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Marked Differences in the Antigenic Structure of Human Respiratory Syncytial Virus F and G Glycoproteins

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Monoclonal antibodies directed against the glycoproteins of human respiratory syncytial virus were used in competitive enzyme-linked immunosorbent assays for topological mapping of epitopes. Whereas epitopes of the F glycoprotein could be ascribed to five nonoverlapping antigenic sites, anti-G antibodies recognized unique epitopes, many of whose competition profiles overlapped extensively. Variant viruses selected with a neutralizing (47F) anti-F antibody lost the binding for only 47F and 49F antibodies, which mapped in the same antigenic area. In contrast, viruses selected with an anti-G antibody lost the capacity to bind most of the anti-G antibodies, and their G protein was not recognized by an anti-virus antiserum, indicating major changes in the antigenic structure of the G molecule. Finally, we found great antigenic variation of the G protein among viral isolates. This occurred even within viruses of the same subtype with only limited divergence of amino acid sequence between strains. All of these data indicate marked differences in the antigenic organization of the G and F glycoproteins of respiratory syncytial virus; we discuss these differences in terms of the chemical structure of the glycoproteins.

Human respiratory syncytial (RS) virus is a major cause of severe lower respiratory infections in infants and young children (17). Until recently, it was considered a monotypic virus, although early cross-neutralization data with animal hyperimmune sera indicated antigenic differences among isolates (8). With the use of monoclonal antibodies, antigenic heterogeneity is now well documented, and viral isolates have been classified in two subtypes (3, 10, 19, 20).

Strain differences are potentially important both for vaccine development and for the understanding of clinical and epidemiological characteristics of RS virus. This might be particularly relevant in the case of the two external antigens (F and G). The G protein is synthesized as a 33-kilodalton precursor (22, 28), which is then extensively glycosylated to yield a mature protein of 80 to 90 kilodaltons. The F 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 disulfide bridges (11).

The genetic basis of antigenic variation in RS virus remains unknown. However, sequence data provide some quantitative estimation of genetic heterogeneity among isolates. When strains (A2 and CH18537) of different subtypes are compared, the G glycoproteins share only 53% amino acid identity, whereas the homology for other proteins is much higher (1A, 76%; 1C, 87%; F, 89%; N, 96%) (12). The comparison of strains within the same subtype, however, shows a more restricted variation: 94% amino acid identity for the G protein (12), 97 to 98% identity for the F protein (6, 16), and 98 to 99% identity for the phosphoprotein (P) (13, 16).

In agreement with the sequence data, the greatest antigenic variation between strains of RS virus subtypes was detected in the G protein, but changes were also observed in the F, NP, P, M, and 22-kilodalton protein epitopes (3, 19). Only occasional antigenic differences have been reported within strains of the same subtype (1, 18). We have extended these studies with a larger panel of monoclonal antibodies to better characterize RS virus glycoproteins. Extensive changes of the G epitopes are reported even within strains of the same subtype. In addition, topological mapping of epitopes and isolation of variants resistant to antibody neutralization revealed marked differences between the F and G glycoproteins.

MATERIALS AND METHODS

Cells and viruses. The Long strain of human RS virus, isolated in Baltimore, Md., in 1956, was used as the RS virus prototype throughout this work. It was grown in HEp-2 cells and purified from culture supernatants as previously described (9). The origins and years of isolation of other strains, including viruses isolated in this laboratory during 1984 and 1986 epidemics, are indicated in Fig. 6.

Preparation of monoclonal antibodies. Purified RS virus (Long strain) was used to immunize female BALB/c mice. Immune splenocytes were fused to Sp2-O myeloma cells (23) as previously described (21). In some cases, mice were immunized or boosted with purified F glycoprotein. The identification of antibody-containing supernatants was done by an enzyme-linked immunosorbent assay (ELISA) with purified virus or F protein as the antigen. The antibody isotypes were determined by double immunodiffusion using class and subclass immunoglobulin-specific antisera (Nordic). The determination of antibody specificities was done by one of the following two procedures.

(i) Western blotting. Proteins from purified virus were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (25) and electrotransferred to nitrocellulose papers (26), which were cut into strips and incubated with individual antibodies. The antigen-antibody complexes were developed by using biotinylated anti-mouse immunoglobulin, streptavidin-peroxidase, and 4-chloro-1-naphtol as recommended by the manufacturer (Amersham Corp.).

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(ii)Antigen-binding assays. The antigen-binding assay is an alternative procedure to radioimmunoprecipitation (15). In short, individual antibodies were bound to polyvinylchloride microdilution wells via protein A and anti-mouse immunoglobulin serum (Calbiochem-Behring). Radiolabeled cell extracts were then added and allowed to adsorb to the antibodies. The bound material was eluted in SDS-containing buffer and analyzed by SDS-PAGE (25) and fluorography.

The antibodies are referred by number followed by a letter denoting their specificity throughout the text.

Preparation of radiolabeled cell extracts. HEp-2 monolayers were infected with RS virus (1.0 PFU per cell) as previously described (9). Cultures were labeled from 24 to 36 h after infection with either 300 μCi of [^{3}H]glucosamine per ml or 100 μCi of [^{35}S]methionine per ml. After labeling, cells were washed with phosphate-buffered saline and solubilized in lysis buffer (10 mM Tris hydrochloride [pH 7.5], 140 mM NaCl, 5 mM EDTA, and 1% octyl glucoside).

