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Protection Against Lethal Viral Infection by Neutralizing and Nonneutralizing Monoclonal Antibodies: Distinct Mechanisms of Action In Vivo

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Monoclonal antibodies (MAb) reactive with the glycoprotein of vesicular stomatitis virus (VSV) serotypes Indiana (VSV-Ind) and New Jersey (VSV-NJ) were used to protect mice against lethal infection. MAb which reacted with a number of distinct epitopes and which could neutralize the virus in vitro could also protect against infection in vivo. MAb which could not neutralize the virus in vitro but which were specific for the glycoprotein of a single serotype were also able to protect mice against lethal VSV challenge. Interestingly, a group of MAb which cross-reacted with the glycoproteins of VSV-Ind and VSV-NJ could passively protect against challenge with either serotype. It was shown that as early as 2 h after infection, neither neutralizing nor nonneutralizing MAb could protect. Nonneutralizing MAb were found to be less effective at in vivo protection than neutralizing MAb. Furthermore, nonneutralizing MAb demonstrated a much lower binding efficiency to intact virions than did neutralizing MAb. These observations, plus the fact that the nonneutralizing MAb could lyse virus-infected cells in the presence of complement, suggested that in vivo protection by these antibodies may involve cell-associated viral determinants. To compare the mechanisms by which neutralizing and nonneutralizing MAb protected in vivo, $F(ab')_2$ fragments were used in protection experiments. Although the $F(ab')_2$ of a neutralizing MAb was still able to protect animals against lethal virus challenge, the $F(ab')_2$ of a cross-reactive nonneutralizing MAb was unable to do so. The reactivity of nonneutralizing MAb with virions and the apparent necessity of an intact Fc portion for protection further distinguish these antibodies from those MAb that are able to neutralize VSV solely by binding to the glycoprotein.

The development of monoclonal antibodies (MAb) directed toward virus-coded proteins has allowed the definition of antigenic determinants on these proteins in both operational and structural terms (8, 16, 23). Whereas some MAb which bind to certain viral proteins have been shown to affect in vitro measurements of biological function (i.e., hemagglutination, infectivity), other MAb which bind to the same or other proteins do not appear to affect in vitro functional assays. For example, certain MAb which bind to the influenza virus hemagglutinin are unable to inhibit hemagglutination or infectivity (2). However, some MAb of this latter type are able to lyse infected target cells or prevent in vivo viral pathogenesis (1, 15, 17).

Vesicular stomatitis virus (VSV) is an enveloped *Rhabdovirus* capable of producing infections in many species and occasionally has been transmitted to humans (6). The single surface glycoprotein (G-protein) of VSV, which is responsible for inducing neutralizing antibody, distinguishes the two major serotypes of the virus, Indiana (VSV-Ind) and New Jersey (VSV-NJ) (10). Recently, an antigenic map of the G-protein was generated with MAb in competitive binding assays and variant analysis (12–14). In addition to G-protein-directed neutralizing MAb, a group of nonneutralizing MAb were isolated, some of which bound to the G-proteins of both VSV-Ind and VSV-NJ. These MAb could lyse target cells infected with either serotype in the presence of complement (15). Although the role of neutralizing antibody in protection from viral disease seems clear, the in vivo relevance of nonneutralizing antibody is less obvious. Nonneutralizing MAb specific for surface proteins of other viruses, including rabies virus (7) and Sindbis virus (20), have been reported. In the case of Sindbis virus, MAb to protein E1 did not cause in vitro virus neutralization but could protect mice against a lethal infection. However, the mechanism by which protection occurred was not determined. A number of possible explanations for in vivo protection by nonneutralizing MAb are apparent, including complement-dependent lysis or antibody-dependent, cell-mediated lysis of virus-infected cells.

The present study was undertaken to determine the in vivo protective effects of serotype-specific as well as cross-reactive nonneutralizing MAb against the G-protein of VSV. Cross-protection could be achieved by administration of MAb to animals challenged with viral serotypes that are totally distinct by in vitro neutralization assays. In addition, the relative efficiencies of in vivo protection and the differential binding properties to intact virions of neutralizing versus nonneutralizing MAb indicated interesting differences in the reactivities of these antibody subsets with a single viral protein. Significantly, an intact Fc portion of a nonneutralizing MAb was shown to be required for in vivo protection, whereas the $F(ab')_2$ of a neutralizing MAb could still prevent infection, thus distinguishing the mechanisms by which these MAb protect against viral infection.

MATERIALS AND METHODS

Virus. Stocks of VSV-Ind and VSV-NJ were prepared by infecting baby hamster kidney (BHK) cells at a multiplicity of 0.01. Virus was then purified and concentrated by sucrose gradient centrifugation (21).

