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Neutralization of Respiratory Syncytial Virus by Individual and Mixtures of F and G Protein Monoclonal Antibodies

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We identified several types of neutralization effected by F and G protein monoclonal antibodies (MAbs) reacted individually or as mixtures against respiratory syncytial virus (RSV). Neutralizing activity was identified by a microneutralization test in which virus replication was determined by enzyme immunoassay. Complete neutralization was seen only with MAbs against the F protein. Strain-specific neutralization, complete neutralization against some strains of RSV, and no neutralization against other strains were seen with an additional MAb against the F protein. Partial neutralization, virus replication significantly reduced but still present, and no neutralization were seen with MAbs against both the F and G proteins. Enhanced neutralization, enhanced efficacy of neutralization, or increased neutralizing titer with a mixture of two MAbs over that for the individual MAbs was seen with all MAbs against the F protein and all but three MAbs against the G protein. Most (10 of 13) of the MAbs that exhibited neutralizing activity reacted with some but not all strains of RSV in an enzyme immunoassay. The epitopes corresponding to these 10 MAbs probably contribute to the strain-specific component of the neutralizing antibody response to RSV. Our results suggest that interpretation of RSV neutralization with MAbs is complex and that studies of such neutralization should include mixtures of MAbs and multiple RSV strains.

Since respiratory syncytial virus (RSV) is a major cause of lower respiratory tract illness in infants and young children, it has been the focus of numerous efforts to develop a vaccine (31). Earlier failures in developing a vaccine have made it clear that to improve our chances of success, we need a better understanding of the properties of the virus and the immune response it induces. Several studies have demonstrated variability in the protective capacity of the immune response. For example, the first RSV vaccine, a Formalin-inactivated vaccine, induced good complement-fixing and neutralizing-antibody titers but failed to protect children from infection and was associated with more severe illness after subsequent natural infection (5). High levels of maternal antibody correlate with but do not always confer protection from RSV disease (8, 10, 13, 19, 22). The cellular immune response also appears to be important in the host response to infection (14, 17).

In an attempt to better understand which part of the virus induces protective immunity, we have studied the antigenic structure of the two surface glycoproteins, F and G, at the level of their epitopes. The F and G proteins appear to be the most important of the RSV proteins for inducing protective immunity (23, 28, 34, 36). As part of these studies, we have characterized the in vitro function of many epitopes as manifested by the ability of their respective monoclonal antibodies (MAbs) to neutralize the virus. In this report, we describe the distinct types of neutralization we found with these MAbs when tested individually and as mixtures of two MAbs.

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MATERIALS AND METHODS

MAbs. Most of the MAbs used in these studies have been described previously (2, 3); others were developed by fusing SP2/0 myeloma cells with spleen cells from a BALB/c mouse immunized with RSV A2 (12) or RSV isolate 82-776 (isolated in Boston in 1982). Each hybridoma was cloned by limiting dilutions at least twice, and cells from the last cloning were injected into BALB/c mice to make ascitic fluid. Unpurified ascitic fluid was used for all tests unless otherwise stated. The isotypes of the MAbs were determined by an enzyme immunoassay (EIA). The antigenic sites and epitopes corresponding to the MAbs were determined by blocking antibody assays or strain reaction studies (2, 3).

Virus. RSV Long (4), A2 (12), 18537 (7), 8/60, and 82-776 were used in these studies. Long, A2, and 82-776 are group 1 strains; 8/60 (9) and 18537 are group 2 strains (13).

EIA infectivity test. The infectivity titer was determined by adding serial dilutions of virus to wells (100 μl per well) in a sterile 96-well microtiter plate (three wells per dilution) followed by 15,000 to 20,000 HEp-2 cells per well. The plates were then incubated at 35.5°C in a CO_2-humidified incubator for 5 days, and the cells were fixed with an 80% acetone–20% phosphate-buffered saline solution. Replication of the virus was detected by EIA (1). The EIA was performed by adding biotinylated MAb 131-4g, a MAb against the major nucleocapsid protein, to the fixed cells (initial tests were performed with a bovine anti-RSV serum from Burroughs Wellcome Co., Research Triangle Park, N.C.) and incubating the cells for 1 h; after the cells were washed, peroxidase-conjugated streptavidin from Amersham Corp., Arlington Heights, Ill. (peroxidase-conjugated goat anti-bovine antibody was used with the bovine anti-RSV antibody from Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.), was added and incubated for 10 min. After the plates were washed, substrate (*o*-phenylenediamine dihydrochloride [0.4 mg/ml] and hydrogen peroxide [0.015%] diluted in 0.15 M citrate phosphate buffer at pH 5.5) were added. The reaction was stopped with 3.5 M HCl, and the A_{490} was read. Specific

