JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 1986, p. 475–480
0095-1137/86/030475-06$02.00/0
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Vol. 23, No. 3

Identification of Epitopes on Respiratory Syncytial Virus Proteins by Competitive Binding Immunoassay

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Received 13 September 1985/Accepted 5 December 1985

To characterize the interrelationship of monoclonal antibodies (MAbs) against respiratory syncytial virus (RSV) and their respective epitopes, we developed a competitive binding assay based on the biotin-avidin system and a tissue culture enzyme-linked immunosorbent assay. The competitive binding assay clearly distinguished between competing and noncompeting MAbs. Eight MAbs against the fusion protein (F protein) demonstrated two blocking patterns consistent with two antigenic sites. MAbs reacting at one site neutralized the virus, while those reacting at the other site did not. Eight MAbs against the large glycoprotein (G protein) demonstrated five blocking patterns consistent with three antigenic sites, one with three epitopes and the other two with one each. None of the MAbs against G protein neutralized the virus. The reaction pattern of the MAbs against three strains of RSV identified three additional epitopes on the F protein and no additional epitopes on the G protein. The epitopes on G protein showed the greatest antigenic diversity among the three strains. These results help us better understand the functional and antigenic structure of the two surface glycoproteins of RSV.

Differences among strains of respiratory syncytial virus (RSV) have been demonstrated by neutralization studies (4, 8–10, 12, 17, 21, 23, 29), migration patterns of certain viral proteins (6), peptide maps of the nucleoprotein (N protein) (28), and reaction patterns against monoclonal antibodies (MAbs) (3, 15, 28). Neither the extent of these differences relative to the overall structure and function of the virus nor their importance in the clinical and epidemiologic picture of RSV disease has yet been clearly established. Limited evidence suggests that strain differences are not a major factor in one aspect of RSV disease, reinfection. Several studies have shown that infection with one strain usually produces antibodies that react broadly against strains of RSV and that serum-neutralizing antibodies are poorly correlated with protection from infection (4, 8, 12, 23). We have developed MAbs to characterize differences among RSV strains and to study the importance of these differences in RSV disease (3).

As part of these studies, we developed a competitive binding assay to further characterize the interrelationships among the MAbs and their respective epitopes. With this assay, based on the biotin-avidin system, we have presumptively characterized distinct antigenic sites, and epitopes on the fusion protein (F protein) and the large glycoprotein (G protein) of RSV (18).

MATERIALS AND METHODS

Monoclonal antibodies. Sixteen MAbs directed against RSV (Table 1) were evaluated in a competitive binding assay. Twelve have been previously described (3, 11, 13), and the others were produced by fusion of Sp 2/0 myeloma cells with spleen cells from 12- to 20-week-old immunized BALB/c mice by an adaption of a protocol described by Oi and Herzenberg (20). Hybridomas producing antibodies to RSV were cloned two to four times by limiting dilutions. The cells from the final clone were injected into pristane-primed BALB/c mice, and the MAbs in the resultant ascites fluid were purified by ammonium sulfate precipitation. The antibody class and subclass were determined by an enzyme-linked immunosorbent assay (ELISA) with a subtype-specific antiserum (Litton Bionetics, Kensington, Md.). The protein specificity was

TABLE 1. Characteristics of MAbs against RSV

MAb	Immunoglobulin G subclass	Neutralization titer[a]	Immunizing strain of RSV
Anti-F			
13-1	1K	<1:20	Long
130-2E	1K	<1:20	A2
131-2A	2A	<1:20	A2
92-11C	2AK	<1:20	Long
102-10B	1K	<1:20[b]	18537
130-2A	1K	1:1,280	A2
130-8F	1K	1:40,960	A2
133-1H	2AK	1:2,560	A2
Anti-G			
63-10F	3K	<1:20	Long
91-4	1	<1:20	Long
130-6D	1K	<1:20	A2
132-5G	1K	<1:20	A2
132-10C	1K	<1:20	A2
131-2G	1K	<1:20	A2
130-2G	1K	<1:20	A2
232-1F	1K	<1:20	A2
Anti-N			
100-7G	1K	<1:20	18537

[a] Median of three tests against the A2 strain of RSV. The three neutralizing MAbs also neutralized the Long and 18537 strains RSV.
[b] Tested against strain 18537.