Mice. BALB/cByJ female mice, 7 to 8 weeks old, were obtained from Scripps Clinic and Research Foundation or The Jackson Laboratory, Bar Harbor, Maine.

Production and characterization of MAb. Production of the MAb used in this study has been described previously (6). Briefly, a hybridoma variant cell line, SP2/0 Ag-14, which produces no immunoglobulin chains, was fused with spleen cells which came from BALB/c mice which were either

hyperimmunized over a 6-month period or given a single intravenous (i.v.) injection of 10^6 PFU of VSV followed by a second injection 3 days before fusion.

The neutralizing ability of the MAb was assessed as described previously (6) with a microneutralization assay. VSV (10 PFU) was added to dilutions of MAb in 96-well microtiter plates, which were then incubated at 4°C for 30 min, followed by the addition of 10^4 BHK cells to the mixture. The reciprocal of the dilution that prevented the cytopathic effect of the virus after 48 h of incubation at 37°C was considered the endpoint titer.

Determination of immunoglobulin class. MAb in culture supernatants were concentrated 10 times by $(NH_4)_2SO_4$ precipitation (50%). This concentrate was then tested against isotype, subclass, and light-chain-specific antisera (Bionetics, Kensington, Md.) by Ouchterlony double diffusion.

ELISA. The enzyme-linked immunosorbent assay was modified from that described by Voller et al. (22). Purified virus (0.5 μg) in 100 μl of coating buffer ($NaHCO_3$-Na_2CO_3 [pH 9.6]) was added to each well of 96-well polyvinyl chloride microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va.) or Immulon plates (Microbiological Associates, Bethesda, Md.) and allowed to sit overnight at 4°C. The plates were then washed three times with phosphate-buffered saline (PBS) containing 0.1% Tween 20 (PBST). The MAb (diluted in PBST) was then added and allowed to react for 2 h at 37°C. The plates were then washed five times, and 100 μl of a peroxidase-labeled goat antimouse immunoglobulin (Bionetics) was added and reacted for 2 h at room temperature. The plates were washed five times in PBST and developed with 0.2 mg of *O*-dianisidine per ml–0.012% H_2O_2 in Tris-citrate buffer (pH 6.0) containing 0.5% Triton X-100. The reaction continued for 10 min and was stopped with 20 μl of 2% NaN_3. The optical density (OD) at 492 nm was then determined.

Immunization and passive transfer of MAb. Three approaches were used in the protection studies. (i) MAb or control ascites fluid was mixed with the virus (1:1) in PBS and allowed to incubate at 4°C for 30 min. Mice were injected i.v. with 200 μl of the mixture. (ii) Mice received 200 μl of ascites fluid intraperitoneally (i.p.) 24 h before i.v. inoculation of the virus. (iii) Mice were given i.v. injections of virus and 2 h later were given 200 μl of MAb either i.v. or i.p.

Absorption of MAb by live virus. Dilutions of MAb were mixed with a standard amount of virus (10^{10} PFU) and allowed to incubate for 1 h at 4°C. The mixture was then centrifuged at 27,000 × *g* for 2 h to remove virus. The resulting supernatant was tested for binding activity by ELISA as described above.

Preparation of F(ab')$_2$ fragments. MAb purified by protein A-Sepharose CL-4B (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) chromatography was digested with pepsin (2 mg of enzyme per 100 mg of antibody) in 0.1 M sodium acetate buffer (pH 4.5) for 18 to 24 h (9). After adjustment of the pH to neutrality, the digest was applied to a column of Sephadex G-100 equilibrated with 0.02 M PBS (pH 7.2). F(ab')$_2$-containing fractions were pooled and concentrated with Aquacide (Calbiochem-Behring, La Jolla, Calif.). Any remaining undigested immunoglobulin G (IgG) was removed by adsorption to protein A-Sepharose CL-4B. The final preparation was analyzed for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (11). F(ab')$_2$ preparations were tested for binding by ELISA and neutralization.

Statistical analysis. Differences in survival of groups of mice were analyzed for statistical significance by hypothesis testing using the difference between the proportions of survivors in control and test groups.

RESULTS

Characteristics of MAb employed. Infection of mice with high doses of VSV results in hind limb paralysis followed by fatal meningoencephalitis (18). Experiments were performed with a diverse panel of MAb reactive with the VSV G-protein to determine the in vivo protective ability of MAb against distinct G-protein epitopes. Table 1 lists the MAb employed and their functional characteristics. Twelve MAb, reactive with VSV-Ind, VSV-NJ, or both serotypes, were used. All of the MAb are directed to distinct epitopes on the G-protein, as previously determined by competitive radioimmunoassay and antigenic variant analysis (12–14). MAb I1, I14, and I15 are able to neutralize only VSV-Ind in vitro, whereas MAb I17 binds to the G-protein of VSV-Ind but does not cause in vitro neutralization. MAb N5 and N9 neutralize VSV-NJ in vitro, whereas MAb N13 and N14 bind to the VSV-NJ G-protein but do not neutralize the virus in vitro. MAb IN2, IN5, IN6, and IN7 bind to the G-protein of both serotypes but cannot affect in vitro infectivity, with the exception of IN7, which consistently neutralizes only VSV-Ind to a low level.