TABLE 1. Absorbance versus virus inoculum and production of infectious virus

Reciprocal of dilution of inoculum[a]	Absorbance[b]	SD of absorbance[c]	Infectious virus produced[d]
1	0.922	0.049	≥16,384
4	0.778	0.035	≥16,384
16	0.620	0.047	≥16,384
64	0.456	0.090	1,024
256	0.297	0.073	4,096
1,024	0.249	0.049	256
4,096	0.238	0.036	0

[a] EIA infectivity titration of the A2 strain of RSV.
[b] Mean absorbance for six wells. The mean background absorbance (11 wells with uninfected HEp-2 cells) is 0.211 (SD = 0.038).
[c] From readings for the six wells at each dilution of virus.
[d] Reciprocal titer of infectious virus in the supernatant of wells at each dilution of virus. A sample of the supernatant was taken just before the plates were fixed for the EIA infectivity assay. Titer of virus in the supernatant was determined by an EIA infectivity assay.

absorbance (absorbance minus the mean absorbance of uninfected control cells) of ≥0.100 was considered indicative of virus replication, and higher absorbance readings correlated with greater replication of virus. Specific absorbance of 0.100 was usually ≥3 standard deviations above the mean absorbance of uninfected control cells. The endpoint dilution was calculated by the Reed-Muench method (27) and was considered to contain 1 50% tissue culture infective dose ($TCID_{50}$) of virus.

EIA neutralization assay. Serial dilutions of the MAbs, individually or as mixtures, were added to approximately 100 $TCID_{50}$ of virus. A range of 32 to 320 $TCID_{50}$ was considered acceptable. After the virus-antibody mixture was incubated for 2 h at room temperature, 15,000 to 20,000 HEp-2 cells were added (the virus-antibody mixture was left in the wells) and the plate was incubated for 5 days at 35.5°C in a humidified CO_2 incubator. The cells were then fixed, and virus replication was determined by EIA as described above. Dilutions of a MAb that gave a mean or median (three to eight wells per dilution) specific absorbance of <0.100 were considered to give complete neutralization.

Correlation between absorbance and inoculum/production of virus. The correlation between absorbance and input virus was estimated from back titrations of the virus inoculum in the EIA infectivity assay. The maximum absorbance was that for wells with cells infected with the undiluted inoculum; the percentage of maximum specific absorbance (specific absorbance for a well [mean or median for multiple wells] divided by maximum specific absorbance) was correlated with the dilution for that well. The correlation between absorbance and production of infectious virus was estimated by determining the infectivity titer of a sample of the supernatant from wells just before they were fixed for the EIA neutralization or infectivity assay. The percentage of maximum specific absorbance for a well was then correlated with the titer of infectious virus for that well. An example of the relationship of absorbance to both virus inoculum and production of infectious virus is given in Table 1.

RESULTS

MAbs. The MAbs reacted at six epitopes representing three antigenic sites on the F protein and at 10 epitopes representing at least four antigenic sites on the G protein (Table 2). Since MAbs at G2, G3a, and G3b did not react with the same strains of RSV as did MAbs at G5a, G5b, and G6, we could not perform blocking antibody assays to determine if they react at the same or different antigenic sites.

Neutralization. MAbs 133-1h (epitope F2) and 143-6c (epitope F3), reacting at two antigenic sites on the F protein,

TABLE 2. MAbs against F and G proteins of RSV

MAb	Epitope[a]	Antibody subclass	EIA reaction[b]		EIA titer[c]	Fusion inhibition[d]
			Group 1	Group 2		
131-2a (A2)[e]	F1a	G2ak	+	+	10^6	<20
92-11c (Long)	F1b	G2ak	+	−	$>10^6$	<20
102-10b (18537)	F1c	G1k	−	+	$>10^6$	<20
130-7e (A2)	F1d	G2bk	+/−	+/−	10^6	<20
133-1h (A2)	F2	G2ak	+	+	$>10^6$	50
143-6c (82-776)	F3	G1k	+	+	10^6	>500
63-10f (Long)	G11	G3k	+	−	10^5	ND
130-6d (A2)	G12	G1k	+	−	$>10^6$	ND
131-2g (A2)	G13	G1k	+	+	10^5	ND
130-2g (A2)	G2	G1k	+/−	−	10^6	ND
232-1f (A2)	G3a	G1k	+/−	−	10^6	ND
130-9g (A2)	G3b	G1k	+/−	+/−	10^3	ND
130-5f (A2)	G4	G2bk	+/−	+/−	10^6	ND
143-5a (82-776)	G5a	G2ak	+/−	+/−	10^6	ND
142-12g (82-776)	G5b	MK	+/−	−	10^3	ND
143-4f (82-776)	G6	G1k	+/−	−	10^3	ND

