the right and their locations in the protein diagram are indicated by arrowheads.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Nucleotide change</th>
<th>Amino acid change</th>
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<tbody>
<tr>
<td>5G/9A/1</td>
<td>439 (G→U)</td>
<td>142 (Q→K)</td>
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<td>5G/2B/1</td>
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<td>263 (E→G)</td>
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<td>145 (K→R)</td>
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<td>239 (T→A)</td>
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<tr>
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<td>239 (T→A)</td>
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<td>9G/9A/4</td>
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<td>21 (N→T)</td>
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<td>244 (R→S)</td>
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<tr>
<td>7G/9A/6</td>
<td>708 (U→C)</td>
<td>282 (T→A)</td>
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**SS**  
**Epitope category**

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**GS**

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**C**
13 amino acid changes. This virus represents the escape mutant of HRSV with the greatest number of A–G transitions reported to date. These changes involved the loss of the last two cysteines (Cys^{182}Arg and Cys^{186}Arg) of the cluster of cysteines. Thus, neither of the two disulphide bonds proposed by Langedijk et al. (1996) (173–186 and 176–182) to occur in the native G protein could be formed in mutant 19G/8B/1. However, data on reactivity of MAbs with modified synthetic peptides indicate that disulphide bridge formation is not an absolute requirement for the conserved G protein central segment to adopt the appropriate epitope conformation (Langedijk et al., 1996).

Finally, mutant 19G/9A/2 has five nucleotide changes, all of them A–G transitions. These changes were restricted to the central region of the G protein ectodomain where most of the nucleotide changes in the other two mutants of Fig. 5 accumulated preferentially. The nucleotide changes in mutant 19G/9A/2 were translated into four amino acid changes, one of them again involving one of the cysteines (Cys^{182}Arg).

Residues 171 (Val), 172 (Pro) and 173 (Cys) are conserved in the hypermutated viruses of Fig. 5, and in those previously described for the Long strain (Rueda et al., 1994). The same three amino acids are conserved in the G protein of human, bovine (Lerch et al., 1990) and ovine (Alansari & Potgieter, 1993) respiratory syncytial viruses. This may reflect severe structural and/or functional restrictions at these sites. In fact, residues 171 and 172 are found at the bottom and residue 173 at the left of the putative receptor binding pocket proposed by Doreleijers et al. (1996). In contrast, some of the amino acid changes selected in the hypermutated viruses are also found in bovine and ovine G protein sequences. This indicates that changes at certain positions of the G protein central segment are tolerated, even if they are conserved among HRSV isolates, explaining the viability of hypermutated viruses.

It is significant that the three hypermutated viruses of Fig. 5 have lost both the conserved and the group-specific epitopes of the G glycoprotein (Fig. 3). Some of the amino acid changes selected in these mutants coincide or are very close to residues that are changed in mutants selected with antibody 021/IG. This might explain the lack of reactivity of the hypermutated viruses with antibodies that identify conserved epitopes. In addition, mutants 18G/9A/4 and 19G/8B/1 have changes near the C-terminal end of the G molecule, in close proximity to changes selected in mutants resistant to antibody 021/7G,
explaining the lack of reactivity of this antibody with the former mutants (Fig. 3).

The F protein mRNA of the three hypermutated viruses was also sequenced by the dideoxy method, using specific primers and reverse transcriptase. No sequence differences were found with respect to the parental Mon/3/88 virus, indicating that the multiple A–G transitions were not scattered throughout the viral genome.

Multiple reiterative A–G changes have been found also in two escape mutants of the Long strain, selected with an antibody (c793) that recognized a conserved epitope (Rueda et al., 1994). Considering together the statistics for Long and Mon/3/88 mutants, 5 out of 14 viruses selected with antibodies reacting with either conserved or group-specific epitopes have A–G hypermutations. In contrast, this type of change has not been observed in any of the 37 viruses resistant to 15 different MAbs that recognized strain-specific epitopes.