Protection by serotype-specific MAb. Initially, serotype-specific neutralizing or nonneutralizing MAb were used to attempt passive protection of VSV-infected mice (Table 2). MAb were injected i.p. 24 h before i.v. virus inoculation. When 10^7 PFU of VSV-Ind were inoculated into mice that had been previously injected with an anti-VSV-NJ MAb, N5, no protection was afforded (Table 2). However, MAb to three distinct neutralizing epitopes of VSV-Ind (A_{Ind}, B_{Ind}, and C_{Ind}) were able to protect VSV-Ind-infected mice. Additionally, antibody I17, which does not neutralize the virus in vitro, was able to protect against lethal infection. Similar experiments using VSV-NJ were also performed (Table 2). A dose of VSV-NJ ca. 5 to 10 times greater than that of VSV-Ind was necessary for a lethal infection in all experiments. The reason for this is unknown, but each virus preparation is prepared from thrice-cloned virus isolates. Anti-VSV-Ind neutralizing MAb did not protect against

TABLE 1. Characteristics of MAb used in protection studies

MAb designation[a]	G-protein reactivity[b]	ELISA titer to:		Neutralizing titer
		Immunizing virus	Heterologous virus	
I1	Ind	6.5×10^5	<40	5.1×10^5
I14	Ind	1.6×10^5	<40	5.1×10^4
I15	Ind	6.5×10^5	<40	5.1×10^5
I17	Ind	1.6×10^5	<40	<20
N5	NJ	2.6×10^6	<40	5.1×10^5
N9	NJ	6.5×10^5	<40	5.1×10^4
N13	NJ	2.6×10^6	<40	<20
N14	NJ	1.6×10^5	<40	<20
IN2[c]	Ind, NJ	6.5×10^5	1.6×10^5	<20
IN5[c]	Ind, NJ	4.1×10^4	4.1×10^4	<20
IN6[c]	Ind, NJ	1.6×10^5	4.1×10^4	<20
IN7[d]	Ind, NJ	1.3×10^6	1.3×10^6	128[e]

[a] Immunoglobulin class 2a, light-chain type K for all MAb tested, except IN5, which is γ2b, K. All MAb exhibited complement-dependent cytolysis.
[b] Determined by immunoprecipitation of [^{35}S]methionine-labeled VSV proteins from infected cell lysates.
[c] VSV-NJ was the immunizing virus.
[d] VSV-Ind was the immunizing virus.
[e] Neutralization of VSV-Ind only.

lethal VSV-NJ infection, whereas antibodies reactive with two distinct neutralizing epitopes, A_{NJ} and C_{NJ}, did confer protection. MAb N13 and N14, which are specific for distinct nonneutralizing epitopes on the VSV-NJ G-protein, were also able to protect VSV-NJ-inoculated mice (Table 2). Therefore, serotype-specific antibodies that are specific for the G-protein of VSV-Ind or VSV-NJ and do not cause in vitro neutralization of the virus are capable of preventing lethal infection in vivo.

Ability of cross-reactive MAb to protect against infection by either serotype. Since serotype-specific nonneutralizing MAb could protect against lethal VSV infection, it was of interest to determine the protective abilities of MAb which could bind to the G-proteins of both VSV-serotypes but could not neutralize the virus in vitro. Groups of mice were given i.p. injections of cross-reactive MAb followed 24 h later by an i.v. injection of VSV-Ind or VSV-NJ (Table 3). Neutralizing MAb to the inappropriate virus did not protect mice, whereas nearly all of the cross-reactive nonneutralizing MAb did confer some degree of protection. As previously stated, the four cross-reactive MAb employed in these experiments bind to four nonoverlapping determinants on the G-protein. Although MAb IN2 did not completely protect animals against VSV-NJ infection, it was able to prevent lethal VSV-Ind infection in nearly all cases. It should be noted that the VSV-NJ lethal dose is significantly greater than the VSV-Ind lethal dose, thus decreasing the sensitivity of the experiments with VSV-NJ. MAb IN6 and IN7 protected a majority of the animals challenged with either VSV-NJ or VSV-Ind. Although MAb IN5 was the only antibody of the IgG2b subclass and did not appear to protect effectively against lethal infection, it was able to lyse VSV-infected cells in the presence of complement (Table 1). The lack of protection by this MAb is likely to be due to the lower concentration of antibody in the ascitic fluid (Table 1). Thus, the results indicate that cross-reactive nonneutralizing MAb can protect against lethal infection with either VSV serotype, although these serotypes are totally distinct by in vitro neutralization assays.