Purification and labeling of antibodies. Hybridomas were grown as ascitic tumors in pristane-primed BALB/c mice. The ascitic fluids were mixed with equal volumes of buffer A (1.5 M glycine, 3.0 M NaCl [pH 8.9]) and passed through a protein A-Sepharose column (Pharmacia Fine Chemicals) equilibrated in the same buffer. More than 90% of all immunoglobulin G subclasses and variable amounts of immunoglobulin M were absorbed under these conditions. Bound antibodies were eluted with buffer B (0.1 M citrate [pH 5.0]), neutralized with saturated Tris, and dialyzed against phosphate-buffered saline.

Purified antibodies were peroxidase labeled by the glutaraldehyde procedure (4). Alternatively, some antibodies were peroxidase labeled after periodate oxidation (29).

Purification of the F glycoprotein. Purified 2F antibody (see Fig. 3) was bound to CNBr-activated Sepharose (Pharmacia) according to the instructions of the manufacturer. Cell extracts, made as described above, were applied to immunoaffinity columns, and the bound material was eluted with 0.1 M glycine (pH 2.5) as described by Walsh et al. (27).

Virus neutralization. The ability of individual monoclonal antibodies to inhibit RS virus infectivity was assayed by one of the following two procedures.

(i) Microneutralization. Tenfold serial dilutions (20 μl) of virus in Dulbecco medium containing 2.5% fetal calf serum (DMEM2.5), were mixed with an equal volume of antibody-containing supernatant and incubated for 60 min at 37°C. The virus-antibody mixtures were then added to monolayers of HEp-2 cells grown in 96-well tissue culture plates. After 2 h of adsorption at 37°C, the inoculum was replaced by DMEM2.5. Plates were incubated for 3 days at 37°C, fixed with 10% formaldehyde in phosphate-buffered saline, and stained with crystal violet. Using this basic protocol, three modifications were also tested: (i) individual antibodies were included in the growth medium after virus adsorption as a 1/5 dilution of hybridoma supernatants, (ii) a 1/10 dilution of rabbit serum was added to the virus-antibody mixtures as a source of complement, and (iii) a 1/10 dilution of heat-inactivated rabbit anti-mouse immunoglobulin was added to the virus-antibody mixtures. In the last two cases the antibodies were not included in the DMEM2.5 added after virus adsorption.

(ii) Plaque assay. Virus dilutions (100 μl) were prepared and mixed with antibodies as before and then added to HEp-2 monolayers grown in 35-mm plates (Costar). After the adsorption period the inoculum was replaced by DMEM2.5 containing 0.7% agar. Plates were incubated for 5 days at 37°C, and then an overlayer of medium containing 0.7% agar and 0.01 μg of neutral red per ml was added. Viral plaques were counted after a further incubation at 37°C for 24 h.

Isolation of neutralization-resistant variants. (i) RS viruses resistant to 47F antibody. Five 75-μl samples of plaque-purified Long virus (4×10^6 PFU) were incubated for 60 min at 37°C with an equal volume of culture supernatant containing 47F antibody (see Fig. 3). In parallel, the same amount of virus was treated with control antibody. The antibody-virus mixtures were used to infect HEp-2 cells grown in 24-well plates as mentioned above. No cytopathic effect was observed after 3 days at 37°C with 47F-treated virus; however, after three consecutive cycles under identical conditions, RS viruses that were resistant to 47F neutralization emerged in the five independently passed culture supernatants. These viruses were cloned in agar plates. Several viral plaques were isolated, and their resistance to 47F neutralization was confirmed. One plaque coming from each of the five supernatants was selected for further analysis.

(ii) RS viruses resistant to 63G antibody. The basic protocol was essentially as described above except that 63G antibody (see Fig. 4) was used throughout the selection procedure. In this case, the amount of virus in the initial inoculum was lowered (2×10^3 PFU), and a 1/10 dilution of rabbit serum (complement source) was added to the virus-antibody mixture; furthermore, a 1/5 dilution of 63G antibody containing supernatant was added to the culture medium. Under these conditions, no cytopathic effect was observed after 3 days at 37°C, but resistant viruses emerged after seven cycles under identical conditions. These were cloned as indicated above.

RESULTS

Preparation of monoclonal antibodies against the Long strain of RS virus. Hybridomas were obtained after fusion of immune splenocytes with Sp2-O myeloma cells. Most immunizations were done with purified RS virus, but in some cases purified F protein was used to stimulate the production of antibodies directed against this protein (see below).

The supernatants of hybridoma cultures producing virus-specific antibodies, as determined by ELISA, were tested in an antigen-binding assay with either [^{35}S]methionine- or [^{3}H]glucosamine-labeled cell extracts (Fig. 1). Both the anti-G and anti-F monoclonal antibodies were more easily identified with sugar-labeled extracts; anti-G antibodies (lanes a through d) recognized a characteristic wide band of 90 kilodaltons, whereas the anti-F monoclonal antibody (lanes e through g) bound both F1 and F2 subunits, which were separated upon electrophoresis under reducing conditions.

