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Monoclonal Antibodies to the Glycoprotein of Vesicular Stomatitis Virus: Comparative Neutralizing Activity

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Nineteen independently isolated hybridomas producing monoclonal antibodies to the glycoprotein of vesicular stomatitis virus were isolated and studied for their capacity to neutralize viral infectivity. By measuring competitive binding of ^{125}I-labeled monoclonal antibodies in a radioimmunoassay, 11 different, non-cross-reacting antigenic determinants were identified on the vesicular stomatitis virus G protein. All monoclonal antibodies reacting with determinants 1, 2, 3, and 4 resulted in viral neutralization, whereas those binding to the other seven determinants did not neutralize infectivity. A mixture of two monoclonal antibodies binding to different determinants resulted in a more rapid neutralization than either antibody alone, suggesting that different antibodies can exert a synergistic effect on viral neutralization. Kinetic experiments revealed biphasic neutralization curves similar to those expected for heterologous antibody. No evidence could be obtained to relate biphasic kinetics of viral neutralization to heterogeneous populations either of antibody molecules or of virus. The possible significance of the kinetic data with monoclonal antibodies is discussed.

Vesicular stomatitis (VS) virus is a membrane-enclosed, negative-strand RNA virus which buds from the infected cell membrane (28). Although the nucleocapsid core is itself infectious at low efficiency, a glycoprotein (G protein) spike embedded in the membrane enhances infectivity at least 10^5-fold (2). VS virus has two major antigens: a group-specific nucleocapsid antigen and a type-specific glycoprotein antigen (3). Purified G protein serves as the antigen that gives rise to and reacts with neutralizing antibody (15). The major antigenic reactivity of the G protein resides in its protein and not in the carbohydrate chains. Since the VS viral G protein has a molecular weight of ~67,000 (28), one would predict that this protein has multiple antigenic determinants.

In the present experiments, we produced 19 clones of mouse hybridomas, each of which secreted a monoclonal antibody that reacted with VS viral G protein. By definition, each monoclonal antibody is of a single isotype, possesses a specific avidity, and presumably binds to a single antigenic determinant. Each monoclonal antibody has been tested for its capacity to neutralize the infectivity of VS virus.

MATERIALS AND METHODS

Virus and cells. The Indiana serotype (San Juan strain) of cloned VS virus was produced by infecting monolayer cultures of BHK-21 cells at a multiplicity of 0.1 PFU/cell. As previously described (22), virus harvested at 18 to 20 h postinfection was pelleted through a 50% glycerol cushion and suspended in 20 mM Tris (pH 7.4) containing 1 mM EDTA and 1 M NaCl. The virus was then subjected to rate-zonal centrifugation in a 0 to 40% sucrose gradient followed by equilibrium centrifugation in a 15 to 50% tartrate gradient. Purified VS virus from the tartrate gradient was dialyzed against 10 mM Tris (pH 7.4) and stored at −80°C. Virus infectivity was determined by plaque assay on monolayer cultures of L cells (22).

Isolation of virion glycoprotein. As described by Petri and Wagner (25), the G protein was extracted from purified VS virions at a protein concentration of 1 mg/ml in 10 mM Tris (pH 7.4) by incubation for 1 h at room temperature with 0.06 M β-D-octylglucoside (Calbiochem, La Jolla, Calif.). After removal of nucleocapsids by centrifugation at 150,000 × *g*, supernatant fluids containing G protein were dialyzed for 24 h at 4°C against 4 liters of 10 mM Tris (pH 7.4) and then dried by lyophilization. This G protein was at least 97% pure and free of other viral proteins as determined by polyacrylamide gel electrophoresis and staining with Coomassie brilliant blue. Undialyzed supernatant fluid in 0.06 M octylglucoside was used in radioimmunoassays (RIAs).

Immunization procedures. BALB/c mice were injected subcutaneously with 100 μg of G protein suspended in 0.2 ml of complete Freund adjuvant. Four weeks later each mouse received a subcutaneous booster of 100 μg of G protein suspended in 0.2 ml of incomplete Freund adjuvant. After an additional 3 to 5 weeks, and 4 days before sacrificing for hybridoma cell fusion, each mouse received 50 μg of G protein in 10 mM Tris intravenously.