TABLE 2. Passive protection by serotype-specific neutralizing and nonneutralizing MAb against distinct epitopes[a]

Virus	MAb	Epitope specificity[b]	No. of survivors/total no. tested[c]	Mean day of death
VSV-Ind	N5	A_{NJ}	0/5	6.2
	I1	A_{Ind}	5/5	—[d]
	I14	B_{Ind}	5/5	—
	I15	C_{Ind}	5/5	—
	I17	—[e]	4/5	5.0
VSV-NJ	I1	A_{Ind}	0/5	5.8
	N5	A_{NJ}	5/5	—
	N9	C_{NJ}	5/5	—
	N13	—[f]	5/5	—
	N14	—[f]	5/5	—

[a] Virus (200 μl; 10^7 PFU of VSV-Ind or 10^8 PFU of VSV-NJ) in saline was injected i.v. 24 h after i.p. injection of 200 μl of ascites fluid containing the indicated MAb.
[b] Determined previously by competitive radioimmunoassay (8).
[c] $P < 0.01$ for all groups as compared with groups receiving control MAb specific for the inappropriate serotype plus virus.
[d] —, All animals survived >60 days.
[e] MAb I17 binds to a distinct epitope on the VSV-Ind G-protein as previously shown (13).
[f] MAb N13 and N14 bind to distinct epitopes on the VSV-NJ G-protein as previously shown (13).

TABLE 3. Cross-protection of mice inoculated with neutralization-distinct serotypes[a]

MAb	VSV-NJ			VSV-Ind		
	No. of survivors/ total no. tested	Mean day of death	P^b	No. of survivors/ total no. tested	Mean day of death	P^b
I1	1/10	6.2		ND[c]	ND	
N5	ND	ND		0/10	6.6	
IN2	3/5	8.0	<0.05	9/10	6.0	<0.01
IN5	2/8	4.5	>0.05	3/8	8.4	<0.05
IN6	8/10	6.0	<0.01	8/9	5.0	<0.01
IN7	7/10	7.3	<0.01	8/10	8.0	<0.01

[a] Virus (200 μl; 10^8 PFU of VSV-NJ or 10^7 PFU of VSV-Ind) in saline was injected i.v. 24 h after i.p. injection of 200 μl of ascites fluid containing the indicated MAb.
[b] Value for the difference in survival between the test group and the group receiving control MAb specific for the inappropriate serotype plus virus.
[c] ND, Not done.

Necessity of circulating antibody for protection. Because protection occurred if injection of MAb preceded virus inoculation, attempts were made to protect animals that had an ongoing VSV infection. Mice were injected i.v. with 10^7 PFU of VSV-Ind and 2 h later were given injections of MAb (Table 4). Animals given an anti-VSV-NJ MAb were not protected. Interestingly, an anti-VSV-Ind neutralizing MAb, I14, could not protect infected animals whether injected either i.v. or i.p. Likewise, two cross-reactive nonneutralizing MAb, IN2 and IN6, were unable to protect previously infected mice. This suggests that circulating antibody must be present at the time of infection in order to protect, possibly because the virus travels to immunologically privileged sites, the antibody is at a concentration such that it is unable to overcome the spread of virus in an ongoing infection, or both. In comparison, passive protection studies in which MAb to herpes simplex virus G-proteins were used also indicated that the time at which MAb were administered in relation to virus inoculation was critical (5).

Comparison of protective effects of neutralizing versus nonneutralizing MAb. The relative protective effects of neutralizing versus nonneutralizing antibodies were studied by incubating dilutions of MAb premixed with various doses of VSV-Ind and then injecting the mixture i.v. (Table 5). All control mice which had received a mixture of an anti-VSV-NJ neutralizing MAb and doses of VSV-Ind died. When dilutions of an anti-VSV-Ind neutralizing MAb, I15, were premixed with virus and inoculated into mice, total protection occurred even at a dilution of 1:1,000. At a final dilution of 1;8,000, protection was achieved at the lowest virus dose, 10^7 PFU, but not at doses of 2.5×10^7 or 5.0×10^7 PFU. In contrast to this result, much higher concentrations of a cross-reactive nonneutralizing MAb were required to protect against lethal VSV-Ind infection. MAb IN2, which has a titer comparable to that of MAb I15 by ELISA, could protect at a final dilution of 1:10 (although not completely at high doses of virus) but did not effectively protect mice when used at a dilution of 1:100. The results indicate that neutralizing MAb appear to be much more efficient at protecting against lethal virus infection than nonneutralizing MAb.