In addition to antibodies directed against the external glycoproteins, other antibodies were identified which reacted with the internal components of RS virus present in [^{35}S] methionine-labeled cell extracts. Anti-P antibodies (lanes j and l) bound both ^{35}S-labeled P and NP proteins, which, as reported by others (19), suggests the presence of a protein complex in the extracts. Anti-NP antibodies (lane k) also bound small amounts of P protein in addition to the NP polypeptide. Consequently, the specificities of anti-NP and -P antibodies were better determined by immunoblotting (lanes n and o) after SDS-PAGE separation of the viral polypeptides. Anti-M antibodies recognized the matrix polypeptide in both antigen-binding and Western blotting (immunoblotting) tests (lanes m and p). Finally, antibodies against the 22-kilodalton M2 protein reacted best in Western blotting (lane r). Of 56 monoclonal antibodies raised against the Long

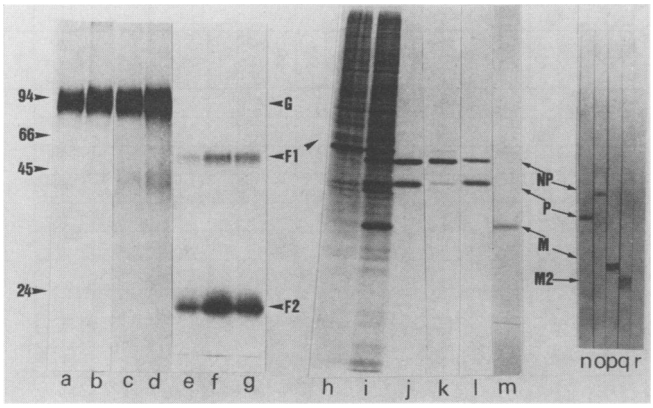

FIG. 1. Determination of antibody specificities. Monoclonal antibodies were tested in the antigen-binding assay (described in Materials and Methods) with either [^{3}H]glucosamine (lanes a through g)- or [^{35}S]methionine (lanes j through m)-labeled extracts of RS virus-infected cells. The bound material was eluted from the antibodies and analyzed by SDS-PAGE and fluorography. The following antibodies were tested (lanes): a, 25G; b, 63G; c, 28G; d, 78G; e, 2F; f, 47F; g, 52F; j, 1P; k, 42NP; l, 11P; m, 29M. Antibodies were also tested by immunoblotting against the following separated viral proteins (lanes): n, 1P; o, 42NP; p, 29M; q, M2; r, negative control antibody. Lanes h and i correspond to [^{35}S]methionine-labeled cell extracts from uninfected and RS virus-infected HEp-2 cells, respectively. Molecular weight markers, shown in the left-hand margin, are expressed in thousands.

strain of RS virus, 19 were specific for the G protein, 14 were specific for the F protein, and the rest recognized other internal viral components.

Identification of antigenic areas in RS virus glycoproteins. Monoclonal antibodies reacting with RS virus glycoproteins were purified, enzyme labeled, and used in ELISAs either alone or in the presence of increasing amounts of unlabeled antibodies of the same specificity. Figure 2 shows representative results obtained with antibodies 71G (Fig. 2A) and 2F (Fig. 2B). In both cases, some unlabeled monoclonal antibodies inhibited the binding of the labeled antibody in a dose-dependent manner; other monoclonal antibodies, however, did not interfere with the binding of the test antibody. These results then identified groups of antibodies that compete for simultaneous binding to the antigen. The epitopes recognized competing antibodies were considered to be operationally within the same antigenic area of the protein molecule.

Similar competitive ELISAs were done with the panel of 14 anti-F antibodies (Fig. 3). All epitopes could be clustered into four antigenic areas according to the competition profiles of their specific antibodies. However, area I could be further subdivided into two subareas, since antibodies 75F and 70F (area Ib) showed nonreciprocal competition with the other antibodies of the same group. It is noteworthy that no overlapping was observed among antibodies recognizing epitopes of different antigenic domains.

A similar approach was used to test the panel of 19 anti-G antibodies. In this case, most monoclonal antibodies presented individual competition profiles (Fig. 4). For instance, 62G and 26G antibodies competed to a similar degree with several labeled monoclonal antibodies but differed in their