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determined by immunoprecipitation of radiolabeled or biotinylated RSV proteins or by transblot electrophoresis (1, 13, 14, 16). The protein concentration was determined by a protein assay (Bio-Rad Laboratories, Richmond, Calif.) (5).

Biotinylation. For the biotinylation procedure, the purified MAb was dialyzed against 0.1 M $NaHCO_3$ (pH 8.4) and adjusted to a concentration of 1 mg of protein per ml. A sample (1 ml) of this solution was reacted with 0.2 ml of dimethyl sulfoxide with 1.4 mg of *N*-biotinyl-ω-aminocaproic acid–*N*-hydroxysuccinimide ester (Enzo Biochem, Inc., New York, N.Y.) for 4 h at room temperature. The biotinylated MAb was then dialyzed overnight at 4°C against 0.01 M phosphate-buffered saline (PBS; pH 7.2), divided into aliquots, and stored at −20°C.

Viruses. Three strains of RSV—Long (7), A2 (19), and 8/60 (12)—were used in the study. These strains were chosen because their reactions against a panel of MAbs placed them into distinct subgroups of RSV strains (3).

Preparation of the plates. Antigens for the strain reaction studies and competitive binding assays were prepared by growing and fixing RSV-infected and -uninfected cells in microtiter plates. First, 100 μl of minimal essential medium–2% fetal calf serum with or without virus was added to sterile 96-well, flat-bottom tissue culture microtiter plates (Costar, Cambridge, Mass.), and approximately 15,000 HEp-2 cells in 100 μl minimal essential medium–5% fetal calf serum were added to each well. The plates were incubated at 35.5°C under CO_2 until a two to three plus cytopathic effect was evident in the virus-infected cells; the plates were then fixed by aspirating the contents, washing three times with PBS (pH 7.2)–0.5% Tween 20 and by adding 75 μl of an 80% (vol/vol) solution of acetone-PBS for 15 min at 4°C. The plates were air dried and stored at −20°C until they were used.

Strain reaction assay. The reactivity of the MAbs against the three strains was determined by a tissue culture ELISA (2). The plates, prepared as described above, were precoated with 300 μl of PBS with 0.5% gelatin and 2% normal goat serum (PBS-G-NGS) for 30 min at 35°C; 75 μl of the MAb diluted in PBS-G-NGS was added and incubated for 60 min at 35°C; 75 μl of a 1:3,333 dilution in PBS-G-NGS of peroxidase-conjugated goat anti-mouse immunoglobulin G (Kirkegaard and Perry Laboratories Inc., Gaithersburg, Md.) was added and incubated for 60 min at 35°C; and 125 μl of substrate (*o*-phenylenediamine [0.4 mg/ml] and hydrogen peroxide [0.015%] diluted in 0.15 M citrate phosphate buffer [pH 5.5]) was added. The reaction was stopped after 30 to 45 min with 25 μl of 3.5 M HCl. A_{490} was measured with an MR 580 Microelisa Auto Reader (Dynatech Laboratories, Inc., Alexandria, Va.).

The MAbs were reacted against the three strains of RSV at the highest dilution that gave the best combination of a high absorbance against infected cells and low absorbance against uninfected cells. This dilution ranged from 1:3,000 to 1:30,000. Strain 8/60 was substituted for strain 18537 (10) because the former grew to a higher titer and had the same reaction pattern against the MAbs (3).

Competitive binding assay. The plates, prepared as described above, were precoated with PBS-G-NGS for 30 min at 35°C. After the plates were washed, 75 μl of the competing MAb, diluted in PBS-G-NGS with 0.02% sodium azide, was added and incubated overnight in a humidified chamber at room temperature. Without aspiration of the wells, 25 μl of the biotinylated-detector MAb was added and incubated for 1 h at 35°C. After washing, 75 μl of a 1:10,000 dilution of avidin peroxidase (Sigma Chemical Co., St. Louis, Mo.) was added and incubated for 20 min at room temperature. Substrate was added, and absorbance was read as described for the strain reaction assay.