Fusion of spleen and myeloma cells. Spleen cells, obtained as described by Mishell et al. (23), were treated with Gey solution (23) to destroy erythrocytes.

SP2/O myeloma cells were harvested by centrifugation and washed twice in calcium-free and serum-free RPMI 1640 medium. Fusion of the spleen cells (from three mice) and myeloma cells (5×10^7 cells) was carried out by the addition of 1 ml of 37% polyethylene glycol by the procedure of Oi and Herzenberg (24). After gently washing the fused cells three times in serum-free RPMI 1640 medium, the cells were suspended in 60 ml of serum-free medium and incubated at 37°C for 30 min. After centrifugation, the thymocytes obtained from three 6-week-old BALB/c mice were added, and the cells were suspended in 300 ml of RPMI 1640 medium containing 15% horse serum. The cells were then distributed into 20 96-well Costar flat bottom plates (150 μl/well) and incubated at 37°C.

After incubation for 24 h, 100 μl of 2.5× HAT (hypoxanthine-aminopterin-thymidine) medium (24) was added to each well. About one-half of the medium in each well was exchanged every 2 to 3 days thereafter, and after 14 days of growth, aminopterin was omitted from the medium.

RIA. Irradiated VS virus (10 μg/ml) was suspended in 10 mM Tris (pH 7.4) and 100 μl of suspended virus was placed in each of the 96 wells of a polyvinyl chloride microtiter plate (Dynatech Laboratories, Inc., Alexandria, Va.). After 8 to 12 h, the wells were washed with 1% γ-globulin-free horse serum, and 50 μl of supernatant culture fluid from the cell fusion was added to each well. The wells were washed again after 3 h of incubation at room temperature, and ^{125}I-labeled rabbit anti-mouse $F(ab)_2$ was added to each well. The wells remained at room temperature for 12 to 15 h before being thoroughly washed to remove unreacted ^{125}I. Wells were then excised with a hot wire, and radioactivity was counted in a Beckman II gamma counter. All radioimmune assays were controlled by placing an identical amount of the material to be tested into a well that had been saturated with 10% horse serum but contained no virus.

Subcloning. Clones producing antibodies to the G protein were diluted in RPMI 1640 medium plus 15% horse serum, and serial dilutions were made before reinoculating Costar wells. Each well was observed daily, and those containing single cells were scored. After assaying for antibody to VS virus, the cell suspension in a single well was again diluted, and single-cell clones were again selected for the final hybridoma.

Isotype determination. Monoclonal isotypes were determined by double diffusion with a cell lysate made from approximately 10^7 cells in 0.2 ml of 0.5% Nonidet P-40 in phosphate-buffered saline (pH 7.0). Isotype-specific antisera were purchased from Meloy Laboratories, Springfield, Va.

Ascites. To obtain larger amounts of each monoclonal antibody, approximately 10^7 cloned hybridoma cells were injected intraperitoneally in BALB/c mice which had been primed 2 weeks before with an intraperitoneal injection of 0.5 ml of 2,6,10,14-tetramethylpentadecane (pristane). Ascites fluid was collected after 5 to 7 days, and lipoproteins were precipitated by the addition of 1 ml of 5% sodium dextran sulfate to each 20 ml of ascites fluid. After 1 h at 4°C, 1 ml of 11.1% $CaCl_2$ was added to each 20 ml of dextran sulfate-treated ascites fluid, and the mixture was again maintained at 4°C for 1 h before centrifuging at 10,000 × *g* for 60 min. Immunoglobulins were then precipitated from the clear supernatant fluid by the addition of an equal volume of saturated $(NH_4)_2SO_4$ (pH 7.4). Precipitated immunoglobulins were collected by centrifugation and dissolved in water, followed by dialysis against 10 mM Tris (pH 8.0). Protein concentration was determined by absorption at 280 nm by using an extinction coefficient of 1.4 per mg of protein.

