Characterization of two antigenic sites recognized by neutralizing monoclonal antibodies directed against the fusion glycoprotein of human respiratory syncytial virus

Juan Arbiza, Geraldine Taylor, Juan A. López, Julie Furze, Sara Wyld, Paul Whyte, E. James Stott, Gail Wertz, Wayne Sullender, Michel Trudel and Jose A. Melero

Two antigenic sites recognized by neutralizing monoclonal antibodies (MAbs) directed against the fusion (F) glycoprotein of human respiratory syncytial virus were mapped on the primary structure of the protein by (i) the identification of amino acid substitutions selected in antibody-escape mutants and (ii) the reactivity of synthetic peptides with MAbs. The first site contained several overlapping epitopes which were located within the trypsin-resistant amino-terminal third of the large F1 subunit. Only one of these epitopes was faithfully reproduced by a short synthetic peptide; the others might require specific local conformations to react with MAbs. The second antigenic site was located in a trypsin-sensitive domain of the F1 subunit towards the carboxy-terminal end of the cysteine-rich region. One of these epitopes was reproduced by synthetic peptides. In addition, mutagenized F protein with a substitution of serine for arginine at position 429 did not bind MAbs to the second site. These results are discussed in terms of F protein structure and the mechanisms of virus neutralization.

Introduction

Human respiratory syncytial virus (RSV) is the major cause of lower respiratory tract infections in infants and young children (McIntosh & Chanock, 1990). Early attempts at vaccination with formalin-inactivated virus failed to protect young vaccinees against infection by RSV during subsequent epidemics, despite the induction of a serum neutralizing antibody response. Moreover, vaccinated children experienced an exacerbated disease after a natural infection more frequently than control children who received an inactivated parainfluenza virus type 3 virus vaccine (Kim et al., 1969). Experimental live vaccines made with attenuated RSV also fail to induce protection in children (Belshe et al., 1982). However, seroepidemiological data indicate that high levels of naturally acquired neutralizing antibodies (maternally derived or generated after a natural infection) correlate with less severe infection or complete protection (Lamprecht et al., 1976; Glezen et al., 1981, 1986; Hall et al., 1991).

The failure of formalin-inactivated RSV to induce a protective immune response might be due, at least in part, to modification of selected epitopes on the fusion (F) or attachment (G) viral glycoproteins (Prince et al., 1986). Thus, a disproportionately high ratio of antibodies that bind/neutralize virus, as judged by ELISA, has been observed in the sera of children who received the inactivated vaccine (Murphy et al., 1986). However, the augmentation of disease observed in vaccinated children appears to involve cell-mediated immune mechanisms. These results emphasize the importance of a detailed characterization of RSV antigens in order to design and evaluate alternative safe vaccines.

The two major RSV external glycoproteins (G and F) represent presumptive targets of the protective immune response. Consistent with this hypothesis, polyclonal antisera raised against purified G or F protein (Walsh et al., 1987), or sera from animals inoculated with vaccinia virus recombinants expressing G or F antigens (Olmsted et al., 1986; Stott et al., 1987; Portela et al., 1989) neutralize RSV in tissue culture. Furthermore, the immunized animals are protected against a challenge with live RSV. The neutralizing and protective responses against the G protein are subgroup-specific, whereas the anti-F protein responses are broadly cross-reactive with
strains of the two (A and B) antigenic subgroups (Olmsted et al., 1986; Stott et al., 1987; Walsh et al., 1987).

Monoclonal antibodies (MAbs) with the highest neutralization index recognize epitopes of the RSV F glycoprotein (Trudel et al., 1987; Anderson et al., 1988; Beeler & van Wyke Coelingh, 1989; Garcia-Barreno et al., 1989). This protein is synthesized as an inactive precursor which is glycosylated and processed proteolytically during maturation to generate two subunits (F1 and F2) that remain linked by disulphide bridges (Gruber & Levine, 1983). Virus-binding competition assays between MAbs have identified several antigenic sites on the F protein molecule which are recognized by both the murine and the bovine immune systems (the latter being a natural host of bovine RSV, which is closely related to human RSV) (Taylor et al., 1992).