[a] The uppercase letter designates the protein; the first numeral designates an antigenic site determined by blocking antibody assays; the second letter designates an epitope distinguished from other epitopes at an antigenic site by blocking antibody assays; the lowercase letter designates an epitope distinguished from other epitopes at an antigenic site by reaction patterns against strains of RSV.
[b] MAbs were reacted against fixed (80% acetone-20% phosphate-buffered saline), RSV-infected HEp-2 cells, and the reaction was detected with a peroxidase-conjugated anti-mouse immunoglobulin G antibody. The MAbs were tested against 15 group 1 strains (RSV Long is a group 1 strain) and 10 group 2 strains (RSV 18537 is a group 2 strain). +, Positive reaction; −, negative reaction; +/−, positive reaction for some strains and negative reaction against others.
[c] Determined against the immunizing strain.
[d] Fusion was measured by an assay adapted from Walsh and Hruska (35). After the virus was incubated with the cells for 2 h, the medium was aspirated and replaced with fresh medium. After a 3-h incubation period, dilutions of the MAb were added. The cells were fixed at 4 days and tested for RSV antigens by immunofluorescence. The presence of virus-infected but unfused cells was considered positive for fusion inhibition.
[e] The strain of RSV used to make the MAb is given in parentheses.

TABLE 3. Neutralization, partial neutralization, and enhanced neutralization of RSV with MAbs

MAb	Epitope	Neutralization[a]	Partial neutralization[b]	Enhanced neutralization[c] with:		
				F1a/F1b	F2/F3	G MAbs
131-2a	F1a	<20	+/−	ND	10	+
92-11c	F1b	<20	+/−	ND	10	+
102-10b	F1c	<20	<20	ND	3	0
130-7e	F1d	+/−[d]	<20	ND	3	0
133-1h	F2	2,560		+	ND	+
143-6c	F3	20,480		+	ND	+
63-10f	G11	<20	10,240	+	3	ND
130-6d	G12	<20	<20	0	3	ND
131-2g	G13	<20	<20	0	0	ND
130-2g	G2	<20	20,480	+	10	ND
232-1f	G3a	<20	10,240	+	10	ND
130-9g	G3b	<20	<20	0	0	ND
130-5f	G4	<20	<20	0	0	ND
143-5a	G5a	<20	+/−	+	3	ND
142-12g	G5b	<20	>100	+	3	ND
143-4f	G6	<20	10,240	+	3	ND

[a] Complete neutralization in an EIA infectivity assay. Titer is taken as the highest dilution of the MAb that gave a specific absorbance of <0.100. The reciprocal neutralizing titer listed is the median titer against the A2 strain. MAbs at epitopes F1a, F1b, F1d, F2, and F3 were tested against the Long, A2, 18537, 8/60, and 82-776 strains of RSV. The MAb at F1c was tested against the 18537 and 8/60 strains of RSV.

[b] Neutralization in an EIA assay in which no dilution gave complete neutralization (specific absorbance of <0.100) but some dilutions gave a specific absorbance of <50% of maximum specific absorbance. All MAbs were tested against RSV A2 except MAbs 143-5a (epitope G5a), 142-12g (epitope G5b), and 143-4f (epitope G6), which were tested against RSV 82-776, and MAb 102-10b (epitope F1c), which was tested against RSV 18537. +/−, Positive result in some but <50% of four to eight tests.

[c] +, Enhancement of neutralization from none or partial to complete. Numbers indicate median (three to four tests) fold increase in the complete neutralization titer; 0, no enhancement of neutralization; ND, not done.

[d] 130-7e neutralized strain 18537 to a median titer of 1:640 but did not neutralize strain A2, Long, 8/60, or 82-776.

neutralized RSV in the EIA neutralization assay to a specific absorbance reading of <0.100 (complete neutralization) against all strains tested (Table 3). One of the MAbs, 130-7e (epitope F1d), at the other antigenic site on the F protein, gave complete neutralization against the 18537 strain of RSV to a median titer of 1:640 but had no neutralizing activity against strain A2, Long, 82-776, or 8/60 (Fig. 1). We designated this as strain-specific neutralization. No G protein MAb gave complete neutralization.