Iodination of ascites immunoglobulin. Approximately 10 mg of $(NH_4)_2SO_4$-precipitated ascites fluid (pH 8.0) was passed over a column (1 by 10 cm) of protein A linked to Sepharose CL-4B (Sigma Chemical Co.). After thorough washing with 0.14 M phosphate buffer (pH 8.0), the absorbed immunoglobulin was eluted by lowering the pH to 6.0, 4.5, or 3.0 as described by Ey et al. (7). Iodination was accomplished by placing 100 μl of purified immunoglobulin eluted from the protein A-sepharose column (~100 μg of protein) into a tube that had been coated with 10 μg of Iodo-Gen (Pierce Chemical Co., Rockford, Ill.). Forty microcuries of carrier-free $Na^{125}I$ (New England Nuclear Corp., Boston, Mass.) was added, and the reaction was allowed to proceed for 15 min at room temperature. The mixture was desalted by passage through a Sephadex G-25 column (1 by 10 cm) which had been equilibrated with Dulbecco phosphate-buffered saline (pH 7.4). The void volume, which contained the desalted, iodinated immunoglobulin, was made 1% with respect to γ-globulin-free horse serum and used for the competition experiments described below.

Competitive binding of monoclonal antibodies. To ascertain which monoclonal antibodies bound to the same or similar antigenic determinants on the G protein, radioimmune assays were carried out in which 0.1 mg of each unlabeled monoclonal antibody per 100 μl was mixed with 30,000 to 40,000 cpm of a specific ^{125}I-labeled antibody. This mixture was then added to a polyvinyl RIA well in which 1 μg of G protein had been adsorbed to the plastic surface. After 3 h, the wells were thoroughly washed, excised with a hot wire, and counted as described above. Those unlabeled monoclonal antibodies that reduced binding to G protein of ^{125}I-labeled monoclonal antibody by 90 to 100% were assumed to be recognizing an identical determinant or one close enough to sterically hinder the binding of the ^{125}I-labeled antibody.

Neutralization assays. Serial twofold dilutions of sterile $(NH_4)_2SO_4$-precipitated ascites fluid (0.4 mg/ml) were mixed with an equal volume (0.3 ml) of buffer containing 300 PFU of Indiana VS virus; after interaction for 1 h at room temperature, 0.2-ml samples were plated in duplicate on 60-mm plates of L-cell monolayers. After 1 h each plate was covered with 6 ml of Eagle basal medium (BME) containing 1% agar. After an additional 48 h, 6 ml of BME containing 1% agar and 0.003% neutral red was added, and viral plaques were counted 12 to 14 h later.

RESULTS

Characterization of monoclonal antibodies secreted by different hybridomas. Spleen cells from BALB/c mice immunized with G protein were fused with SP2/O myeloma cells. After screening the resulting hybridoma clones, we selected those producing antibody demonstrable by RIA with whole intact VS virions firmly attached in wells of microtiter plates. Of these, 19 recloned

hybridomas were selected for producing ascites in BALB/c mice. These immunoglobulins were tested for specificity of binding to purified G protein. As nonspecific controls, the same immunoglobulins were tested for binding to purified M protein (Table 1). In almost all cases, some ^{125}I-labeled rabbit anti-mouse F(ab)$_2$ was present in association with M protein, which we interpret as nonspecific binding. Quite clearly, however, the immunoglobulin of two ascitic fluids with relatively high titers of G antibody (i.e., clone 9 and clone 17) showed more radioactivity in the wells containing M protein. One possible explanation for this finding is that our original G-protein immunogen contained traces of M protein and that these two hybridomas secrete antibody directed to M-protein determinants. However, this would not explain the marked cross-reactivity with G protein of these two unique monoclonal antibodies; hybridoma clones 9 and 17 react with a G-protein antigenic determinant different from that of our other 17 hybridomas (see Table 3). Although never described previously to our knowledge, VS viral G and M protein may share some antigenic determinants. Dietzschold et al. (5), however, were not able to detect cross-reactivity between VS viral purified G and M protein with their respective hyperimmune rabbit antisera by immunodiffusion or complement fixation, tests which are less sensitive than the RIA used in our studies. The immunoglobulins secreted by the 17 hybridomas that could be tested were all immunoglobulin G (IgG) (Table 2). The predominant isotype was IgG1, but the IgG2a and IgG2b isotypes are well represented.