Several laboratories have located a few epitopes on the primary structure of the F protein by (i) using synthetic peptides to examine the binding of MAbs (Trudel et al., 1987; Bourgeois et al., 1991; Martin-Gallardo et al., 1991) or polyclonal antibodies (Scopes et al., 1990), or (ii) isolating viruses that escape neutralization by anti-F protein MAbs in order to identify amino acid residues essential for epitope integrity (Lopez et al., 1990). Since both approaches have limitations (see Discussion), we have used the two complementary strategies to locate the epitopes recognized by several anti-F protein neutralizing MAbs on the primary structure of the protein.

Methods

Cells and viruses. Wild-type and neutralization escape mutant viruses were grown in HEp-2 cells and purified from culture supernatants as described previously (Garcia-Barreno et al., 1988). The Long and A2 reference strains of RSV were plaque-purified before being used for the selection of MAb-resistant mutants. These were selected in two different ways.

(i) Escape mutants of the Long strain were isolated as described previously (Garcia-Barreno et al., 1989). Briefly, virus stocks were enriched in mutant viruses by four or five consecutive passages in the presence of the selecting antibody (47F, 7C2 or AK13A2). The viruses were then plaque-purified in agar plates containing antibody. Several virus plaques were isolated, and their resistance to antibody neutralization was confirmed. A single plaque originating from each aliquot of the virus stock was chosen for further analysis.

(ii) Escape mutants of the A2 strain were selected for resistance to MAbs B4, 11, 19 or 20 by direct plaquing of the virus in agar plates containing antibody as described (Taylor et al., 1992).

Purification of the F glycoprotein and trypsin treatment. The F protein was purified by immunoaffinity chromatography from extracts of HEp-2 cells infected with the Long strain using the method of Walsh et al. (1985) as described previously (Garcia-Barreno et al., 1989). The purified protein was digested with different amounts of trypsin for 4 h at 37 °C. Digestion was terminated by the addition of electrophoresis sample buffer (Studier, 1973) and boiling of the samples for 3 min. The binding of MAbs to the trypsin fragments was tested by Western blotting (Towbin et al., 1979).

Antibody binding to RSV. The isolation and characterization of the MAbs used in this study have been documented (Taylor et al., 1984, 1992; Trudel et al., 1987; Garcia-Barreno et al., 1989). Antibody AK13A2, raised against the Long strain F protein, was a generous gift from Dr. P. Coppe (Centre d’Economie Rurale, Marloie, Belgium). B4 is a bovine MA produced by a heterohybridoma of bovine splenocytes and murine myeloma cells (Kennedy et al., 1988). All other MAbs were of murine origin. The antibodies were purified from ascitic fluids by Protein A-Sepharose chromatography and peroxidase-labelled as described (Garcia-Barreno et al., 1989).

The different viruses were tested for reactivity with anti-F protein MAbs in a direct ELISA using non-saturating amounts of each antibody previously titrated against the Long strain (Palomo et al., 1990). The competition between two antibodies for simultaneous binding to the virus was determined by using a peroxidase-labelled antibody mixed with increasing amounts of an unlabelled antibody (Garcia-Barreno et al., 1989). The capacity of an anti-idiotypic rabbit antiserum, raised against MAb 47F, to inhibit the binding of MAbs to RSV was tested by ELISA as described (Palomo et al., 1990).

Peptides. The peptides indicated in Table 2 were synthesized according to the sequence of the Long strain F protein in an Applied Biosystems 430 instrument, using the solid-phase technology and t-Boc chemistry developed by Merrifield (1986). The peptides were cleaved off the resin with trifluoroethyl sulphonic acid, and purified from protecting groups and scavengers by Sephadex G-25 chromatography. The amino acid sequence of each peptide was confirmed by automated Edman degradation in an Applied Biosystems 477 protein sequencer.

The binding of MAbs to synthetic peptides or RSV cell lysate was tested by ELISA in PVC microtitre plates coated overnight with 1 to 2 μg of peptide. PBS containing 5% pig serum was used as a blocking reagent to eliminate spurious cross-reactions (Taylor et al., 1992).