Partial neutralization. Among the MAbs that did not individually give complete neutralization, five had no neutralizing activity and eight demonstrated some neutralizing activity (Table 3). This neutralizing activity was demonstrated by a >50% decrease in percentage of maximum specific absorbance in the EIA neutralization assay and was defined as partial neutralization. A 50% decrease in maximum specific absorbance was usually ≥3 standard deviations below maximum specific absorbance (the mean absorbance of the virus-infected control cells). This decrease in maximum specific absorbance correlated with an 85 to 95% decrease in virus inoculum in 10 back titrations and a 75 to 95% decrease in production of infectious virus for 48 wells as described in Materials and Methods. Figure 2 illustrates the pattern of neutralization for several MAbs that gave partial neutralization and one that gave no neutralization. Note the constant decrease in absorbance to a relatively high titer for MAbs 130-2g (epitope G2), 232-1f (epitope G3a), and 143-4f (epitope G6) but never a decrease to a level consistent with

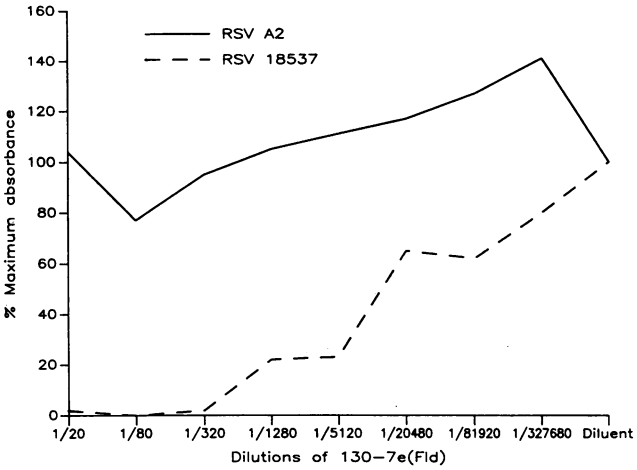

FIG. 1. Neutralization of RSV by MAb 130-7e (epitope F1d) in an EIA neutralization assay. Absorbance readings from the EIA are expressed as the percentage of maximum specific absorbance (maximum specific absorbance = mean absorbance for eight wells with virus-infected control cells − mean absorbance for eight wells with uninfected control cells); a higher absorbance signal indicates greater replication. Each point represents the mean value of two wells. The MAb was reacted against 96 $TCID_{50}$ of RSV A2 and 128 $TCID_{50}$ of RSV 18537. The dilution listed is that of the MAb added to the virus; the effective dilution after the MAb had been added to the virus was increased by 33%. One standard deviation in absorbance for uninfected cells was 1 to 2%. A value of ≤13% for strain 18537 and ≤10% for strain A2 corresponds to complete virus neutralization (≤0.100 absorbance units). Note that 130-7e neutralized RSV 18537 to a titer of 1:320 but had no neutralizing activity against RSV A2.

complete neutralization even at low dilutions (Fig. 1). MAb 130-6d (epitope G12) demonstrated no significant neutralizing activity. MAbs 63-10f (epitope G11), 130-2g (epitope G2), and 232-1f (epitope G3a) against RSV A2 and MAbs 142-12g (epitope G5b) and 143-4f (epitope G6) against RSV 82-776 gave partial neutralization in most tests (>60% of three to eight tests) (Table 3). MAbs 131-2a (epitope F1a) and 92-11c (epitope F1b) against RSV A2 and MAb 143-5a (epitope G5a) against RSV 82-776 gave partial neutralization in some tests (≤50% of four to eight tests). MAbs 130-7e (epitope F1d), 130-6d (epitope G12), 131-2g (epitope G13), 130-9g (epitope G3b), and 130-5f (epitope G4) had no neutralizing activity against RSV A2, Long, or 82-776, and MAb 102-10b (epitope F1c) had none against RSV 18537 or 8/60. Five units of complement did not increase neutralization with MAbs at F1a, F1b, G11, G12, G13, G2, or G3a.

To rule out the possibility that partial neutralization may have been caused by the presence of two populations of virus, one neutralized and the other not neutralized by the MAb, we recovered and characterized the breakthrough virus. In this test, approximately 100 $TCID_{50}$ of RSV A2 was grown in the presence of 1:100 dilutions of MAb 92-11c (F1b), 63-10f (G11), 130-2g (G2), or 232-1f (G3a). Breakthrough virus was inoculated onto HEp-2 cells and harvested at 3+ to 4+ cytopathic effect, and approximately 100 $TCID_{50}$ of virus was reacted against serial dilutions of the MAbs in the EIA neutralization assay. The breakthrough viruses gave the same patterns of partial neutralization against these four MAbs as did the original virus.