TABLE 1. Specificity of binding to VS viral G and M protein by monoclonal antibodies from 19 hybridoma clones[a]

Clone no.	^{125}I-labeled rabbit anti-mouse F(ab)$_2$, cpm bound to 1 μg of:	
	G protein	M protein
1	7,058	313
2	7,848	401
3	4,522	44
4	7,158	297
5	5,935	380
6	6,732	967
7	5,600	469
8	1,692	327
9	6,383	9,744
10	5,110	430
11	2,177	359
12	9,628	937
13	6,951	292
14	4,582	422
15	5,571	367
16	6,664	292
17	6,714	10,564
18	5,967	385
19	7,213	1,626

[a] As described in the text, RIAs were carried out with a 1:200 dilution of an $(NH_4)_2SO_4$-precipitated ascites fluid (containing 0.4 mg of protein per ml); 50 μg was added to wells to which 1 μg of either G or M protein had been absorbed. Counts were corrected by subtracting control counts from wells to which no ascites fluid had been added.

TABLE 2. Comparative VS virus neutralization titers with antibody in ascitic fluid obtained from the intraperitoneal injection of hybridomas into BALB/c mice[a]

Clone no.	Plaque neutralization titer[b]	Isotype[c]
1	16	IgG1
2	<2	ND[d]
3	8	IgG1
4	<2	ND
5	<2	IgG1
6	16	IgG2a
7	<2	IgG2a
8	<2	IgG1
9	<2	IgG2b
10	128	IgG1
11	<2	IgG2a
12	128	IgG2b
13	32	IgG1
14	<2	IgG1
15	64	IgG1
16	64	IgG1
17	<2	IgG2b
18	8	IgG2a
19	<2	IgG2a

[a] All ascitic fluid was clarified, and the antibodies were precipitated with 50% saturated $(NH_4)_2SO_4$ as described in the text. The $(NH_4)_2SO_4$ precipitate was dissolved in 12 mM Tris (pH 7.4) to yield a final concentration of 0.4 mg of protein per ml.

[b] Values represent the reciprocal of the dilution of antibody causing a 50% reduction in plaque formation.

[c] Isotypes were determined by double diffusion of hybridoma cell lysates (as described in the text) and class-specific immunoglobulin antisera.

[d] ND, Not determinable.

Comparative RIA titers and neutralization titers of the hybridoma immunoglobulins. It was of interest to determine the comparative ability of the 19 hybridoma immunoglobulins to bind antigen and to neutralize the infectivity of VS virus. To this end, an $(NH_4)_2SO_4$-precipitated immunoglobulin from ascitic fluid was tested for its neutralizing activity against VS virus plated on L-cell monolayers. Table 2 shows a comparison of the dilution endpoints for 50% neutralization of VS virus. Ten of the 19 anti-G ascitic fluids did not neutralize VS viral infectivity despite very high titers by the RIA (Table 1). Also, there was no obvious correlation between immunoglobulin binding to antigen and its capacity to neutralize viral infectivity; for exam-

ple, ascitic fluid from clones 2 and 4 possess higher RIA titers than most neutralizing antibodies, yet they completely failed to neutralize VS virus. The anti-G ascitic fluids, which contained immunoglobulin capable of neutralizing VS virus, varied in their neutralization titer from 8 to 128.