Sequencing of F mRNA's. HEp-2 cells were infected with the different viruses and harvested 30 to 40 h post-infection, when c.p.e. was evident by the formation of syncytia. Total RNA was isolated by the isothiocyanate–CsCl method (Chirgwin et al., 1979) and poly(A)+ RNA was selected by oligo(T)- cellulose chromatography. These mRNA preparations were used for sequencing by the dideoxynucleotide chain termination method (Sanger et al., 1977) using reverse transcriptase and 5'-32P-labelled oligonucleotides, followed by a chase with terminal deoxynucleotidyl transferase (DeBorde et al., 1986). The primers used for sequencing were synthesized according to the reported sequence of the Long strain F protein gene (Lopez et al., 1988) and their composition can be obtained from the authors upon request.

For mutants selected with MAb 11, cytoplasmic extracts were prepared from virus-infected HEp-2 cells treated with actinomycin D (Huang & Wertz, 1983). After centrifugation through CsCl, poly(A)+ RNA was selected as above. Primer extension sequencing was performed using avian myeloblastosis virus reverse transcriptase (Air, 1979) and a synthetic oligonucleotide complementary to bases 983 to 906 in the strain A2 F protein gene sequence (Collins et al., 1984). The sequence between bases 758 and 847 was determined for the parental strain and each mutant selected with MAb 11.

Generation and expression of mutated F glycoproteins. Plasmid pSLF88 (Lopez et al., 1988), which contains a cDNA copy of the entire F mRNA from the Long strain, was used as a template to mutagenize nucleotide 1298 by polymerase chain reaction (PCR) technology (Ho et al., 1989), as illustrated in Fig. 4(a). Oligonucleotides F1306 (5’ GATTCACATTTTATGGAATG, anti-mRNA sense) and F1287 (5’ CCAATAAAATAGTGGAATC, mRNA sense), which included the transcription C to A at position 1298 (shown in bold), were used in conjunction with oligonucleotides F11 (5’ ACAATGGAGTTGCCAG, mRNA sense) and F1287 (5” GATTCCACTATTTTTATTGG, anti-mRNA sense) and poly(A) + mRNA from the Long strain, was used as a template to mutagenize.

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Epitopes on RSV fusion protein

Table 1: MAb used for selection

<table>
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<th>7C2</th>
<th>B4</th>
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<th>20</th>
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<td></td>
<td>2F</td>
<td>44F</td>
<td>45F</td>
<td>55F</td>
<td>60F</td>
<td>75F</td>
</tr>
<tr>
<td>Ib</td>
<td></td>
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<td>49F</td>
<td></td>
<td></td>
<td>55F</td>
<td>76F</td>
</tr>
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<td>7C2</td>
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<td>59</td>
</tr>
<tr>
<td>III</td>
<td></td>
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<td></td>
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<td>75F</td>
<td>79</td>
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<tr>
<td>IV</td>
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<td>19</td>
<td>20</td>
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</table>

Fig. 1. Binding of anti-F protein MAbs to RSV Long strain and escape mutant viruses. The MAbs were tested in a direct ELISA using the purified viruses indicated at the top to coat microtitre plates. <25% (□), 25% to 50% (■) and >50% (anyak) of the absorbance values obtained with the Long strain.

ATCC, mRNA sense) and F1899-Eco R1 (5' CGGAATTCCGATATCCTTAACTATAAACTAGG, anti-mRNA sense) for the first amplification step. Oligonucleotides F743 (5' ACTACACCTGTAAGCACT) and F1899-Eco R1 were used for the second amplification step.

The amplified DNA was digested with BclI and EcoRI and the intervening segment was isolated after electrophoresis in agar plates. This segment was ligated to the largest segment of the LF1 plasmid (Cristina et al., 1990) digested with the same enzymes. Bacterial colonies containing the recombinant plasmid (LF1/1298) were identified by hybridization to radiolabelled F1287 primer, and the mutated nucleotide was confirmed by direct sequencing of the insert. The recombinant LF1/1298 was used in the transient expression system of Fuerst et al. (1986), using CV1 cells infected with a vaccinia virus-T7 recombinant (10 p.f.u./cell) and subsequently transfected with plasmid DNA (1 ng/10⁶ cells) by the lipofectin procedure (Bethesda Research Laboratories). The cells were incubated at 37°C for 48 h, fixed with methanol:acetone and stained for immunofluorescence as described (Garcia-Barreno et al., 1988).