Neutralization with mixtures of MAbs. We found that mixtures of two MAbs sometimes enhanced the efficacy of neutralization (from partial or no neutralization to complete

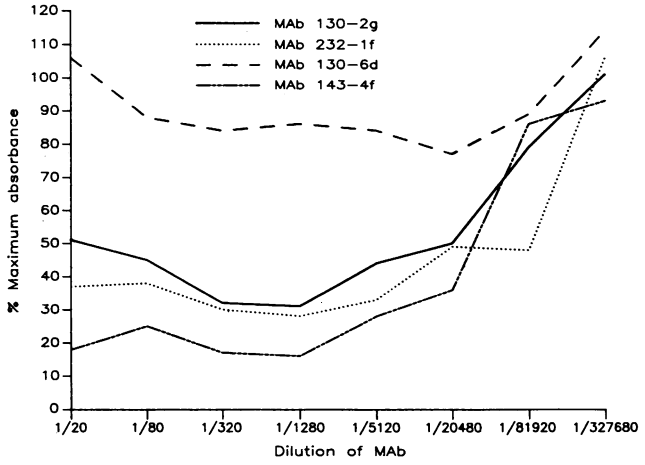

FIG. 2. Neutralization of RSV by MAbs 130-6d (epitope G12), 130-2g (epitope G2), 232-1f (epitope G3a), and 143-4f (epitope G6) in an EIA neutralization assay. Absorbance readings from the EIA are expressed as the percentage of maximum specific absorbance (maximum specific absorbance = mean absorbance for eight wells with virus-infected control cells − mean absorbance for eight wells with uninfected control cells); a higher absorbance signal indicates greater replication. MAbs 130-6d, 130-2g, and 232-1f were reacted against 256 $TCID_{50}$ of RSV A2, and MAb 143-4f was reacted against 48 $TCID_{50}$ of RSV 82-776. The dilution listed is that of the MAb added to the virus; the effective dilution after the MAb had been added to the virus was increased by 33%. One standard deviation in maximum specific absorbance was 6 to 10% for RSV A2 and 30% for RSV 82-776. One standard deviation in absorbance for uninfected cells was 1 to 2%. Note that MAb 130-2g gave <50% maximum absorbance (indicative of partial neutralization) to a titer of 1:20,480, MAb 232-1f gave <50% maximum specific absorbance to a titer of 1:81,920, and MAb 143-4f gave <50% maximum specific absorbance to a titer of 1:20,480. MAb 130-6d showed no neutralization activity at the dilutions tested.

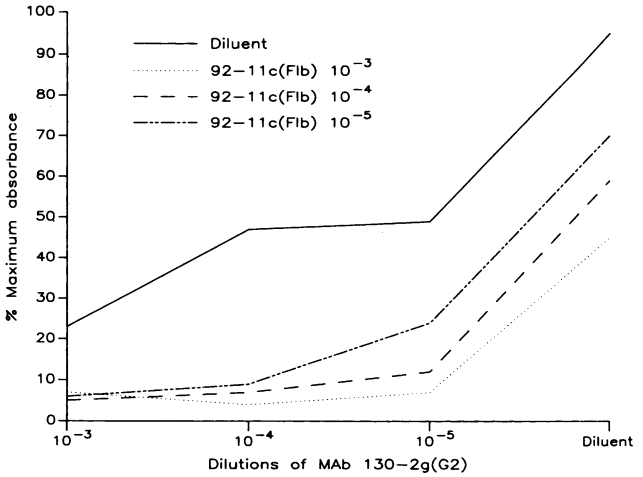

FIG. 3. Enhanced neutralization between MAbs 92-11c (epitope F1b) and 130-2g (epitope G2). Neutralization was detected in an EIA neutralization assay. Absorbance readings from the EIA are expressed as the percentage of maximum specific absorbance (maximum specific absorbance = mean absorbance for four wells with virus-infected control cells − mean absorbance for four wells with uninfected control cells); a higher absorbance signal indicates greater replication. One standard deviation in maximum specific absorbance was 10%, and 1 standard deviation in background absorbance was 1%. The MAbs were reacted against 128 $TCID_{50}$ of RSV A2. The dilutions are those of the MAbs as added to the virus-MAb mixture; the effective dilution after the MAb had been added to the mixture was increased by 167%. Neither MAb 130-2g nor MAb 92-11c gave complete neutralization (absorbance reading of <0.100, which corresponds to <10% in this test) by itself. The diluent curve at 10^{-3}, 10^{-4}, and 10^{-5} dilutions of MAb 130-2g gives the values for MAb 130-2g by itself. The diluent dilutions for the 10^{-3}, 10^{-4}, and 10^{-5} dilution curves of MAb 92-11c give the values for MAb 92-11c by itself. The two MAbs together gave complete neutralization of the virus, as demonstrated by a percentage of maximum absorbance of <10%.