Specificity of antigenic determinants for each monoclonal antibody. The 19 monoclonal antibodies were classified on the basis of shared versus independent antigenic determinants by means of competitive binding of paired monoclonal antibodies to purified G protein. In each test an excess of one unlabeled immunoglobulin was assayed for its capacity to inhibit binding to G protein of another immunoglobulin labeled with ^{125}I. When binding of ^{125}I-labeled immunoglobulin was inhibited by 90% or greater, the two immunoglobulins were considered to share the same (or closely adjacent) antigenic determinants. The G protein of VS virus possesses at least 11 different antigenic determinants (Table 3). Only four of these, however, are involved in viral neutralization as a result of binding their specific monoclonal antibody. Interestingly, different monoclonal antibodies that apparently bind to the same determinant vary considerably in their ability to neutralize infectivity. This might reflect differences in antibody class (e.g., clones 6 and 10 or clones 16 and 18) or differences in the avidity with which the antibody binds to its determinant or might merely indicate that they bind to adjacent determinants which sterically block each other and vary in their ability to neutralize infectivity.

TABLE 3. G-protein antigenic determinants and neutralization activity of monoclonal antibodies derived from 19 hybridoma clones[a]

Determinant no.	Clone no.[b]	Neutralizing activity[c]
1	6, 10, 12, 15	+
2	1, 16, 18	+
3	13	+
4	3	+
5	2, 4	0
6	9, 17	0
7	8, 14	0
8	5	0
9	7	0
10	11	0
11	19	0

[a] Antigenic determinants (arbitrarily numbered) were identified by competitive inhibition of binding to purified G protein of ^{125}I-labeled monoclonal antibody by each of the 19 unlabeled monoclonal antibodies. Antibody clones were classified in the same antigenic determinant if the unlabeled antibody inhibited the binding of the ^{125}I-labeled antibody by 90 to 100% in pair-wise competitive binding experiments. All antibodies binding to determinants 1, 2, 3, and 4 resulted in neutralization; no other monoclonal antibodies neutralized viral infectivity.

[b] See Table 1 for clone numbers.

[c] See Table 2 for neutralization titers.

Comparative kinetics of VS virus neutralization by different monoclonal antibodies. A customary method for determining the neutralizing potency of antiviral sera is to test the rate at which they neutralize infectivity. This kinetic analysis has been a standard procedure for quantitating serum antiviral activity. We used such a procedure to compare the kinetics of VS virus neutralization by anti-G mouse serum with that of monoclonal G antibody present in ascitic fluids of two non-cross-reacting hybridomas, clone 15, clone 16, and clones 15 and 16 combined (Tables 2 and 3). One milliliter of VS virus containing 6×10^5 PFU was rapidly mixed with an equal volume of $(NH_4)_2SO_4$-precipitated ascitic fluid containing a total of 0.4 mg of protein per ml or a mixture of the two antibodies each with 0.2 mg of protein per ml. These mixtures were incubated at 37°C; at various intervals thereafter, 0.1 ml was removed and diluted in 1% γ-globulin-free horse serum to stop the neutralization reaction. Various dilutions were then plated on monolayer cultures of L cells, and plaques were counted after incubation for 72 h.

Figure 1 shows similar initial rates of VS virus neutralization by anti-G mouse serum and monoclonal G antibody to levels of 90% plaque reduction during the first minute. However, anti-G mouse serum continued its exponential neutralization of virus to undetectable levels of infectivity by 10 min, whereas each monoclonal antibody exhibited biphasic kinetics of viral neutralization. Antibody from clone 15 neutralized VS virus more efficiently than did antibody of clone 16, but the mixture of antibodies of clones 15 and 16 exhibited a steeper slope, showing a more enhanced neutralization in the secondary phase than did antibody of clone 15 alone, even though total protein concentration was the same for all monoclonal antibody neutralization reactions. These data suggest a synergistic neutralization action of clone 15 mixed with clone 16 monoclonal antibodies, each of which reacts with independent antigenic determinants.