Results

Isolation and characterization of neutralization escape mutants

The Long and A2 strains of human RSV were plaque-purified before being used to select viruses that escaped neutralization by MAbs 47F, AK13A2, 7C2, B4, 19 or 20 (see Methods). Each escape mutant was used as antigen in an ELISA to test its reactivity with a panel of anti-F protein MAbs (Fig. 1). The epitopes recognized by most MAbs had been previously grouped into five antigenic areas (Ia, Ib, II, III and IV) according to their competition for simultaneous binding to the virus (Garcia-Barreno et al., 1989). The epitopes recognized by MAbs AK13A2, B4 and 7C2 were included in antigenic area II owing only to their reactivity with escape mutants; similarly, the epitopes of MAbs 19 and 20 were included in antigenic area IV by the same criteria. The A2 strain reacted identically to the Long strain with the MAbs tested in Fig. 1 (not shown).

The mutations selected in the escape viruses affected only epitopes from the antigenic area which included the epitope recognized by the selecting MAb. For instance, mutant 4/4 did not react with any of the MAbs recognizing area II, whereas other mutants selected with the same MAb (11/3, 4, 5 and 7) reacted with MAbs 7C2 and B4, but not with MAbs 47F, 49F or AK13A2. Similarly, the mutants selected with MAbs 19 and 20 did not bind the MAbs recognizing epitopes grouped in antigenic area IV, except MAb 52F. However, in all
cases the mutant viruses did bind MAbs recognizing other antigenic areas.

The different reactivities of the MAbs from antigenic area II with the escape mutants indicated that their epitopes might overlap on the F protein molecule, but were not identical. To differentiate these epitopes further, we tested whether the corresponding MAbs would compete for simultaneous binding to the virus, and if their binding could be inhibited by an antiidiotype antiserum raised against MAb 47F (Palomo et al., 1990). The results obtained (Fig. 2) indicated extensive competition between these MAbs for virus binding; however, MAb AK13A2 inhibited the binding of MAbs 47F and 49F in a non-reciprocal manner. In addition, the anti-idiotype antiserum inhibited the virus binding of MAbs 47F and 49F, but not that of MAbs AK13A2, 7C2 and B4 (Fig. 2d). Thus, the epitopes included in antigenic area II could be distinguished by at least one of the following criteria: (i) the reactivity of MAbs with escape mutants, (ii) the competition of MAbs for virus binding and (iii) the inhibition of virus binding by an anti-idiotype antiserum. Only the epitopes recog-
nized by MAbs 47F and 49F could not be distinguished by the above criteria, but these MAbs differ in both neutralizing capacity and susceptibility to denaturing agents. Thus, MAb 49F is a non-neutralizing antibody, the binding activity of which is lost in Western blots, whereas MAb 47F is a highly neutralizing antibody which resists Western blot treatment (Garcia-Barreno et al., 1989).

Location of amino acid changes selected in neutralization escape mutants

As an initial step toward locating the epitopes recognized by the MAbs used in the selection of mutant viruses, the MAbs were tested against trypsin fragments of purified F protein by Western blotting (Fig. 3). Increasing amounts of trypsin generated smaller fragments of the F1 subunit which were stained with Coomassie blue (Fig. 3a). Four F1 fragments of 30K, 20-5K, 19K and 15K were recognized by MAb AK13A2 (Fig. 3b). The 20-5K and 19K fragments have been mapped previously (Lopez et al., 1990) to the N-terminal end of the F1 subunit (Fig. 3d). MAbs 47F, 7C2 and B4 recognized the same set of fragments as AK13A2 (not shown). Thus, the epitopes of antigenic area II could be ascribed to amino acid sequences included within the N-terminal third of the F1 fragment.

Antibody 19 reacted with a different set of F1 fragments (Fig. 3c). Only large fragments (26K and 22K), generated with low amounts of trypsin, reacted with MAb 19 (MAb 20 reacted less efficiently with the same set of fragments; data not shown). Thus, the epitopes recognized by MAbs 19 and 20 contain trypsin-sensitive amino acid sequences which were tentatively located within the C-terminal two-thirds of the F1 subunit (Fig. 3d), outside the region covered by the fragments recognized by MAb AK13A2. The N-terminal end of the 26K and 22K fragments could not be determined by direct protein sequencing owing to a low yield after trypsin treatment.