Differential binding properties of neutralizing versus nonneutralizing MAb. Virus adsorbed to the polyvinyl chloride plates used in the ELISA is at least partially denatured, since MAb that are reactive against matrix or internal nucleocapsid proteins bind very effectively (data not shown). There-

TABLE 4. Inability of MAb to protect mice with an ongoing VSV infection[a]

MAb	No. of survivors/ total no. tested[b]	Mean day of death
N5	1/5	5.0
I14	1/5	5.8
I14[c]	0/5	5.6
IN2	0/5	5.4
IN6	1/5	6.0

[a] VSV-Ind (10^7 PFU) was inoculated i.v. into BALB/c mice; 2 h later, 200 μl of ascites fluid containing the indicated MAb was injected i.p.
[b] $P > 0.05$ for all groups as compared with groups receiving anti-VSV-NJ control MAb plus virus.
[c] MAb (200 μl) injected i.v. 2 h postinfection.

fore, the possibility exists that MAb that were binding to the solid-phase immunosorbent were binding to sites not exposed on the intact virion. Thus, if differences in binding to native antigen were present they might not be detected with this assay. To test this possibility, dilutions of neutralizing or nonneutralizing MAb containing equivalent antibody concentrations were mixed with a standard amount of live virus and incubated at 4°C for 1 h. Virus-MAb complexes were then removed by centrifugation. The resulting supernatant was tested in an ELISA and compared with unabsorbed controls or controls absorbed with the heterologous virus (Fig. 1). Panels A and B show the results of absorption of two anti-VSV-Ind neutralizing MAb directed against distinct epitopes by 10^{10} PFU of VSV-NJ or VSV-Ind. Absorption by VSV-Ind of neutralizing MAb I1 or I14 (which bind to distinct epitopes) resulted in a nearly complete removal of

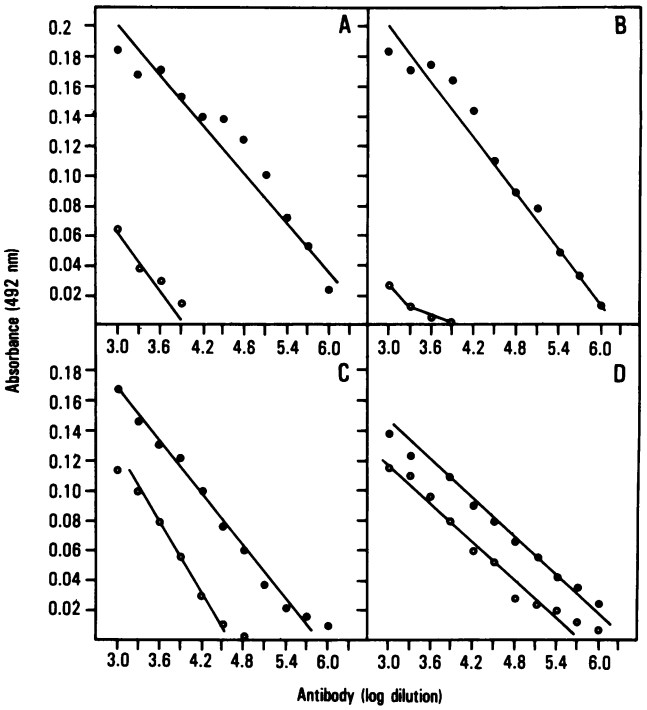

FIG. 1. Differential binding properties of neutralizing versus nonneutralizing MAb. Dilutions of MAb were incubated with 10^{10} PFU of VSV-Ind or VSV-NJ and incubated for 1 h at 4°C. The mixture was then centrifuged at 27,000 × *g* for 2 h. The supernatant was removed and assayed for binding to VSV-Ind immunosorbents by ELISA. Symbols: ●, 10^{10} PFU of VSV-NJ (panels A through C) or buffer only (panel D); ○, 10^{10} PFU of VSV-Ind bound to Mab I1 (panel A), MAb I14 (panel B), MAb I17 (panel C), and MAb IN2 (panel D).

TABLE 5. Relative efficiency of in vivo protection by neutralizing versus nonneutralizing MAb[a]

MAb	Final dilution	Virus dose (PFU × 10^7)	No. of survivors/ total no. tested	Mean day of death	*P*[b]
N5	1:10	5.0	0/3	5.7	
		2.5	0/3	6.0	
		1.0	0/3	7.3	
I15	1:100	5.0	3/3	—[c]	<0.05
		2.5	3/3	—	<0.05
		1.0	3/3	—	<0.05
	1:1,000	5.0	3/3	—	<0.05
		2.5	2/2	—	<0.05
		1.0	3/3	—	<0.05
	1:8,000	5.0	1/5	6.8	>0.05
		2.5	1/3	6.0	>0.05
		1.0	2/2	—	<0.05
IN2	1:10	5.0	6/8	6.5	<0.05
		2.5	7/11	9.0	<0.05
		1.0	8/9	6.0	<0.01
	1:100	5.0	1/4	6.0	>0.05
		2.5	0/3	5.3	>0.05
		1.0	1/5	7.0	>0.05