The similar initial rate of VS virus neutralization by anti-G mouse serum and clone 15 antibody raised the question of whether a limiting amount of monoclonal antibody was the causal factor in divergence from linearity of virus neutralization by clone 15 antibody. To test this possibility, clone 15 ascitic fluid was mixed with 6×10^5 PFU of VS virus, and infectivity was scored by plaque assay during incubation at room temperature for 30 min. Residual neutralized and unneutralized virus was removed from this antibody-virus mixture by centrifugation at

50,000 × g for 90 min. The supernatant fluid was then mixed with a separate fresh sample of 6×10^5 PFU of VS virus, and the plaque neutralization was tested a second time. Figure 2 reveals similar biphasic kinetics of VS virus neutralization for the two successive incubations of virus with the same immunoglobulin preparation. The lesser degree of overall plaque reduction during the second neutralization can be explained by twofold-greater dilution of the antibody. These results strongly suggest that clone 15 monoclonal antibody does not contain two populations of neutralizing antibody with different antigen-binding affinities.

Another possible explanation for biphasic kinetics of VS virus neutralization by monoclonal antibody is the existence of two virus populations, one of which might have fewer or modified antigenic determinants in its G protein. This possibility was tested by recloning from picked plaques of VS virus that had survived neutralization by a 30-min incubation with clone 15 monoclonal antibody (Fig. 2). Two separate recloned preparations of surviving virus and the original wild-type virus were grown to the same titer, and the kinetics of their neutralization by the same clone 15 antibody was compared in parallel experiments. After 1 min, 29% of wild-type virus survived as compared with 33 and 37% survivors for plaques 1 and 2, respectively (data not shown). These results argue against a hypothesis of two virus populations with antigens differentially susceptible to neutralization by a single monoclonal antibody.

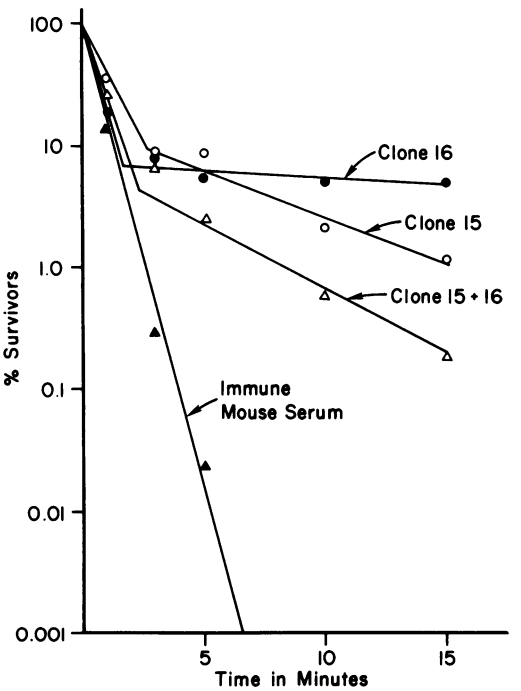

FIG. 1. Comparative kinetics of VS virus neutralization by immune mouse serum, clone 15 antibody, clone 16 antibody, and a mixture of clone 15 and clone 16 antibodies. All neutralization experiments with monoclonal antibodies were carried out with ascitic fluids containing a total of 0.4 mg of ammonium sulfate-precipitated protein per ml. Neutralization with immune mouse serum was carried out with a 1:20 dilution of the serum. As described in the text, 0.3 ml of twofold dilutions of antibody was mixed with an equal volume of buffer containing 300 PFU of VS virus. Surviving virus was titered at intervals after incubation by plating on L-cell monolayers.