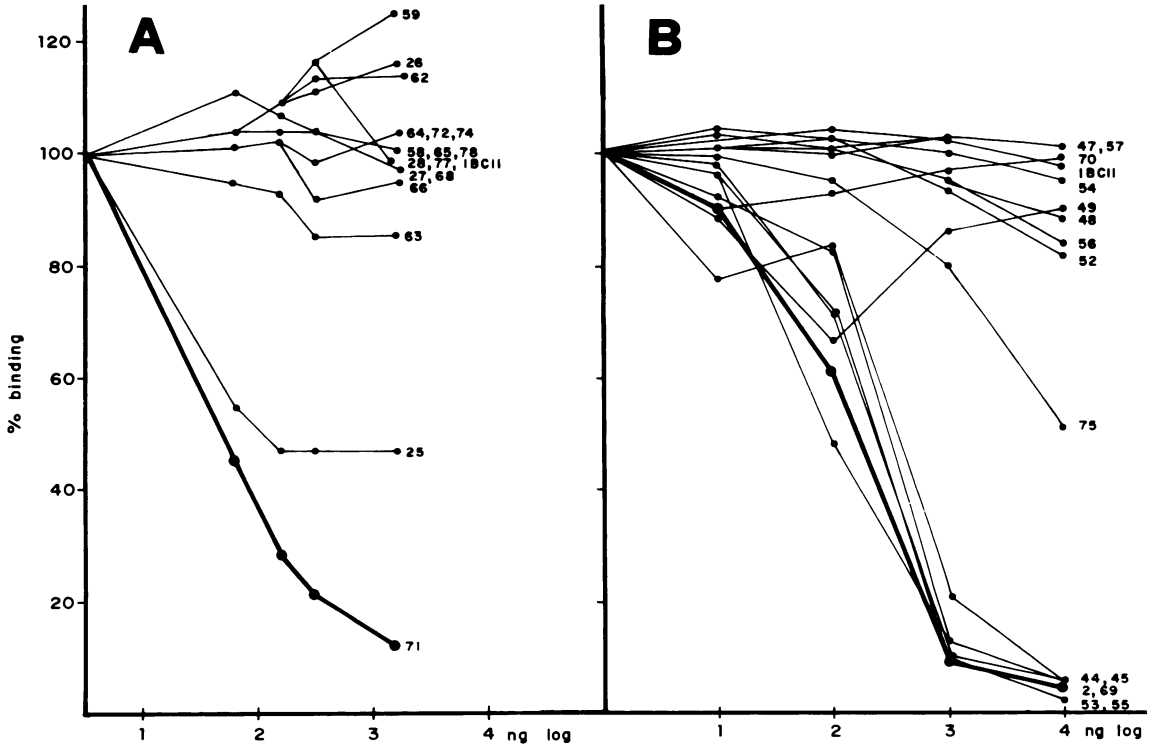

FIG. 2. Competitive ELISAs. Peroxidase-labeled 71G (part A) or 2F (part B) antibodies (shown by the reinforced lines) were mixed with increasing amounts of unlabeled antibodies (shown in abscissae) of the same specificity. They were added to virus-coated microdilution plates, and after incubation at 37°C for 1 h the labeled antibody bound to the wells was developed with the enzyme substrate. The results are expressed as percentages of the value obtained with the labeled antibody in the absence of competitors. 1BC11 is a negative control antibody.

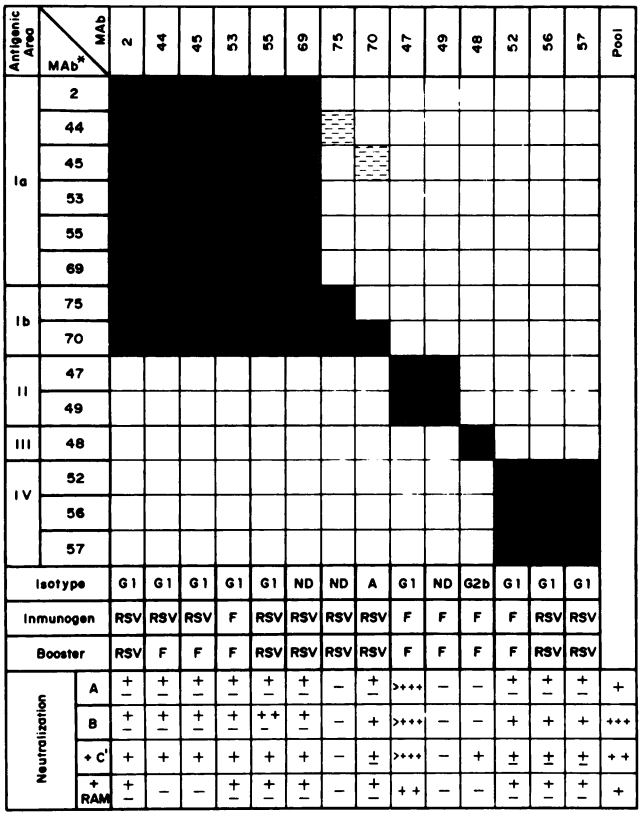

FIG. 3. Epitope mapping and neutralization tests done with anti-F antibodies. Fourteen anti-F antibodies were peroxidase labeled (indicated by the asterisk) and tested in competitive ELISAs against the same panel of unlabeled monoclonal antibodies (see legend to Fig. 2). Symbols: less than 25% (■), 25 to 50% (▣), or more than 50% (□) remaining bound at the highest amount of competitor antibody tested. The isotypes and the source materials used for immunization and boosting of mice are also indicated. The antibodies were tested in the microneutralization assays (Materials and Methods) after mixing with the virus only (A), including the antibodies also in the culture medium (B), including complement (+C'), or including a rabbit anti-mouse immunoglobulin reagent (+RAM) in the test. Each plus sign (+) denotes a reduction of 1 log unit virus titer compared with a negative antibody control; ± indicates 50% reduction of the cytopathic effect only at the highest viral dilution tested.