[a] Dilutions of MAb and the indicated dose of VSV-Ind were mixed 1:1 and incubated for 30 min at 4°C. An inoculum of 200 μl of the mixture was then injected i.v. into BALB/c mice.
[b] *P* value for the difference in survival between the test group and the group receiving anti-VSV-NJ control MAb plus virus.
[c] —, All animals survived >60 days.

antibody. Interestingly, when nonneutralizing MAb were tested in this fashion a much less dramatic removal of antibody was observed. MAb I17, a VSV-Ind-specific nonneutralizing MAb, was only reduced in titer by a factor of 8 after absorption by VSV-Ind (Fig. 1C). Similarly, binding of the cross-reactive nonneutralizing MAb IN2 was reduced only fourfold after absorption (Fig. 1D). Two other nonneutralizing MAb were also tested but are not shown in Fig. 1. MAb IN5 and IN6 exhibited four- and twofold reductions in titers, respectively, after absorption by VSV-Ind. Similar results were obtained when the absorption was carried out at 37°C (data not shown). Thus, it would appear that nonneutralizing MAb that react with multiple, distinct epitopes bind much less effectively to the intact virion than neutralizing MAb do. Preliminary results indicate that the reduced binding of nonneutralizing MAb versus neutralizing MAb to whole virus can be due both to decreased affinity and to a lower number of binding sites for nonneutralizing MAb (L. Lefrancois and D. S. Lyles, unpublished data).

Requirement for Fc in protection by nonneutralizing MAb. The relatively reduced binding of the nonneutralizing MAb to live virus in vitro and the fact that these MAb are able to lyse infected target cells in the presence of complement suggested that their action in vivo could be due to an interaction with infected host cells. In this way, complement-dependent lysis or antibody-dependent, cell-mediated cytotoxicity could occur. Thus, the Fc portion of the MAb would be necessary for protection. Several attempts were

made to produce $F(ab')_2$ of a number of the antibodies, in some cases resulting in degradation of the antibodies (even at low enzyme concentrations and with reduced incubation times) or in other instances the production of what appeared to be an intact $F(ab')_2$, which upon testing could no longer bind to antigen. Finally, functional $F(ab')_2$ fragments of a neutralizing MAb (I1) and a cross-reactive nonneutralizing MAb (IN2) were obtained. These preparations were tested for binding activity by ELISA, and in the case of MAb I1, also for neutralizing ability (Table 6). The binding values at saturation for both $F(ab')_2$ preparations were considerably lower than those obtained with the intact MAb due to the loss of antigenic determinants on the Fc portion which would be recognized by the second-stage peroxidase-labeled goat antimouse IgG reagent. For comparing the binding of the intact MAb with that of the $F(ab')_2$, the amount of antibody required to achieve 50% of the maximal value at saturation was used. Approximately equivalent concentrations of the $F(ab')_2$ of MAb I1 and of the intact MAb were required to reach the 50% level of binding with VSV-Ind as immunosorbent. Furthermore, the intact IgG and the $F(ab')_2$ of MAb I1 had similar neutralizing ability, indicating that the removal of the Fc portion did not significantly affect the binding characteristics of this MAb. The binding efficiency of the nonneutralizing MAb IN2 was affected somewhat upon removal of the Fc portion by treatment of the antibody with pepsin. In attempts to produce $F(ab')_2$ of other nonneutralizing MAb, a loss of binding ability after enzymatic treatment was a common occurrence, suggesting that the binding of these MAb is more easily affected by structural changes in the molecule. For MAb IN2 a concentration of $F(ab')_2$ ca. two- to threefold higher than that of the intact MAb was required to achieve the 50% level of maximum binding (Table 6). This result may be due to (i) a proportion of the antibody protein being rendered inactive by enzymatic treatment, (ii) a relative decrease in binding ability after removal of the Fc portion of the antibody, or (iii) both. This difference was compensated for by employing a large excess of $F(ab')_2$ in protection studies (see below).