To identify the amino acid changes selected in the escape mutants, we sequenced F mRNAs obtained from cells infected with the different viruses. The F mRNAs of viruses selected with MAbs AK13A2, 7C2 and B4 were sequenced between nucleotides 420 and 920, which encode the trypsin-resistant fragments recognized by these MAbs (Fig. 3). Similarly, the F mRNAs of viruses selected with MAbs 19 and 20 were sequenced between nucleotides 1100 and 1680, which encode the region of the putative 26K trypsin-resistant fragment recognized by these MAbs.

The changes detected in the different mutants are listed in Table 1, including two previously reported mutants selected with MAb 47F (Lopez et al., 1990). Also included in Table 1 is the sequence change observed in the prototype (C4389/1) of five mutants selected with

<table>
<thead>
<tr>
<th>MAb used for selection</th>
<th>Nucleotide at position*</th>
<th>Amino acid at position*</th>
<th>Loss of binding with antibodies</th>
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<tr>
<td>Long and A2</td>
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<td>AK13A2</td>
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<tr>
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*Only nucleotide (mRNA sense) and amino acid changes from the sequence of the Long and A2 strains of RSV are shown.
†ND, Not done.
MAb 11. This MAb competed for virus binding with other MAbs recognizing epitopes from antigenic area II (Taylor et al., 1992) and selected mutants with a single transversion (A to U) at nucleotide 816, which changed the asparagine residue at position 268 to isoleucine. This change is identical to that found in mutant 7 selected with MAb 47F, which led to the loss of all the epitopes included in antigenic area II (Fig. 1).

Four viruses selected with MAb AK13A2 (11/3, 4, 5 and 7) had a single transversion (A to U) at nucleotide 797 which changed the asparagine at position 262 to tyrosine. This change eliminated the binding sites for antibodies 47F, 49F and AK13A2 (see Fig. 1) and is identical to the change observed in mutant 4 selected with MAb 47F. A fifth virus selected with MAb AK13A2 (4/4) had, in addition, a transition (A to G) at
Table 2. Reactivity of MAbs with synthetic peptides

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* log_{10} titre of MAb binding to synthetic peptides dried onto wells or RSV antigen tested in an ELISA.

nucleotide 659 which changed the asparagine at position 216 to aspartic acid. This second amino acid change led to the loss of all the epitopes from antigenic area II (Fig. 1). The last mutant selected with MAb AK13A2 (4') had a single transition of A to G at nucleotide 827, leading to the replacement of the lysine at position 272 with glutamic acid and the loss of all the epitopes from area II.

All mutants selected with MAb 7C2, except mutant 4, contained single nucleotide changes (A to G or A to C) at positions 827 or 828 which changed Lys 272 to Glu or Thr, respectively. These changes eliminated the reactivity of all MAbs with the epitopes in antigenic area II. Mutant 4 had two nucleotide substitutions, at positions 583 (C to A) and 786 (U to C), which changed amino acids 190 (Ser to Arg) and 258 (Leu to Ser). The last mutant lost only the binding site for MAb 7C2, but retained its reactivity with all the other anti-F protein antibodies (Fig. 1).

The two mutants selected with MAb B4 had a single nucleotide transversion at position 828 (A to C) which changed Lys 272 to Thr. Thus, all the amino acid changes selected with MAbs recognizing epitopes from antigenic area II were clustered in a small segment of the F protein, between amino acids 262 and 272, except the changes at amino acids 258, 216 and 190, which were detected only in viruses with two amino acid substitutions.

All mutants selected with MAbs 19 or 20 contained a single C to A transversion at nucleotide 1298 which changed Arg 429 to Ser (Table 1). This amino acid change, located towards the C-terminal end of the cysteine-rich region of the F1 subunit (Fig. 3d), eliminated the reactivity of all the MAbs recognizing epitopes in antigenic area IV, except that of MAb 52F. The sequence results confirmed that the epitopes included in antigenic areas II and IV did not overlap in the primary structure of the F protein, as inferred from the results in Fig. 1 and 3.