TABLE 2. ELISA results of MAb reactions against three strains of RSV

MAb	Specific A_{490}[a] by RSV strain:		
	Long	A2	8/60
Anti-F			
13-1	1.417	1.249	0.792
130-2E	1.301	1.362	0.245
131-2A	1.286	1.336	0.695
92-11C	1.401	1.386	0.067[b]
102-10B	0.008[b]	0	0.821
130-2A	1.177	1.198	0.691
130-8F	1.284	1.315	0.753
133-1H	1.343	1.380	1.069
Anti-G			
63-10F	0.963	1.202	0
91-4	1.334	1.318	0
130-6D	1.162	1.315	0
132-5G	1.362	1.364	0
132-10C	0.746	1.048	0
131-2G	1.211	1.160	1.166
130-2G	0.079[b]	1.224	0.006[b]
232-1F	0.798	1.173	0.051[b]
Anti-N			
100-7G	0.908	0.875	0.916

[a] Median of three tests.
[b] Not significantly above background absorbance.

The biotinylated MAb was used at the dilution that gave the best combination of low background and high absorbance with no competing MAb. The competing antibody was used at a concentration of 32 μg/ml to screen for competition. At this concentration, all tested MAbs blocked at least one biotinylated detector MAb. We tested the G-protein MAbs against the A2 strain, since they all reacted against A2. We tested all but one of the F-protein MAbs against the Long strain. This MAb, 102-10B, reacted only with group 2 strains and therefore was tested against strain 8/60.

The percentage of blocking was calculated according to the formula $(1-A/A_{max}) \times 100$, where A is the mean of the specific absorbance for three pairs of wells with the competing MAb, and A_{max} is the mean of the specific absorbance for 11 pairs of wells with diluent in place of the competing MAb. The specific absorbance is the absorbance of a well with virus-infected cells minus the absorbance of the adjacent well with uninfected cells. The mean of the specific absorbance was calculated from the log of the specific absorbance, which was then converted back to absorbance units. A MAb was considered to block significantly if it gave a mean specific absorbance of ≤50% of A_{max}. A 50% decrease in A_{max} was 2 and usually 3 or more standard deviations below the mean of A_{max}.

Microneutralization assay. The ability of individual MAbs to neutralize RSV was determined in a microneutralization test described in detail elsewhere (2). Replication of the virus was detected by a tissue culture ELISA similar to that described for the strain reaction assay.

RESULTS

Strain reaction patterns. The tissue culture ELISA gave background absorbance which was unaffected by the cells or fixation (2) and which was low, between 0.020 and 0.200,