Protection experiments utilizing the $F(ab')_2$ preparations were performed with the VSV-Ind serotype (Table 7). Animals receiving either no MAb or an anti-VSV-NJ MAb, N5, were not protected against a lethal VSV-Ind infection. When 300 μg of purified anti-VSV-Ind MAb I1 was given before infection, all animals survived (Table 7, experiment 1). When the same dose of MAb I1 in the form of $F(ab')_2$ was administered, all animals were again protected. In the second experiment an identical protocol was used with the cross-reactive nonneutralizing MAb IN2. Whereas the majority of animals given the intact MAb were protected, 300 μg of the $F(ab')_2$ of this MAb apparently could not protect animals against lethal challenge with VSV-Ind. To further compare the protective abilities of the intact nonneutralizing MAb and its $F(ab')_2$ and to be sure that the lack of protection was not due to the decreased binding ability of the $F(ab')_2$, virus was mixed with an excess of antibody and incubated for 30 min at 4°C before injection. Under these conditions, 100 μg of intact MAb IN2 afforded protection against lethal challenge with 10^7 PFU of VSV-Ind, whereas the same amount of an anti-VSV-NJ MAb, N5, did not (Table 7, experiment 3). In contrast to the intact MAb IN2 IgG molecule, the $F(ab')_2$ of MAb IN2 was unable to protect against lethal infection when 100 μg was used, or more importantly, when a 10-fold excess (1 mg) of the $F(ab')_2$ was administered. For this MAb at least, the mechanism by which it confers protection in vivo appears to require an intact Fc portion on the antibody molecule.

TABLE 6. Binding characteristics of $F(ab')_2$ preparations of neutralizing and nonneutralizing MAb

MAb	OD at saturation[a]	Concn of MAb for 50% of maximum (ng/ml)[b]	Neutralization titer[c]
I1	0.594	32.8	1.26
I1 [$F(ab')_2$]	0.229	44.0	1.27
IN2	0.563	22.5	NT[d]
IN2 [$F(ab')_2$]	0.292	63.0	NT

[a] MAb preparations were assayed by ELISA with VSV-Ind as the immunosorbent, and the OD at 492 nm was determined.

[b] The concentration of MAb required to achieve 50% of the maximum OD at saturation was calculated from a plot of the $\log_{10}$ of the antibody concentration versus the OD obtained by ELISA.

[c] The neutralization titer is the $\log_{10}$ of the concentration of MAb (in nanograms per milliliter) required to achieve a 50% reduction in the infectivity of 100 PFU of VSV-Ind.

[d] NT, Not tested.

TABLE 7. Fc-mediated protection by nonneutralizing antibody

MAb	No. of survivors/total no. tested	Mean day of death	P[a]
Expt 1[b]			
No MAb	0/5	6.0	
N5	0.5	6.0	
I1	5/5	—[c]	<0.01
I1 [$F(ab')_2$]	5/5	—	<0.01
Expt 2[b]			
No MAb	0/5	6.0	
N5	1/5	7.0	
IN2	8/10	5.5	<0.05
IN2 [$F(ab'])_2$]	1/5	6.0	>0.05
Expt 3[d]			
N5 (100 μg)	1/10	5.2	
IN2 (100 μg)	5/5	—	<0.01
IN2 [$F(ab')_2$] (100 μg)	0/5	5.4	>0.05
IN2 [$F(ab')_2$] (1 mg)	2/12	4.7	>0.05

[a] *P* value for the difference in survival between the test group and the group receiving anti-VSV-NJ control MAb plus virus.

[b] Purified MAb or $F(ab')_2$ (300 μg) was injected i.p. into BALB/cByJ mice 24 h before i.v. injection of 10^7 PFU of VSV-Ind in saline.

[c] —, All animals survived >60 days.

[d] Purified MAb or $F(ab')_2$ was mixed with 10^7 PFU of VSV-Ind and incubated for 30 min at 4°C. This mixture, containing the indicated amount of MAb or $F(ab')_2$, was then inoculated i.v. into BALB/cByJ mice.

DISCUSSION

MAb specific for distinct epitopes on the VSV G-protein were used to protect mice against lethal infection. The determinants recognized by the MAb are distinct not only in structural terms (i.e., by competitive binding analysis) but also in functional definition. That is, certain MAb were unable to neutralize virus in vitro but could protect against in vivo infection, whereas a second group had the ability to prevent infection in vitro as well as in vivo (Table 2). These results are similar to those from studies of Sindbis virus (20), Venezuelan equine encephalomyelitis virus (17), and herpesvirus (5). An important distinction in this study is that viral serotypes which are clearly distinct by the in vitro neutralization assay can be prevented from causing infection in vivo

by a single type of MAb which can bind to both serotypes (Table 3).