neutralization) or increased the neutralization titer ≥3-fold. We defined this as enhanced neutralization. Some mixtures (1:300 to 1:1,000 dilutions) of an F plus a G protein MAb gave complete neutralization, while the individual MAbs gave partial neutralization (Table 3). For example, a mixture of 130-2g (epitope G2) and 92-11c (epitope F1b) neutralized A2, whereas the individual MAbs partially neutralized the virus (Fig. 3). Similar results were seen with a mixture of 232-1f (epitope G3a) and 92-11c (epitope F1b) (Fig. 4). Some mixtures of MAbs that gave partial or no neutralization (both F and G protein MAbs) increased the neutralizing titer of that MAb by 3- to 10-fold when mixed with neutralizing MAbs (F protein MAbs) (Table 3). For example, the partially neutralizing G protein MAbs 130-2g (epitope G2) and 232-1f (epitope G3a), when mixed with MAb 133-1h (epitope F2), increased the titer of 133-1h by 3- to 10-fold (Fig. 5). Similarly the partially neutralizing or non-neutralizing F protein MAbs 130-7e (epitope F1d), 131-2a (epitope F1a), and 92-11c (epitope F1b) increased the neutralizing titer of 133-1h by 3- to 10-fold (Fig. 6). Some non-neutralizing MAbs did not give enhanced neutralization (Table 3). Neutralization studies of mixtures of G protein MAbs are in progress.

One concern we had about our results was that they might be an artifact of the EIA test we used to detect virus replication. For example, the test MAb might block the detector antibody in the EIA and decrease the absorbance signal without neutralizing virus. We found no evidence for this type of blocking in the EIA. The F and G protein MAbs used in our neutralizing tests, individually or in pairs, did not block the signal for either the biotinylated detector 131-4g or the bovine anti-RSV serum in our blocking assay. Additionally, the ability to detect virus replication in these neutralization tests was not changed when biotinylated F and G protein MAbs, including the biotinylated version of the test MAbs, were used as the detecting antibodies.

DISCUSSION

In this study, we have demonstrated the complexity of RSV neutralization both in terms of apparent diversity in types of neutralization and in terms of the number of epitopes on both the F and G proteins involved in neutralization. Neutralization has been described for numerous RSV MAbs, but diversity in types of neutralization has not. We saw complete neutralization only with F protein MAbs; MAbs at two sites neutralized all strains, and one MAb, representing one of four epitopes at the third site, gave strain-specific neutralization. This MAb neutralized 18537 but not other strains tested, including the immunizing strain, A2. All of the remaining F protein MAbs and 7 of 10 G protein MAbs exhibited some neutralizing activity, either as partial neutralization individually or as enhanced neutralization in a mixture. This is consistent with previous studies demonstrating neutralizing activity associated with monoclonal and polyclonal antibodies against both the F and G proteins and protective immunity induced after immuniza-

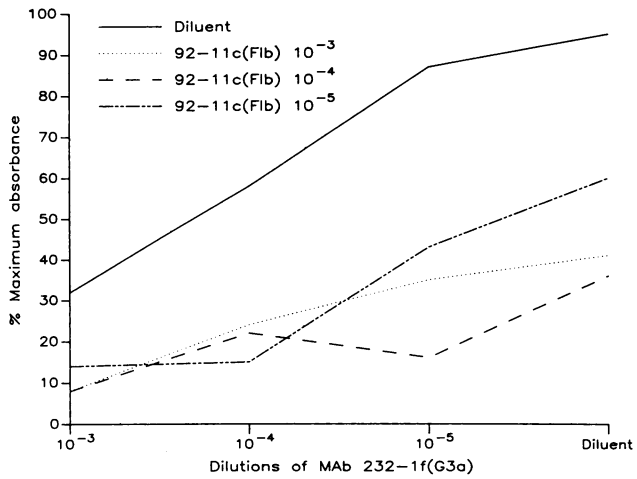

FIG. 4. Enhanced neutralization between MAbs 92-11c (epitope F1b) and 232-1f (epitope G3a). Neutralization was detected in an EIA neutralization assay. Absorbance readings from the EIA are expressed as the percentage of maximum specific absorbance (maximum specific absorbance = mean absorbance for four wells with virus-infected control cells − mean absorbance for four wells with uninfected control cells); a higher absorbance signal indicates greater replication. One standard deviation in maximum specific absorbance was 10%, and 1 standard deviation in background absorbance was 2%. The MAbs were reacted against 128 $TCID_{50}$ of RSV A2. The dilutions are those of the MAbs as added to the virus-MAb mixture; the effective dilution after the MAb had been added to the mixture was increased by 167%. Neither MAb 232-1f nor MAb 92-11c gave complete neutralization (absorbance reading of <0.100, which corresponds to <10% in this test) by itself. The diluent curves at 10^{-3}, 10^{-4}, and 10^{-5} dilutions of MAb 232-1f gave the values for MAb 232-1f by itself. The diluent dilutions for the 10^{-3}, 10^{-4}, and 10^{-5} dilution curves of MAb 92-11c gave the values for MAb 92-11c by itself. The two MAbs together gave complete neutralization of the virus, as demonstrated by a percentage of maximum absorbance of <10%.