Mechanisms implicated in A–G hypermutations and their significance for the natural history of HRSV

Reiterative A–G transitions have been reported in the case of deficient genomes of paramyxoviruses (parainfluenza virus 3, Murphy et al., 1991; measles virus, Cattaneo et al., 1988) and related viruses (vesicular stomatitis virus, O’Hara et al., 1984). However, it is only in HRSV that viable hypermutated viruses
have been isolated. All the nucleotide changes selected in two hypermutated viruses derived from the HRSV Long strain (Rueda et al., 1994) were observed at the earliest stages of the selection process and they were invariably maintained for at least ten more passages even in the absence of selective antibody. These two mutants were not out competed by wild-type virus in five passages of mixed viral populations (Rueda et al., 1994). Thus, hypermutated HRSV viruses seem to be biologically fit and the multiple A–G changes are apparently incorporated during a single replication round of the viral genome.

Bass et al. (1989) proposed that cellular adenosine deaminase could be involved in the generation of A–G hypermutations. They proposed a model of collapsed transcription (an mRNA transcript hybridizing to vRNA) to account for the formation of partially double-stranded RNA molecules that could be substrates for the adenosine deaminase to convert As to Ts (i is equivalent to G in nucleotide pairing). In contrast, intramolecular double-stranded RNA structures have been claimed as substrates for adenosine deaminase activity in editing of cellular RNAs (Melcher et al., 1995; Yang et al., 1995) and hypermutations events in avian retroviruses (Felder et al., 1994) and hepatitis ô virus (Netter et al., 1995). Neither collapsed transcription nor intramolecular base-pairing would be compatible with the established idea that the HRSV genome is always in the form of nucleocapsid complexes with the viral nucleoprotein. However, it is conceivable that dissociation of vRNA from nucleoprotein can occur locally during transcription/replication and that either inter- or intra-molecular double-stranded RNA structures are transiently formed. Highly stable intramolecular double-stranded RNA structures are predicted at the sites where multiple A–G transitions are found in the viruses of Fig. 5 (not shown), lending support to the idea that adenosine deaminase might be involved in the generation of hypermutated viruses.

As postulated for the hypermutated viruses of Fig. 5, double-stranded RNA intermediates might also form during replication of the HRSV genome in its natural host. Thus, an intriguing possibility is that limited hypermutation events could be contributing – in addition to other mutational mechanisms – to generate the genetic variation observed among HRSV isolates. Limited A–G hypermutations might be difficult to distinguish from nucleotide misincorporations when nucleotide sequences of natural isolates are compared. However, a bias in the frequency of nucleotide changes towards A–G transitions would be expected if the changes introduced by hypermutations represent a significant percentage of total nucleotide substitutions. Fig. 6 shows the frequency of different nucleotide changes when 45 sequences of the G protein gene from natural isolates were compared pairwise. A–G and G–A transitions were considered together (and similarly for the other nucleotide changes) since ancestor sequences for each pair of viruses were unknown. As a comparison, 27 partial sequences of the measles virus nucleoprotein gene, 25 sequences of the foot-and-mouth disease virus VP1 protein and 22 sequences of the influenza virus haemagglutinin (HA1 subunit) were included in the analysis. In all cases, transitions were more frequent than transversions, but whereas U–C+ C–U were as frequent as A–G+ G–A transitions in measles, foot-and-mouth and influenza viruses, the last type of change was almost three times more frequent in the G protein gene of HRSV isolates than the former type of change.

Limited hypermutation events occurring during the natural history of HRSV could be more easily observed when sequences of closely related viruses are compared (before nucleotide misincorporations in subsequent replication rounds blur the hypermutated sequence). In agreement with this hypothesis, A–G or G–A transitions are the predominant changes for some pairs of closely related viruses. For instance, in two isolates of HRSV (A/Mon/5/91 and A/Mon/9/92) 7 out of a total of 13 nucleotide differences were A–G transitions (Garcia et al., 1994).

In summary, multiple reiterative A–G transitions are frequently found among HRSV escape mutants selected with antibodies that recognize either conserved or group-specific epitopes of the G protein. Although these epitopes might be hot-spots for hypermutation events, there must be structural and functional restrictions (e.g. receptor-binding) that determine the conserved nature of that protein segment. Nevertheless, limited hypermutation events (probably involving short genome segments) occurring at other sites of the vRNA may account for the bias frequency of A–G and G–A changes among HRSV isolates.

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References


Cane, P. A. (1997). Analysis of linear epitopes recognized by the
primary human antibody response to a variable region of the attachment (G) protein of respiratory syncytial virus. Journal of Medical Virology (in press).


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