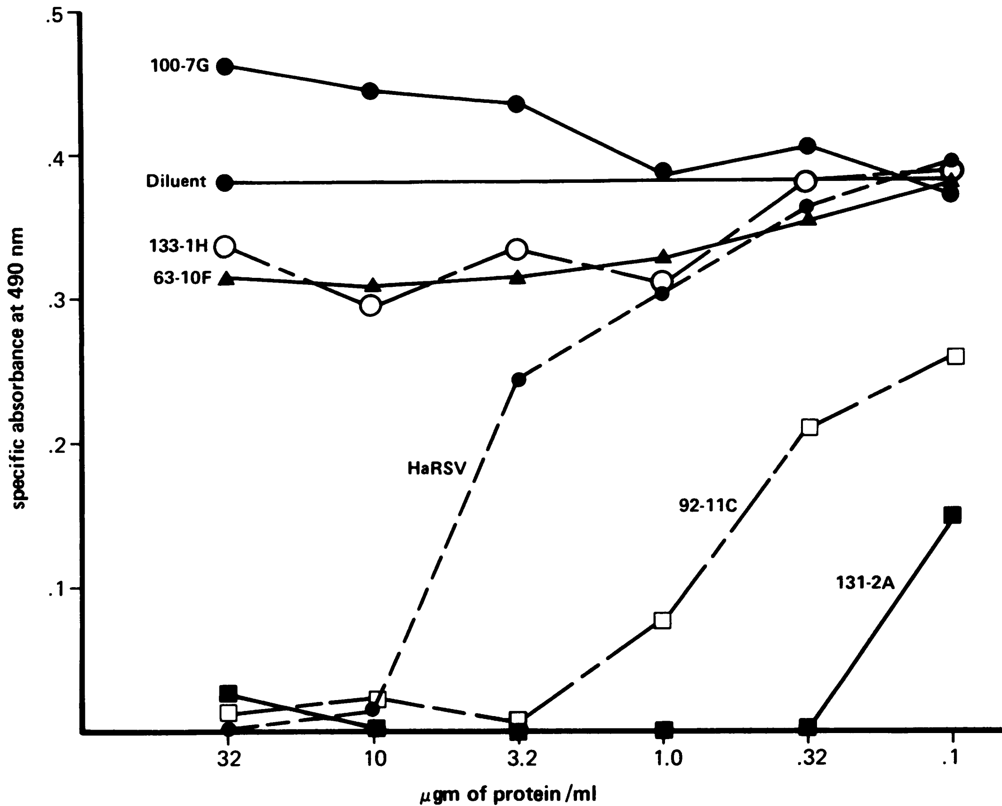

FIG. 1. Titration of competing antibodies. The virus is the Long strain of RSV. The competing antibodies are MAbs against the N protein (100-7G), the G protein (63-10F), and the F protein (92-11C, 131-2A, and 133-1H) of RSV and a polyclonal horse anti-RSV serum (HaRSV). The diluent is PBS plus 0.5% gelatin plus 2% NGS. The biotinylated detector antibody is 92-11C. The specific absorbance is the mean difference in absorbance between wells with RSV-infected cells and wells with uninfected cells. A 50% decrease in the mean absorbance for wells with diluent in place of the competing antibody is considered significant blocking.

depending on the MAb and the test. The qualitative results, i.e., positive or negative, were identical to those previously reported for indirect immunofluorescence (3). There were three distinct patterns of MAb reactions against the F protein (Table 2): one for MAb 92-11C, which reacted against the A2 and Long strains; one for MAb 102-10B, which reacted only against the 8/60 strain; and one for the remaining MAbs, which reacted against all three strains. Similarly, there were three distinct patterns of reaction against the G protein: one for MAb 130-2G, which reacted only against A2; one for MAb 131-2G, which reacted with all three strains; and one for the remaining MAbs, which reacted against the A2 and Long strains.

Epitope characterization by competitive binding. The competitive binding assay distinguished among MAbs against the F, G, and N proteins and among epitopes on the same protein. Absorbance readings were taken for serial dilutions of different competing antibodies, with a biotinylated MAb (92-11C) against F as the detector antibody (Fig. 1). There was a significant decrease in A_{max}, and therefore blocking, when MAbs 92-11C and 131-2A (both anti-F) and a polyclonal horse anti-RSV serum (Flow Laboratories, Inc., McLean, Va.) were the competing antibodies; there was no significant decrease in A_{max}, and therefore no blocking, when MAbs 100-7G (anti-N), 63-10F (anti-G), and 133-1H (anti-F) were the competing antibodies. The screening test gave consistent results, which clearly distinguished between competing and noncompeting MAbs (Table 3). There was only one of many tests in which MAbs which were otherwise noncompeting competed significantly. MAb 131-2A blocked MAbs 130-8F and 133-1H significantly in one of three tests against the Long strain and in none of three tests against the 8/60 strain. The median decrease in A_{max} for the three tests against 8/60 was 1% for 130-8F and 25% for 133-1H. MAbs

TABLE 3. Extent of blocking among competing and noncompeting MAbs

MAb interrelationship (no. of observations)	% Decrease in A_{max}[a]		
	Mean	Median	Range
Noncompeting			
F and G (87)	6	0	0–33
F and other Fs (89)	13	6	0–63[b]
G and other Gs (131)	8	1	0–49[c]
Competing			
F against F (88)	98	100	57–100[d]
G against G (70)	91	100	51–100[e]

[a] A_{max} was taken as the mean of the specific absorbance for 11 pairs of wells (one infected and one uninfected), with diluent replacing the competing MAbs. A 50% or more decrease in A_{max} was considered to represent significant blocking.

[b] The range was from 0 to 40, except for one experiment, when MAb 131-2A decreased A_{max} for 133-1H by 59% and A_{max} for 130-8F by 63%, while 130-8F decreased A_{max} for 131-2A by 47%.