Since the location on the F1 subunit of the trypsin fragments which reacted with MAbs 19 and 20 was deduced indirectly (Fig. 3), direct confirmation of the involvement of amino acid 429 in these epitopes was sought. To this end, plasmid F88 (Lopez et al., 1988) was mutagenized at nucleotide position 1298 using PCR technology (Fig. 4a). A BclI-EcoRI fragment of plasmid LF1, which contained a Long strain F cDNA cloned into the pGEM4 vector (Cristina et al., 1990), was replaced by the in vitro mutagenized DNA. Recombinants with the expected nucleotide change were obtained, and this was confirmed by direct sequencing of the insert. One of these recombinants was used in the vaccinia virus-T7 transient expression system (Fuerst et al., 1986) to test the reactivity of the mutated F protein with MAbs. Fig. 4(b to e) shows the immunofluorescence of cells transfected with one of these recombinants. The transfected cells were stained with MAbs 47F, 7C2 and AK13A2, but not by MAb 19 (nor MAbs 56F and 20; data not shown). This result confirmed that a single change (Arg to Ser) at amino acid 429 of the F glycoprotein was sufficient to confer the antigenic changes observed in the escape mutants selected with MAbs 19 or 20.

Reactivity of antibodies with synthetic peptides

Since the antibodies used to select the escape mutants reacted in Western blots with trypsin fragments of the F1 subunit (Fig. 3), we tested whether synthetic peptides could reproduce the epitopes recognized by these antibodies.

Three peptides were synthesized with sequences corresponding to amino acids 250 to 273, 255 to 275 or 258 to 271 of the F1 subunit, which surrounded the positions changed in the mutants selected with MAbs recognizing antigenic area II. These peptides were tested in an ELISA for reactivity with MAbs (Table 2). Only antibody B4 (and another bovine MAb, B5; not shown) reacted with peptide 255 to 275. The MAb B4 titre with this peptide was similar to that obtained against an RSV-infected cell lysate. However, this antibody did not react with peptide 250 to 273 or 258 to 271, which contained
almost the entire amino acid sequence included in peptide 255 to 275. All other antibodies recognizing area II failed to react with any of the peptides.

Three other peptides, corresponding to the F1 sequences between amino acids 417 and 432, 422 and 438, and 435 and 450 which surround position 429 changed in the escape mutants selected with MAbs 19 or 20, were also tested by ELISA (Table 2). Only MAb 19 reacted with peptide 422 to 438. The titre of MAb 19 with this peptide was similar to that obtained with the RSV-infected cell lysate.

Discussion

Several epitopes on the RSV F glycoprotein recognized by neutralizing antibodies were located using two complementary procedures: (i) isolation and sequencing of mutants which escaped neutralization by MAbs and (ii) synthesis of peptides with sequences containing the amino acids changed in the escape mutants. The results obtained identified two regions of the F protein primary structure in which the epitopes recognized by neutralizing MAbs were located.

The first region mapped within a trypsin-resistant segment of the F1 subunit near its N terminus, and contained the overlapping epitopes recognized by MAbs 47F, 49F, 7C2, AK13A2, 11 and B4, included in antigenic area II (Fig. 1 and 3). Most amino acid changes found in mutants selected with these MAbs were clustered around amino acids 262 to 272. Since these MAbs reacted in Western blots with proteolytic fragments of the F1 subunit, it was originally thought that they recognized linear epitopes determined by sequences of consecutive amino acids. However, it seems that some local conformation(s) is needed for the integrity of certain epitopes. For instance, the change at amino acid 216 (Asn to Asp), selected in mutant 4/4 resistant to MAb AK13A2, eliminated the reactivity with MAbs 7C2 and B4 (which selected changes at position 272). Consequently, some long-range effect of amino acid 216 in the structure adopted by the epitope recognized by MAb B4 in the F1 subunit is likely to occur.