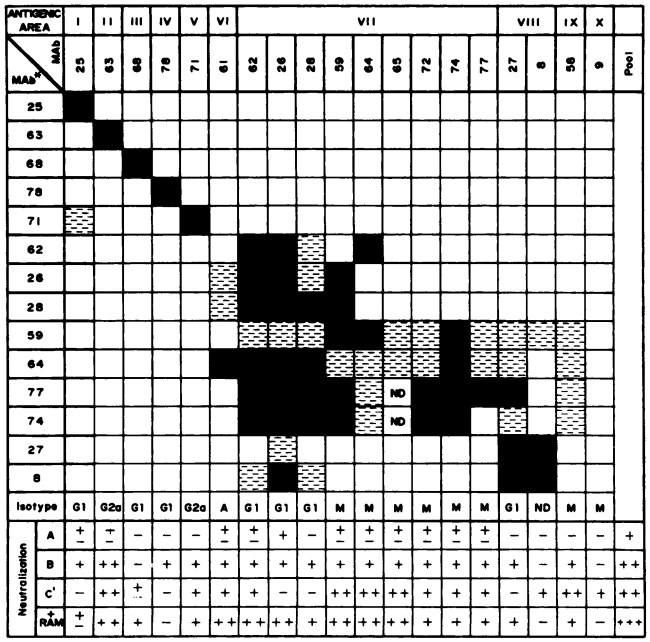

FIG. 4. Epitope mapping and neutralization tests done with anti-G antibodies. Nineteen anti-G antibodies were tested in competitive ELISAs and the neutralization tests (see legend to Fig. 3). Some antibodies were inactivated after peroxidase labeling and were used only as competitors. All symbols are as described in the legend to Fig. 3.

competition with 27G. Even greater differences could be observed among partially overlapping competition profiles; particularly antibodies 25G, 63G, 68G, 78G, and 71G showed only homologous competition. Thus, the clustering of epitopes in antigenic areas was tentatively done on the basis of partial similarities among individual antibodies. Nevertheless, epitopes included in areas VI, VII, VIII, and IX overlapped extensively.

In summary, the patterns that emerge from the results of Fig. 3 and 4 are very different. Whereas the anti-F antibodies identified epitopes that could be ascribed to discrete immunodominant domains, the anti-G antibodies identified unique epitopes, most of them overlapping extensively.

Virus neutralization by anti-F and anti-G antibodies. Figures 3 and 4 also summarize the results of virus neutralization with antibodies tested in the four different assays described in Materials and Methods. Most anti-F antibodies (Fig. 3) had only marginal effects, which in some cases were slightly enhanced by the addition of complement or a rabbit anti-mouse immunoglobulin reagent to the virus-antibody mixtures. The neutralization index was not increased, however, when the virus was incubated with some antibodies concentrated 100 times (data not shown), suggesting that incomplete neutralization is not due to insufficient antibody in the culture supernatants.

Antibody 47F, however, reduced the virus titer by more than 3 log units in the microneutralization test and neutralized 10^6 infectious particles in a plaque-reduction assay (data not shown). Strikingly, 49F antibody, which competed with 47F for binding to the virus, had no effect on virus neutralization. Both antibodies also differed in their reactivity with the F protein in Western blots; whereas 47F recognized the F1 subunit, 49F did not react with the antigen in this type of assay (data not shown).

The anti-G antibodies exerted little or no effect in the neutralization test (Fig. 4). Some monoclonal antibodies, however, like 61G or 59G, showed an increase in neutralization index when complement or rabbit anti-mouse immunoglobulin was included in the assay. It is noteworthy that the pool of anti-G antibodies had an additive effect in the neutralization of RS virus.

Isolation and characterization of virus mutants that were resistant to antibody neutralization. Five viruses were independently isolated from a stock of the Long strain after three selective cycles in the presence of 47F antibody. After cloning, the resistant viruses were tested in a direct ELISA against the panel of anti-F antibodies. The selected variants had lost the capacity to bind not only 47F but also 49F antibody (Table 1). This result suggests, as do the competitive ELISA data, that both epitopes are located within the same antigenic domain of the F molecule. All the other anti-F monoclonal antibodies reacted to the same extent with the Long strain and the 47F resistant viruses.

TABLE 1. Binding of anti-F monoclonal antibodies (MAbs) to Long and 47F-resistant viruses[a]

MAb	Antigenic area	ELISA result (%)					
		Long	R/47/4	R/47/7	R/47/16	R/47/25	R/47/27
2F	Ia	100	110	124	138	126	106
44F	Ia	100	116	125	133	130	108
45F	Ia	100	126	134	151	136	108
53F	Ia	100	122	127	137	137	117
55F	Ia	100	124	126	126	129	115
69F	Ia	100	113	126	132	124	115
75F	Ib	100	124	110	121	131	123
70F	Ib	100	114	119	135	132	117
47F	II	100	3	0	2	38	2
49F	II	100	2	1	4	2	2
48F	III	100	126	168	181	164	133
52F	IV	100	105	111	120	120	103
56F	IV	100	121	126	140	137	108
57F	IV	100	124	139	156	149	124

[a] Viruses were tested by ELISA against the panel of anti-F antibodies. The results are expressed as percentage of the optical density values obtained for each antibody with the Long strain. R/47/4, R/47/7, R/47/16, R/47/25, and R/47/27 refer to 47F-resistant viruses.