[c] For all but four observations the decrease in A_{max} was <40%.

[d] For all but one observation the decrease in A_{max} was >60%.

[e] For all but two observations the decrease in A_{max} was >60%.

TABLE 4. Competitive binding by MAbs against the F protein of RSV

Competing MAb	Epitope[a]	Biotinylated detector MAb (strain of RSV) (% decrease in A_{max})[b]					
		130-2E (Long)	131-2A (Long)	92-11C (Long)	102-10B (8/60)	130-8F (Long)	133-1H (Long)
13-1	F1a	100	100	100	100	0	10
130-2E	F1a	100	100	100	NT[c]	0	29
131-2A	F1a	100	100	100	100	16	26
92-11C	F1b	100	87	100	NT	4	20
102-10B	F1c	NT	100[d]	NT	100	11[d]	25[d]
130-2A	F2	0	5	6	0	90	100
130-8F	F2	14	8	9	0	100	100
133-1H	F2	7	11	1	6	96	97
A_{max}[e]		0.463	0.476	0.257	0.302	0.433	0.394

[a] The number designates the antigenic site and the lowercase letter designates the epitope based on the reaction pattern against different strains of RSV.
[b] Median for three tests.
[c] NT, Not tested because the MAb did not react well enough with that strain of RSV to be tested.
[d] Reacted against strain 8/60.
[e] Median absorbance for three tests.

133-1H and 130-8F never blocked 131-1A significantly. For all other MAbs, if one blocked another, they blocked two ways when tested both ways and showed identical blocking patterns against other MAbs (Table 3 through 5).

F-protein-blocking patterns. Among the eight MAbs against the F protein, there were two patterns of blocking corresponding to two antigenic sites: one for the MAbs in group F1 (represented by 131-2A) and the other for the MAbs in group F2 (represented by 133-1H). At F1 the patterns of reaction with different strains of RSV further defined three epitopes: F1a, F1b, and F1c. F1b and F1c cannot be distinguished by tissue culture ELISA results but can be distinguished by previously published data (3). In those ELISA studies, MAbs 92-11C and 102-10B both reacted against the 8/60 strain, but only 92-11C reacted against the Long. The MAbs reacting at F2 neutralized RSV, and those reacting at F1 did not.

G-protein-blocking patterns. The blocking patterns against the G protein were more complex. There were five distinct patterns corresponding to five epitopes and three antigenic sites (Table 5). One antigenic site had three epitopes: G11 (63-10F), G12 (130-6D), and G13 (131-2G). The other two antigenic sites, G2 and G3, had one epitope each. Although the G12 and G13 MAbs did not compete against each other, both showed two-way blocking with G11, were presumably topologically linked, and therefore reacted at the same antigenic site but at different epitopes. There were three patterns of reaction against the strains: one for the MAbs reacting at G11, G12, and G3; one for the MAb reacting at G13; and one for the MAb reacting at G2. The patterns were identical for MAbs reacting at the same epitope. None of the MAbs against G neutralized RSV.

DISCUSSION

MAbs can be used to define epitopes and antigenic sites on proteins by a number of techniques (30). Epitopes on the same protein can be distinguished from each other by MAbs which give (i) distinct reaction patterns against different strains of a virus, either naturally occurring or laboratory induced; (ii) different blocking patterns in competitive binding studies; or (iii) differences in antibody reaction patterns against the same strain of virus in different tests, e.g., ELISA and neutralization. Antigenic sites, which include one or more epitopes at a topologically distinct area on a protein, can be distinguished by the absence of blocking among their respective MAbs in competitive binding studies or by variant selection studies (30). In this study we used competitive binding studies and strain reaction studies to define distinct epitopes and antigenic sites.