The VSV system is particularly interesting, since a variety of immunological reactions are mediated through a single surface G-protein. Serotype-specific as well as cross-reactive cytotoxic T cells are directed toward the G-protein (15), and the results presented here indicate that antibody-mediated interactions that are functionally distinct can also be G-protein directed. The studies with Sindbis and Venezuelan equine encephalomyelitis viruses suggest that nonneutralizing antibodies react primarily with protein E1, whereas neutralizing antibodies react with protein E2, thus implying discrete functional reactivities assigned to different proteins. However, neutralizing antibodies specific for Sindbis virus E1 have been described (3), and since proteins E1 and E2 are linked in the viral envelope, antibody binding to one could affect function in the other. Overall, the experimental results detailing the antigenic determinants on the VSV G-protein indicate an extremely diverse immune response. The present report shows that at least three subsets of antibodies specific for the G-protein are able to prevent lethal viral infection: (i) antibodies which are able to neutralize virus in vitro and are invariably serotype specific, (ii) nonneutralizing antibodies which are also serotype specific, and (iii) nonneutralizing antibodies that are cross-reactive and are therefore able to bind to the G-proteins of both serotypes. In addition, multiple epitope specificities are contained within each of these subsets. Thus, it seems clear that discrete determinants residing on a single viral protein can induce a variety of host effector functions.

The observation that nonneutralizing MAb were much less efficient in protecting against lethal VSV infection than neutralizing MAb led to the finding that the former MAb did not bind as effectively to live virus in suspension as the latter MAb did (Fig. 1). Neutralizing MAb were bound very efficiently to the surfaces of intact virions, whereas those nonneutralizing MAb tested were bound to virions to a much lower degree. A preliminary report of nonneutralizing MAb specific for Sindbis virus protein E1 suggests a similar finding (A. L. Schmaljohn, K. M. Kokubun, D. S. Stec, and G. A. Cole, Fed. Proc. **42**:24, 1983). These binding characteristics can be due to a generally lower affinity of nonneutralizing MAb as compared with that of neutralizing MAb, the relative scarcity of available binding sites for the nonneutralizing MAb on the surfaces of intact virions (L. Lefrancois and D. S. Lyles, unpublished data), or both. Whether these properties are related to the inability of these MAb to neutralize in vitro or whether the critical factor in neutralization is the determinant specificity is not known. The fact that both of these types of MAb saturate to the same level when bound to an ELISA plate implies that a certain percentage of binding sites reactive with nonneutralizing MAb are masked on the intact virion. These sites could possibly become accessible on the surfaces of infected cells and would facilitate in vivo interaction of nonneutralizing antibodies with the G-protein at the cell surface. Although no quantitative comparison of binding efficiency has yet been made between virus-associated and cell-associated determinants, all of the nonneutralizing antibodies employed could lyse virus-infected cells in the presence of complement. Thus, either nonneutralizing MAb may be characterized by low-affinity binding to virus (and possibly to cell-associated viral antigen), or certain determinants which are relatively inaccessible on free virus exhibit increased expression on infected cells, perhaps allowing in vivo protection, but not in vitro neutralization, to occur.

Because of the distinctions between neutralizing and nonneutralizing MAb, it seemed likely that the mechanisms by which these two types of antibody protect in vivo are different. $F(ab')_2$ fragments of neutralizing and nonneutralizing MAb were made and used in protection studies. The results indicate that an intact Fc portion is required for in vivo protection by nonneutralizing MAb but is not necessary for protection by neutralizing MAb (Table 7). Fc-mediated neutralization could occur through complement-dependent lysis or antibody-dependent, cell-mediated cytotoxicity of virus-infected cells, both of which require an intact Fc portion on the antibody. Alternatively, antibody-coated intact virions could be neutralized by the action of complement or through phagocytosis by macrophages or polymorphonuclear leukocytes, which also may be Fc receptor mediated. These mechanisms could also operate when a neutralizing antibody encounters viral antigen, although these activities do not appear to be absolutely necessary, since $F(ab')_2$ of neutralizing MAb affords protection. Previous work in which neutralizing heterogenous antibody specific for herpes simplex virus was used showed that the Fc fragment was not necessary for in vivo protection when administered before or at the time of virus inoculation (19). However, a recent study in which antisera reactive with Friend leukemia virus were used indicated that $F(ab')_2$ preparations, although retaining their ability to neutralize virus in vitro, could not protect against in vivo virus-induced disease (4). Although these studies employed heterogenous antisera, it is likely that the interaction of host effector mechanisms and antiviral antibody can be influenced dramatically by the particular virus being studied.

The results presented suggest interesting implications for viral immunity in general and for vaccine strategy. Although neutralizing antibody may provide a highly efficient mode of protection against viral infection, Fc-mediated immunity to virus or virus-infected cells could be important in preventing further virus production. The temporal sequence of production of neutralizing versus nonneutralizing antibodies is not known, but early appearance of antibody directed to cell-associated viral determinants could be important in early prevention of viral dissemination. The fact that antibody directed against cross-reactive and possibly conserved epitopes is able to confer protective immunity suggests that synthetic determinants of this nature could be used to induce immunity to related viruses which may be serologically distinct by conventional assays. The relationship between virus-associated and cell-associated determinants recognized by nonneutralizing antibody is currently being investigated.

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