Four viruses were also selected after seven passages of the Long strain in the presence of 63G antibody. In this case, complement had to be included to increase the neutralization index, so that parental virus would be eliminated from the selected progeny. These resistant viruses were also tested by ELISA against the panel of anti-G antibodies (Table 2). In contrast to the results of Table 1, the variants resistant to 63G antibody had lost the capacity to bind most anti-G antibodies, including 63G. Nevertheless, antibodies 71G, 77G, and 9G reacted to a similar extent with the Long strain

TABLE 2. Binding of anti-G monoclonal antibodies (MAbs) to Long and 63G-resistant viruses[a]

MAb	Antigenic area	ELISA result (%)				
		Long	R/63/1	R/63/2	R/63/4	R/63/8
25G	I	100	1	5	1	2
63G	II	100	0	0	0	2
68G	III	100	0	1	0	2
78G	IV	100	12	13	12	12
71G	V	100	87	85	105	111
61G	VI	100	0	1	0	1
62G	VII	100	0	2	0	2
26G	VII	100	1	1	1	1
28G	VII	100	6	11	7	8
59G	VII	100	0	2	2	2
64G	VII	100	0	2	1	2
65G	VII	100	0	3	0	2
72G	VII	100	0	0	1	1
74G	VII	100	0	3	1	1
77G	VII	100	49	42	56	56
27G	VIII	100	0	3	1	2
8G	VIII	100	2	3	2	3
58G	IX	100	4	6	4	6
9G	X	100	65	71	74	81

[a] The binding of antibodies to the indicated virus was assayed by ELISA, and the results are expressed as percentage of the optical density values obtained for each antibody with the Long strain. R/63/1, R/63/2, R/63/4, and R/63/8 refer to 63G-resistant viruses.

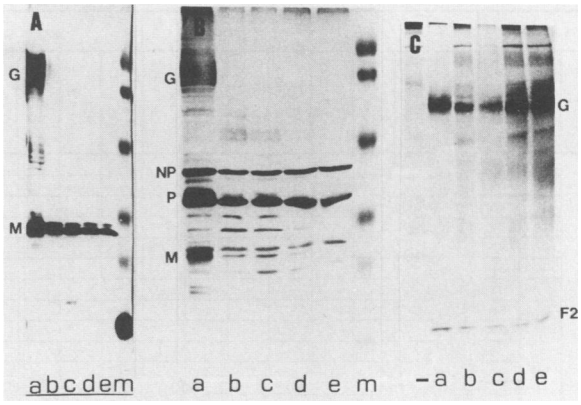

FIG. 5. Immunoblotting of viruses resistant to 63G antibody and SDS-PAGE analysis of [^{3}H]glucosamine-labeled virions. Proteins (30 μg) from purified Long strain (lanes a), R/63/1 (lanes b), R/63/2 (lanes c), R/63/4 (lanes d), and R/63/8 (lanes e) viruses were separated by SDS-PAGE, electrotransferred to nitrocellulose paper, and blotted with either a mixture of the anti-G and 29M antibodies (A) or anti-RS virus antiserum raised against the purified Long strain in BALB/c mice (B). Antibody 29M, directed against matrix protein, was included in part A as an internal control. Part C shows the autoradiogram after SDS-PAGE of virions precipitated from supernatants of [^{3}H]glucosamine-labeled infected cultures with polyethylene glycol (9). Lanes m correspond to molecular weight markers; −, labeled material from mock-infected cells.

and the four variants. Interestingly, these three antibodies were broadly cross-reactive among RS virus isolates (see below).

To confirm the antigenic changes detected in the 63G-resistant viruses, the Long strain and the four variants were assayed by immunoblotting with either a pool of anti-G antibodies or with anti-RS virus antiserum from mice immunized with purified Long virus. The G protein of 63G-resistant viruses reacted very poorly with the anti-Long antiserum (5B) and with the mixture of anti-G antibodies (5A), whereas other structural proteins were recognized to a similar extent in the variants and the parental virus (Fig. 5).

Since the anti-G antibodies 71G, 77G, and 9G, which recognized the epitopes conserved in the G protein of the 63G-resistant viruses, reacted poorly with the Long strain in immunoblots, the presence of the G protein in these viruses was demonstrated after [^{3}H]glucosamine labeling of infected cells followed by precipitation of virions from culture supernatants with polyethylene glycol. SDS-PAGE analysis of the precipitated material (Fig. 5C) revealed similar amounts of two major bands of 90 and 20 kilodaltons in both the Long and the variant viruses, which correspond to the G protein and the F2 subunit, respectively. Hence, the nonreactivity of most anti-G antibodies with the 63G-resistant viruses in the ELISA (Table 2) did not correlate with any significant reduction in the amount of virion-associated G glycoprotein.

Variation of RS virus epitopes among natural isolates. To determine the degree of variation among viral strains of epitopes recognized by the panel of monoclonal antibodies, these were tested in an ELISA against 12 RS viruses isolated during a 30-year period in distant places.