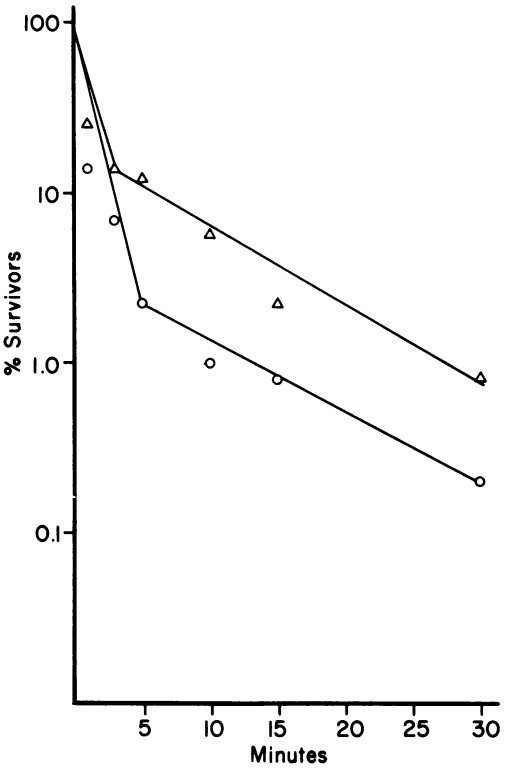

FIG. 2. Comparative kinetics of VS virus neutralization by successive incubations with the same clone 15 monoclonal antibody with two separate preparations of VS virus. A 1:4 dilution of clone 15 ascites fluid was incubated with VS virus, and neutralization kinetics was determined as in Fig. 1. Neutralized and nonneutralized virus were then removed after incubation for 30 min by centrifugation at 50,000 × g. The same clone 15 ascitic fluid (diluted 1:2) was tested for its capacity to neutralize fresh VS virus under the same conditions. Symbols: ○, plaque titers of virus mixed with fresh clone 15 antibody; △, plaque titers of fresh virus mixed with the same ascites fluid after removing the original virus by centrifugation. One-minute neutralizations were 85 and 75% for the two successive assays.

Role of antibody concentration on the kinetics of viral neutralizations. The results of our neu-

tralization studies with monoclonal antibodies and whole immune mouse serum suggest that perhaps the biphasic neutralization curve is merely a reflection of an equilibrium between bound and free antibodies. To test this hypothesis, whole immune mouse serum and monoclonal antibodies from hybridoma clone 15 were each tested at various concentrations to determine the role of antibody concentration on the kinetics of VS viral neutralization. The results of these experiments (Fig. 3) support the conclusion that the biphasic curve with its unneutralized fraction results from an equilibrium between bound and free antibodies.

DISCUSSION

The process by which antibody neutralizes viral infectivity remains a vague concept despite the number of different mechanisms that have been proposed to explain this phenomenon. Some reports have provided data supporting a single-hit process, whereby viral infectivity is lost after the interaction of a virion with a single molecule of antibody (6, 8, 10, 12, 19). Other investigators have presented convincing arguments suggesting a multihit requirement for neutralization to occur (4, 11, 29). Still other studies have concluded that the efficacy of viral neutralization varies with the isotype or avidity of the neutralizing antibody (16). A number of investigations have also provided evidence that some antibodies merely sensitize the virus and that neutralization by such antibodies requires a second mediator, such as complement or antiglobulin (1, 13, 18, 20, 27).

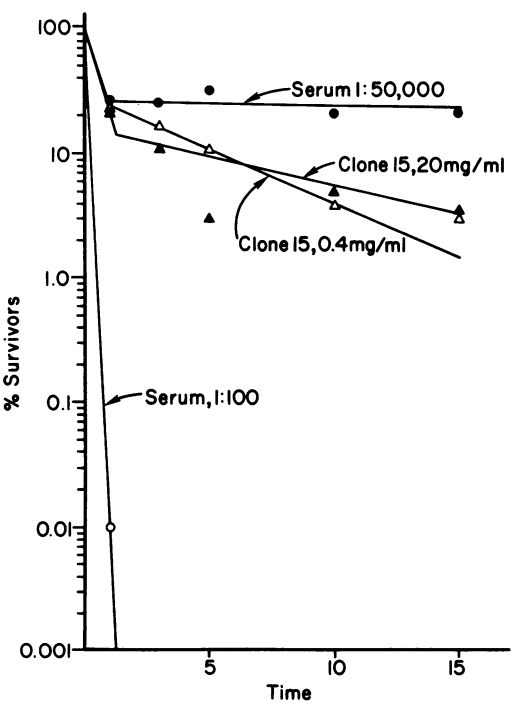

FIG. 3. Comparative kinetics of VS virus neutralization by immune serum and clone 15 ascites fluid. Immune serum had a protein concentration of 60 mg/ml and was tested at dilutions of 1:100 (○) and 1:50,000 (●). Clone 15 ascites fluid was tested at protein concentrations of 20 mg/ml (▲) and 0.4 mg/ml (△). Kinetic neutralization of 300 PFU of VS virus was performed as in Fig. 1 and 2.