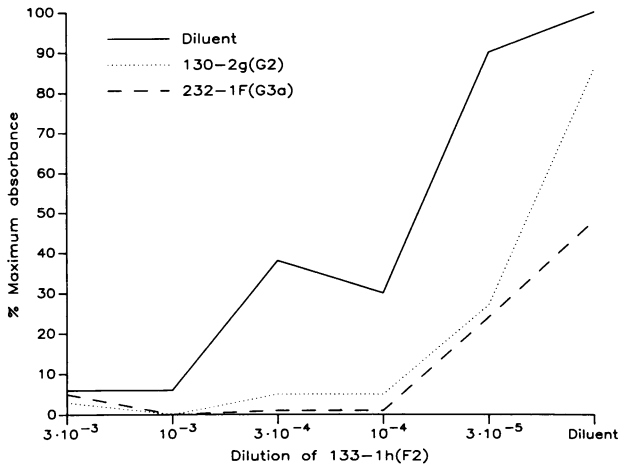

FIG. 5. Enhanced neutralization between MAb 133-1h (epitope F2) and MAb 130-2g (epitope G2) or 232-1f (epitope G3a). Neutralization was determined in an EIA neutralization assay. Absorbance readings from the EIA are expressed as percentage of maximum specific absorbance (maximum specific absorbance = mean absorbance for 4 wells with virus-infected control cells − mean absorbance for 11 wells with uninfected control cells); a higher absorbance signal indicates greater replication. One standard deviation in maximum absorbance was 8%, and 1 standard deviation in background absorbance was 4%. A value of <21% corresponds to complete neutralization (<0.100 specific absorbance). The MAbs were reacted against 128 $TCID_{50}$ of RSV A2. MAbs 130-2g and 232-1f were at a 1:300 dilution. The dilutions are those of the MAbs as added to the virus-MAb mixture; the effective dilution after the MAb had been added to the mixture was increased by 167%. MAbs 130-2g and 232-1f did not neutralize RSV by themselves. The diluent dilutions for their respective curves give the values for the MAbs by themselves. When mixed with MAb 133-1h, they shifted the curve of MAb 133-1h (the diluent curve gives the values for MAb 133-1h by itself) to the right and increased the neutralization titer between 10- and 30-fold.

tion with either protein (11, 21, 23, 24, 28, 30, 33–37). Some investigators have found the G protein to be less effective in inducing neutralizing antibodies or protective immunity than the F protein (16, 23, 29); this is consistent with our finding that G protein MAbs gave only partial and enhanced neutralization, types of neutralization presumably less effective than complete neutralization.

These different types of neutralization can probably best be put into perspective when their mechanisms are understood. The mechanisms for RSV neutralization have not been previously defined and are not defined in this report; our results, however, suggest that RSV may be neutralized by several different mechanisms. For example, several MAbs gave partial neutralization to a high titer yet never gave complete neutralization, even at low dilutions, suggesting that the mechanisms for partial and complete neutralization are different. Neutralization through reactions at different locations on the surface of the virus (that is, different antigenic sites) may occur by different mechanisms; we identified MAbs reacting at multiple antigenic sites on two proteins that gave neutralizing activity.

Our results of neutralization with mixtures of MAbs suggests that at least two mechanisms may be involved in enhanced neutralization, one in which the MAbs act independently and the other in which they act synergistically. If both of the MAbs in the mixture exhibit neutralizing activity, enhancement could be explained by the independent action of the two antibodies. For example, if two MAbs each effect the equivalent of a 90% reduction in virus inoculum, then a mixture of the two acting independently should effect the equivalent of a 99% reduction in inoculum, a decrease consistent with complete neutralization in our assay system. A synergistic interaction could also be present but would need to be documented by further study. On the other hand, since MAbs 130-6d and 130-7e gave no neutralization against RSV A2 individually but gave enhanced neutralization as a mixture, they presumably did so by a synergistic interaction.

Enhanced neutralization of viruses, as evidenced by synergistic neutralization, is not a new concept. Synergy has been described for several viruses, including vesicular stomatitis virus, Newcastle disease virus, and La Crosse virus (15, 18, 32); it has not, however, been previously described for RSV. It is not surprising that MAbs act together to neutralize the virus. The host immune response to infection is certainly polyclonal; therefore, in vivo, antibodies against many epitopes probably react simultaneously with the virus. In fact, the activity of MAbs in pairs or groups may more closely parallel their in vivo neutralizing activity. This interaction may be not only synergistic but also antagonistic, as demonstrated by Massey and Schochetman (20).