The competitive binding test, based on the biotin-avidin system, was simple to perform and gave results that with one exception were consistent and that clearly distinguished between competing and noncompeting MAbs. Others also have used with good results the biotin-avidin system for competitive binding studies (22, 25). Although competitive binding can distinguish between competing and noncompeting MAbs, it does not necessarily identify which

TABLE 5. Competitive binding by MAbs against the G protein of RSV

Competing MAb	Epitope[a]	Biotinylated detector MAb (A2 strain of RSV) (% decrease in A_{max})[b]					
		63-10F	91-4	130-6D	131-2G	130-2G	232-1F
63-10F	G11	100	100	100	100	0	0
91-4	G11	100	85	100	100	0	0
130-6D	G12	100	84	100	0	0	4
132-5G	G12	100	100	100	5	2	11
132-10C	G12	100	100	100	0	11	0
131-2G	G13	65	93	0	100	5	0
130-2G	G2	0	13	23	10	100	22
232-1F	G3	0	0	16	0	1	100
A_{max}[b]		0.444	0.557	0.599	0.365	0.437	0.437

[a] The first numeral designates the antigenic site, and the second numeral represents the epitope based on competitive binding studies.
[b] Median for three tests.

MAbs react at which epitopes and antigenic sites (30). For example, two-way blocking between two MAbs suggests but does not prove that they react at the same or overlapping epitopes. Blocking can occur when MAbs react at distant epitopes through steric hindrance or conformational changes. Similarly, the lack of blocking between two MAbs suggests but does not prove that they react at distinct epitopes or antigenic sites. The lack of one-way blocking could result from differences in avidity while the lack of two-way blocking could be an artifact of the preparation of the virus. It is possible that during the preparation (in this study the PBS-acetone fixation) of the virus, two populations of the same protein could be produced: one with a given epitope altered such that it reacts only with one MAb and the other with the same epitope altered such that it reacts only with another MAb.

Given these limitations, our competitive binding results presumptively identified two antigenic sites on the F protein of RSV and five epitopes and three antigenic sites on the G protein. One of the antigenic sites on F can be further subdivided into three epitopes on the basis of the reaction pattern of their respective MAbs against different strains. The two antigenic sites on F are also distinguished by the ability of their respective MAbs to neutralize and distinguish among strains of RSV. The MAbs which reacted at F2 did neutralize RSV, and those that reacted at F1 did not. Some of the MAbs at F1 had different reactions among the three strains, while the MAbs at F2 reacted with all three strains. These results support our conclusion that F1 and F2 are distinct antigenic sites. Other researchers have identified neutalizing, nonneutralizing, and fusion-inhibiting MAbs against the F protein but have not shown their interrelationship with competitive binding studies (11, 24, 26, 27). One MAb, 13-1, previously reported to neutralize RSV in a plaque reduction test (11, 13), did not neutralize the virus in our test system. This discrepancy, under further study, may result from the greater sensitivity of the plaque reduction test for indicating neutralization compared with our test system, which is based on the complete inhibition of viral replication. The five epitopes on the G protein could not be subdivided on the basis of their reaction patterns against the three strains, and none of their respective MAbs neutralized RSV. Other researchers, however, have reported G-protein MAbs which neutralize RSV in tissue cultures and in experimental infection in animals (24, 26, 27). It is possible that some of our G-protein MAbs might neutralize RSV in a plaque reduction neutralization test. These MAbs showed greater diversity in their reaction patterns against the three strains than did the F-protein MAbs. The reactions of the G-protein MAbs indicated that they could distinguish all three strains, while the F-protein MAbs could distinguish two of the three strains.

The results of this study help to put the antigenic and functional characteristics of the two surface glycoproteins of RSV into perspective. MAbs which neutralize RSV reacted at the antigenic site on the F protein which showed no antigenic variability. If additional studies support this observation, it may simplify the development of vaccines from synthetic peptides or cloning techniques. Four of the five epitopes representing all three antigenic sites on G protein showed antigenic diversity among the strains. Most of this diversity was between 8/60 and Long or A2. This is consistent with our earlier observation that the group 2 strains are the most distinct (3). Additional studies with an expanded pool of MAbs should further clarify the functional and antigenic structure of RSV and the extent and importance of strain differences, as well as help resolve apparent discrepancies among strain studies (17, 21).

ACKNOWLEDGMENTS

This work was supported in part by Public Health Service contract NO1-AI-22665 from the National Institute of Allergy and Infectious Diseases.

We thank Carrie Holley for help in the preparation of this manuscript.

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