Most epitopes present in the, F, NP, P and M proteins were conserved in all viruses (Fig. 6). However, the different isolates could be clustered into two subtypes A and B, by their reactivity with 2F, 44F, 45F, 55F, 69F, and 11P antibodies. It is striking that five of the six epitopes that mapped in antigenic area Ia were subtype specific. We also

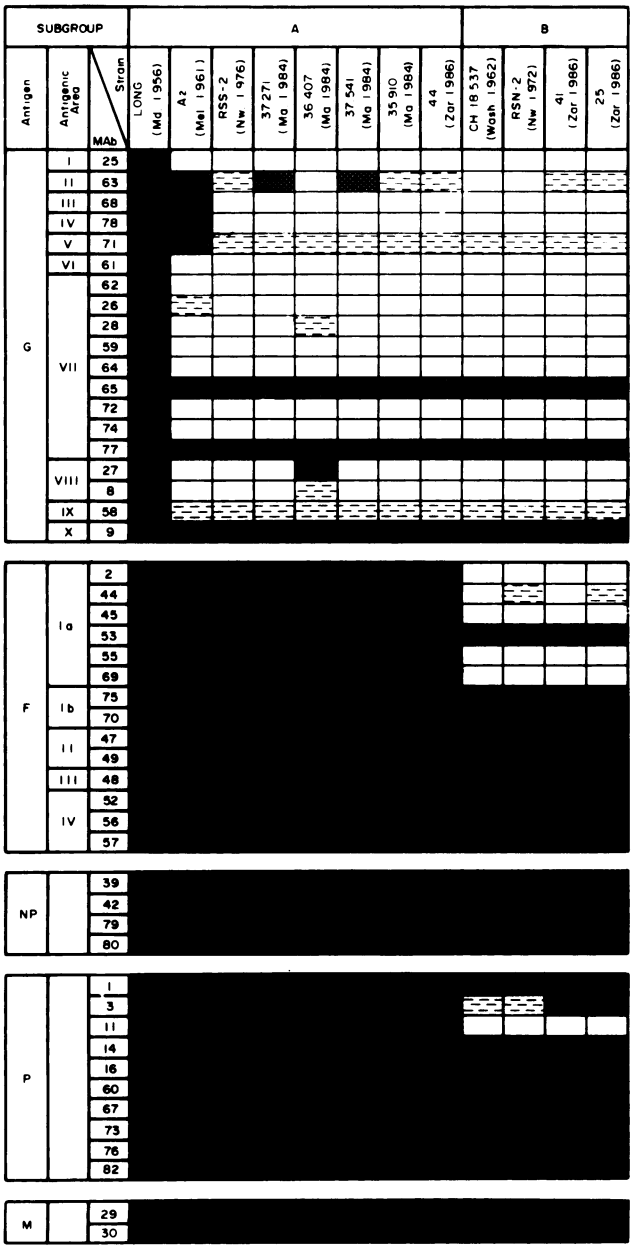

FIG. 6. Reactivity of anti-RS virus monoclonal antibodies with a panel of viral strains. The antibodies, grouped according to their specificities, were assayed in a direct ELISA against a panel of 12 viral isolates. The place and year of isolation are denoted as follows: Mel, Melbourne; Nw, Newcastle-upon-Tyne; Md, Maryland; Wash, Washington, D.C.; Ma, Madrid; Zar, Zaragoza. Symbols: more than 50% (■), 25 to 50% (▨), and less than 25% (□) of the values obtained for each antibody against the Long strain.

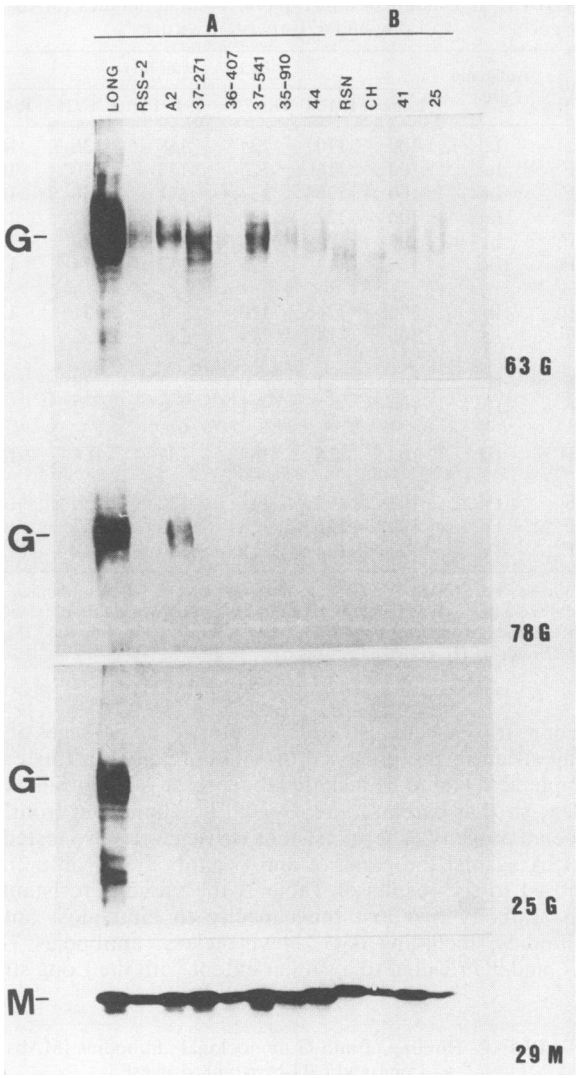

FIG. 7. Immunoblotting of RS viruses with anti-G antibodies. Proteins (30 μg) from each virus were separated by SDS-PAGE, electrotransferred to nitrocellulose paper, and blotted with antibodies 63G, 78G, and 25G. An anti-M antibody (29M) was included as an internal control of uniform transfer of the proteins from all virus tested. Only the relevant parts of the blots are shown.

observed changes in the electrophoretic mobility of some viral proteins (data not shown) which, as reported by others (10), distinguish A and B viruses. Our results confirm previous data (19, 20) which classified the Long, A2, and RSS-2 strains in subtype A and CH18537 and RSN-2 strains in subtype B.