It would seem likely that different antibodies binding to different viruses would show such a spectrum of effects, but it is difficult to formulate a generalized mechanism of antibody neutralization with a heterogeneous antibody mixture induced by immunization of an animal with whole virus or even single viral components; inevitably, such sera contain a number of isotypes and antibodies with various avidities as well as multiple antibody specificities. Most general theories of antibody-mediated neutralization invoke either a steric hindrance resulting from antibody blocking of critical sites on the surface of a virion or a molecular perturbation of the viral capsid after interaction with an antibody.

The data reported here resulted from the use of monoclonal antibodies capable of binding to the VS viral G protein, which contains at least 11 antigenic determinants. Each antibody is of a single isotype, possesses a single avidity, and binds to a single antigenic determinant. Of these, nine were shown to neutralize VS viral activity by binding to four different, non-cross-reacting determinants. This is in contrast to the report by Massey and Schochetman (21), who reported that only a single antigenic determinant on mouse mammary tumor virus was involved in antibody-mediated neutralization. Flamand et al. (9), however, report multiple antigenic determinants on rabies and rabies-like viruses, but their characterization of antigenic determinants is based on the ability of their monoclonal antibodies to cross-react with or neutralize various strains of rabies or rabies-like viruses; these experiments were not designed to evaluate exactly how many different binding sites are actually involved in viral neutralization. However, Flamand et al. (9) do estimate a total of two to nine different antigenic determinants for the various strains of virus used, a figure not at variance with what we have determined for VS virus.

The divergence from linearity in the neutralization curves with monoclonal antibodies is characteristic for viral neutralization curves reported by a large number of investigators using conventional antiserum (10, 14, 16, 17, 26). This divergence has been attributed to antibody heterogeneity in which nonneutralizing or poorly

bound antibodies compete with neutralizing antibodies. Although this explanation may well be correct when using a heterogeneous immune serum, it fails to explain similar results obtained using monoclonal antibodies as shown here in Fig. 1 and 2. The fact that the kinetics of neutralization of virus grown from plaques picked from virus surviving into the late part of the neutralization curve was identical to that of the stock virus indicates that the deviation from linearity is not a result of virus heterogeneity. Thus, the nonlinearity of our monoclonal antibody neutralization curves cannot be explained by the presence of low-avidity antibodies, interfering heterologous antibodies, or competing, nonneutralizing antibodies. Two possible causes can be invoked: (i) the presence of small virus aggregates which could serve as a barrier for antibody penetration, thereby protecting internal virions from reacting with neutralizing antibody; or (ii) an equilibrium between free and bound antibody in which the release of antibody from the viral surface approaches the rate of antibody molecules binding to the virion glycoprotein. The former hypothesis seems unlikely since immune whole mouse serum can rapidly neutralize all infectious virus. The results shown in Fig. 3 could, however, be interpreted as resulting from an equilibrium between bound and free antibodies, particularly if multiple antibodies must bind to effect neutralization. It is surprising that a 50-fold increase in monoclonal antibody concentration caused essentially no change in the biphasic neutralization curve, but it may be that a large number of any one specific monoclonal antibody must bind to a virion to effect neutralization and that an equilibrium between bound and free antibody is quickly established. In contrast, immune mouse serum surely possesses a multiplicity of different antibodies, and even though each antibody may establish its equilibrium between the free and bound state, it would be much more likely that the multiple determinants involved in neutralization would not affect the same antibody-binding equilibrium at any one time.

Our results strongly suggest that multiple neutralizing antibodies reactive with different antigenic sites on G protein exert a synergistic effect on the neutralization of VS virus. We are continuing our studies on this aspect of neutralization since a similar situation with other viruses would greatly influence the value of synthetic vaccines containing only single antigenic determinants.

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