We do not yet know which of these types of neutralization will be most helpful in identifying epitopes important for the induction of protective immunity. It is likely that complete neutralization identifies important epitopes. Our data sug-

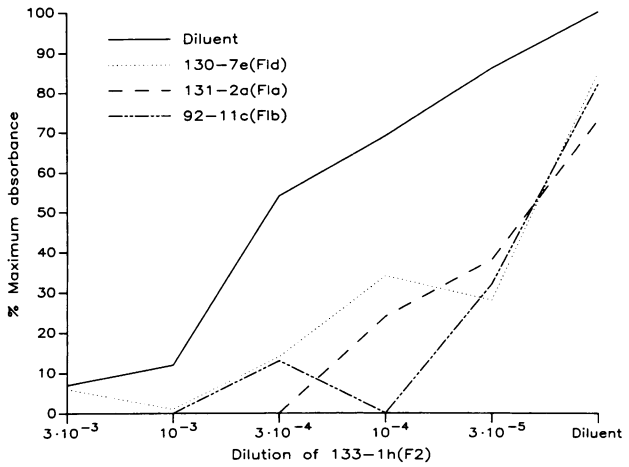

FIG. 6. Enhanced neutralization between MAb 133-1h (epitope F2) and MAb 131-2a (epitope F1a), 92-11c (epitope F1a), or 130-7e (epitope F1d). Neutralization was determined in an EIA neutralization assay. Absorbance readings from the EIA are expressed as percentage of maximum specific absorbance (maximum specific absorbance = mean absorbance for 4 wells with virus-infected control cells − mean absorbance for 11 wells with uninfected control cells); a higher absorbance signal indicates greater replication. One standard deviation in maximum absorbance was 12%, and 1 standard deviation in background absorbance was 6%. A value of <15% corresponded to complete neutralization (<0.100 specific absorbance). The MAbs were reacted against 384 $TCID_{50}$ of RSV A2. MAbs 131-2a, 92-11c, and 130-7e were at a 1:300 dilution. The dilutions are those of the MAbs as added to the virus-MAb mixture; the effective dilution after the MAb had been added to the mixture was increased by 167%. MAbs 131-2a, 92-11c, and 130-7e did not neutralize RSV by themselves. The diluent dilutions for their respective curves give the values for the MAbs by themselves. When mixed with MAb 133-1h, they shifted the curve of MAb 133-1h (the diluent curve gives the values for MAb 133-1h by itself) to the right and increased the neutralization titer between 3- and 10-fold.

gest that partial and enhanced neutralization identify epitopes that contribute to the strain differences seen in cross-neutralization studies and therefore are also likely to be important in inducing protective immunity. Two major groups of RSV strains have been identified by differences in reaction patterns with MAbs, i.e., group 1 and group 2 or subtype A and subtype B (3, 21). Cross-neutralization studies with animal antisera have demonstrated differences of 2- to 32-fold between group 1 and 2 strains (RSV Long and 18537 [7] or RSV Long and 8/60 [9]). Differences of zero to ninefold between group 1 strains (RSV Long and A2 [6] or RSV Long and 159/59 [9]) in cross-neutralization studies have also been reported. The epitopes corresponding to the MAbs that gave complete neutralization were present on all strains, while most (10 of 11) of those corresponding to strain-specific, partial, and enhanced neutralizing MAbs were present on only some strains.

Studies in animals and humans suggest that differences in the ability of serum to neutralize different strains of RSV may be important in protecting the host from disease. In these studies, higher levels of neutralizing antibody correlated with a higher probability of protection (8, 10, 13, 19, 22, 25, 26). It is possible that antibodies induced against epitopes that are not present on all strains and give some neutralizing activity provide the boost in immunity needed to achieve protection. Most of these "variable" epitopes are on the G protein, and studies in mice and cotton rats have demonstrated strain-specific protection after immunization with the G protein but not after immunization with the F protein (16, 28). Further study will be required to determine if, in fact, the strain-specific component of the host immune response is important for protection and for RSV vaccine development.

The different types of neutralization and the number of epitopes involved in neutralization complicate the interpretation of neutralization studies of RSV with MAbs. Comparable differences in types of neutralization with MAbs probably occur with other viruses as well. The enhanced neutralization data suggest that the activity of the individual MAb may be an incomplete measure of the function of the respective epitope. The strain-specific neutralization data suggest that neutralization studies should be done against several and not just one strain of RSV. Studies of the mechanisms of neutralization at the different epitopes should provide a basis for understanding the interaction of neutralization at different epitopes. The merits of these different measures of neutralization, however, will be determined by their value as indicators of protective immunity.

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