A very different situation was observed for antibodies that reacted with epitopes of the G protein. In this case, most antibodies were strain specific. A few antibodies (71G, 65G, 77G, 58G, and 9G), however, reacted broadly with all the isolates tested, whereas other antibodies reacted with individual viruses. For instance, 27G antibody reacted with the 36.407 strain in addition to the Long virus.

The results of Fig. 6 were confirmed by immunoblotting of purified viruses with individual antibodies (Fig. 7); antibody 63G reacted to different degrees with the G protein of A and B viruses, 78G reacted with the Long and A2 strains, and 25G antibody reacted only with the Long strain. In contrast, 29M reacted to the same degree with the M protein of all viruses tested. The results of Fig. 6 and 7 were always compatible, but the ELISA was more quantitative.

In summary, these results emphasize the extensive variation of epitopes of the G protein among RS viruses, even of the same subtype, whereas most other epitopes are very conserved.

TABLE 3. Genetic and antigenic relatedness between the A2 and Long strains of RS virus[a]

Viral gene	% Amino acid identity	% Antigenic homology
G	94	42
F	98	100
P	98	100

[a] Data for amino acid identity are from Johnson et al. (12) for gene G and from López et al. (16) for genes F and P. The antigenic homology was calculated from the data of Fig. 6 by the method of Lee (14).

DISCUSSION

To approach a detailed analysis of RS virus antigenicity, particularly of the external glycoproteins, a large panel of monoclonal antibodies was raised against the Long strain of human RS virus. Then the anti-F and anti-G antibodies were used in competitive ELISAs for topological mapping of epitopes. It is clear that this approach should be taken as an operational classification of epitopes, since antibody binding can be prevented by other antibodies that either recognize overlapping epitopes or induce conformational changes in the antigen molecule.

The results of Fig. 3 and 4 show quite different patterns of antibody competition. Whereas the F epitopes are restricted to only a few separate antigenic sites, the G proteins include a large number of individual epitopes that might be structurally overlapping. This notion is also supported by the results obtained with the antibody-resistant viruses. The variants selected with 47F antibody lost the capacity to bind only the antibodies that mapped in the same antigenic site, whereas viruses resistant to 63G antibody lost the binding of most anti-G monoclonal antibodies; furthermore, the G protein of the 63G-resistant viruses was not recognized by an antiserum raised against the purified Long strain. This is the first report of the isolation of antibody-resistant RS viruses and offers a promising new approach to studying the antigenic organization of RS virus glycoproteins.

Since antibody-resistant viruses were isolated after 3 to 7 selective cycles, it is not possible to estimate the frequency of these mutants in the viral stock. With other RNA viruses the estimated frequencies of antibody-resistant mutants range from 10^{-4} to 10^{-6}; thus, it is likely that in the case of 47F-resistant viruses the three passages were needed to increase the proportion of mutants before a cytopathic effect could be observed. The initial amount of treated virus was 2×10^3 infectious units in the case of the 63G-resistant viruses. Since this antibody only reduces the virus titer by 2 log units, it is likely that resistant viruses emerged during the amplification of the nonneutralized fraction.

The mutations selected in the antibody-resistant RS viruses remain to be determined. In other systems, such as influenza virus, antibody-resistant variants contain one or few mutations that affect the epitopes recognized by the selective antibodies (7). If this were the case for the 63G-resistant viruses, it would imply that few mutations could drastically change G protein antigenicity. This might be related to the uncommon chemical structure of the mature G molecule, in which two-thirds of the molar mass is contributed by oligosaccharide chains. In this sense, it is relevant that carbohydrates may dramatically influence the antigenicity of viral glycoproteins (2) and that specific oligosaccharide chains block the binding of monoclonal antibodies to the influenza virus hemagglutinin (24).

In any case, it seems that few amino acid changes can produce drastic alterations in the G-protein antigenicity. Thus, Table 3 compares the genetic and antigenic relationships between the Long and A2 strains. Both viruses shared 94% amino acid identity of the G protein (12), which is reflected in only 42% antigenic homology. This contrasts with the F and P proteins, in which the genetic and antigenic homologies are very high.

Previous reports have described antigenic differences between RS virus subtypes and only occasional changes detected among viruses of the same subtype (1, 18, 20). The results of Fig. 6 and 7 indicate, however, that antigenic variation of the G epitopes is more extensive than previously recognized, even among strains of the same subtype.

Studies are now in progress to further examine the degree of genetic variation among RS viruses, the genetic alterations selected in the antibody-resistant variants, and the role of carbohydrates in RS virus antigenicity.

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