The results obtained with the synthetic peptides were also indicative of conformational constraints on the epitopes in antigenic area II. The epitope recognized by MAb B4 was reproduced by peptide 255 to 275; however, other closely related peptides failed to react with this MAb. In addition, none of the peptides tested reproduced other epitopes in antigenic area II. It is worth noting that the region of the F1 subunit containing these epitopes is resistant to high doses of trypsin, indicative of a particular three-dimensional conformation which might be preserved in Western blots, but not in short peptides.

Other studies have also identified synthetic peptides closely related to regions of antigenic area II which react with anti-F protein MAbs. Bourgeois et al. (1991) have reported the reactivity of one (RS-348) of nine MAbs with peptide 200 to 225 and Martin-Gallardo et al. (1991) have reported that peptides spanning residues 289 to 298 react with MAb L4. However, no data from mutants escaping neutralization by MAbs RS-348 and L4 has been published to confirm the epitope location deduced using the peptides.

It has been reported previously that MAb 7C2 used in this study reacts with peptides containing regions 221 to 232 and 275 to 288 of the F1 subunit in a dot-blot assay (Trudel et al., 1991). However, this antibody failed to react with peptides containing those regions under the stringent ELISA conditions used in this study (not shown). Thus, caution should be taken in interpreting the results of antibody binding to synthetic peptides, which are prone to show cross-reactions (Trifilieff et al., 1991). The isolation and sequencing of neutralization escape mutants therefore are important to confirm the epitope location inferred from using synthetic peptides. In addition, the escape mutants can be used to locate conformational epitopes, not reproduced by peptides; however, long-range conformational effects of the mutations, similar to those reported for foot-and-mouth disease virus (Parry et al., 1990), should not be overlooked.

The second antigenic region (area IV) of the F protein identified in this study maps towards the end of the cysteine-rich region located in the middle of the F1 subunit. This region is very sensitive to trypsin digestion and is relatively near the amino acid sequence (479 to 490) which Scopes et al. (1991) found to react with human post-infection sera. Peptide 422 to 438 reacted strongly with MAb 19; however, MAbs 52F, 56F, 57F and 20 (having epitopes also included in area IV) did not react with either the synthetic peptides or the denatured F1 subunit. A recombinant F protein which incorporated a single amino acid substitution at position 429 (Arg to Ser) reproduced the antigenic changes observed in the viruses that escaped neutralization by MAbs 19 and 20.

The neutralization of RSV by the MAbs used in this study might be related to their capacity to inhibit the membrane fusion activity of the F glycoprotein (Garcia-Barreno et al., 1989; Taylor et al., 1984). By analogy with other paramyxoviruses (review by Morrison, 1988), it is assumed that the fusion activity of RSV depends upon the proteolytic processing of the F protein precursor. This modification generates the new N terminus of the F1 subunit, proposed to interact with lipid membranes through a short hydrophobic peptide. The antigenic areas of the F glycoprotein identified in this study are located distant to the fusion peptide in a linear map;
however, it is possible that the binding of antibodies to other regions of the F protein influence the activity of the fusion peptide. In this respect, mutants altered in the fusogenic activity of the influenza virus haemagglutinin (Daniels et al., 1985) have been mapped outside the fusion peptide of the HA2 subunit. It should also be emphasized that in vitro protection by MAbs does not always correlate with their capacity for in vitro neutralization.

It is of interest that the murine and bovine MAbs recognizing epitopes in antigenic area II on the F protein molecule were isolated independently in several laboratories, and that human post-infection sera contain antibodies which compete for virus binding with at least some of these MAbs (Palomo et al., 1991). Thus, epitopes in this site of the F protein are antigenic in several hosts. In addition, the epitopes recognized by the antibodies recognizing areas II and IV are conserved in a large number of virus strains (not shown). These results are of special relevance for the design and evaluation of effective RSV vaccines.

We thank Angel del Pozo for the art work. J. A. was the recipient of a training fellowship from the CEE and J. A. L. of a postdoctoral fellowship from "Instituto de Salud Carlos III". This research was supported by grants from Comision Interministerial de Ciencia y Tecnologia, Fondo de Investigaciones Sanitarias and the World Health Organization (J. A. M.), and by a British/Spanish Joint Research Programme (J. A. M. and G. T.).

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(Received 20 March 1992; Accepted 11 May 1992)