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Neutralization of Newcastle Disease Virus by Monoclonal Antibodies to the Hemagglutinin-Neuraminidase Glycoprotein: Requirement for Antibodies to Four Sites for Complete Neutralization

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The neutralizing characteristics of monoclonal antibodies directed to four antigenic sites on the hemagglutinin-neuraminidase glycoprotein of Newcastle disease virus were determined. Neutralization by each antibody resulted in a persistent fraction of nonneutralized virus which varied from 1 to 17% depending on the hemagglutinin-neuraminidase site recognized, but not on the antibody. The addition of antibodies to all four sites on the hemagglutinin-neuraminidase glycoprotein was required to give a level of neutralization comparable with that obtained with polyclonal mouse antiserum. The high persistent fractions were not due to viral aggregates, a high level of variants in the virus stock, the use of insufficient antibody, low antibody avidity, or an effect peculiar to the use of the chicken cells as host. The addition of rabbit anti-mouse immunoglobulin to the persistent fraction left by any of the antibodies resulted in a further reduction in infectivity, often by as much as two logs. Thus, some viral particles are capable of binding antibody while retaining their infectivity. The implications of these findings to the mechanism of neutralization are discussed.

In spite of numerous studies (for reviews, see references 6–8, 10, 22, and 28), we remain without a clear understanding of the mechanism(s) of neutralization of animal viruses by virus-specific antibody. Still to be answered is an important aspect of the problem, namely, the number of antibody molecules required to neutralize the infectivity of a single virion. Can neutralization be effected by a single antibody molecule or is it a multihit phenomenon?

The one-antibody hypothesis is based chiefly on kinetic considerations—inactivation of viral infectivity by antibody proceeds with first-order kinetics (12), suggesting that a single-antibody molecule can neutralize a virus particle. However, there is considerable evidence from several other approaches suggesting that more than one antibody is required (7). The recent development of monoclonal antibody technology (19) has provided a new approach which may ultimately lead to a resolution of this question.

Newcastle disease virus (NDV) is the prototype of the paramyxovirus genus of the *Paramyxoviridae* (17) and was used extensively in the earlier studies on neutralization (11, 26, 27). Monospecific but polyclonal antibodies to both the hemagglutinin-neuraminidase (HN) and fusion (F) glycoproteins of paramyxoviruses have been shown to possess neutralizing capabilities (23). We have prepared a panel of monoclonal antibodies specific for the HN glycoprotein of NDV and, in a previous report (16), have shown by competition antibody-binding radioimmunoassays that these antibodies delineate four antigenic sites on the HN spike. These sites are recognized, respectively, by immunoglobulin G antibodies $HN1_a$, $HN1_b$, and $HN1_c$ (site 1), $HN2_a$ and $HN2_b$ (site 2), $HN3_a$ (site 3), and $HN4_a$, $HN4_b$, and $HN4_c$ (site 4). Antibodies to each site also exhibit specific neutralizing capacities characteristic of that site. Furthermore, neutralization by antibodies to the different sites is additive to the extent that the additive neutralization profile corresponds exactly to the epitope map determined by competition antibody-binding assays and thus confirms these site assignments (16).

The fact that antibodies to each site complement those to the other sites in neutralization assays is certainly suggestive of a multiple-antibody mechanism of viral neutralization. Herein, we have expanded upon this initial finding and have demonstrated (i) that although the vast majority of viral particles bind antibody, only a certain fraction characteristic of each site is neutralized and (ii) that the binding of antibodies to all four sites on HN is required to give the same level of neutralization as polyvalent mouse serum.

MATERIALS AND METHODS

Virus. The Australia-Victoria (1932) (AV-WT) strain of NDV was grown in the allantoic sac of 10-day-old embryonated hen eggs at 37°C from a stock of virus that was one egg passage from cloning (3). After the death of the majority of the embryos, allantoic fluid was harvested and filtered through 0.2-μm filters (Acrodisc; Gelman Sciences, Inc., Ann Arbor, Mich.) before storage at −70°C. For some experiments, virus was purified from unfiltered allantoic fluid as described previously (5, 30).

Cells. Primary and secondary chicken embryo cells were prepared and maintained in standard medium as described previously (3, 14). Confluent secondary cultures (24 to 48 h after plating) in 60-mm tissue culture dishes were used for plaque titrations.

Chinese hamster ovary (CHO) cells were maintained in spinner culture as previously described (24). Vero cells, maintained in minimal essential medium and 10% fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.), were kindly provided by F. Ennis. Both cell types were plated in 60-mm tissue culture dishes and used at confluency for plaque assays.

Hybridomas and monoclonal antibodies. Preparation,

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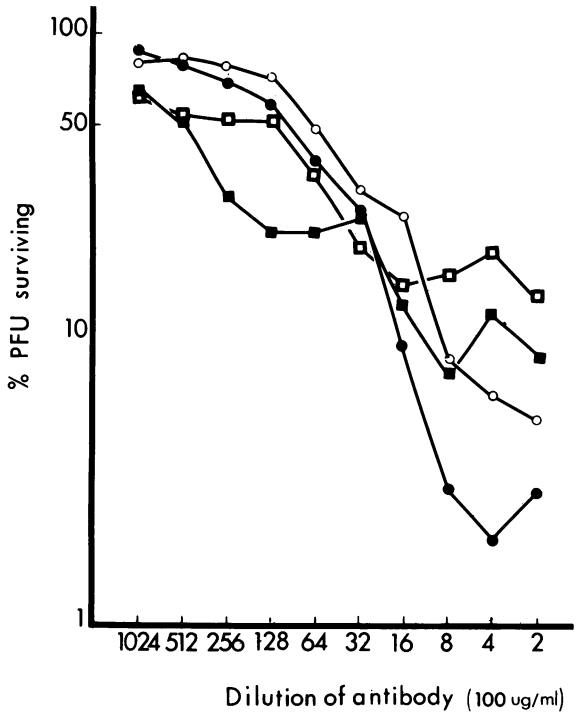

FIG. 1. Neutralizing activity of monoclonal antibodies determined by endpoint dilution assay. Monoclonal antibody (from ascites fluid) was diluted in HBSS to a concentration of 100 μg/ml. Twofold serial dilutions from these preparations were then incubated for 1 h at 25°C with ca. 100 PFU of purified AV-WT virus. Samples (0.2 ml) were plated in duplicate, and the plaque assays were performed as described in the text. The data from one antibody specific for each of the four HN sites are shown: $HN1_b$ (●), $HN2_a$ (○), $HN3_a$ (■), and $HN4_a$ (□). Anti-NP served as a control and was not neutralizing at any concentration (data not shown).

screening, and cloning of hybridomas, purification of antibodies from ascites fluids, and initial characterization of monoclonal antibodies have all been described previously (16). The monoclonal antibody concentration in each ascites preparation was determined by comparing the amount of ^{125}I-labeled second antibody bound in a solid-phase radioimmunoassay (16) to that bound by an affinity-purified preparation (9) of one of the anti-HN monoclonal antibodies (32). Initially, ascites fluids were heat inactivated at 56°C for 30 min to deplete complement, but since no difference was seen in neutralization assays, this treatment was discontinued.

Polyclonal mouse and rabbit sera. BALB/c mice were immunized as described previously (16), except that in some instances the intravenous boost was omitted.

Neutralization assays. For the measurement of neutralizing activity by the method of limiting dilution, purified AV-WT virus (10^3 PFU/ml in Hanks balanced salt solution [HBSS; GIBCO Laboratories]) was mixed with an equal volume of twofold serial dilutions of ascites fluid previously adjusted to an antibody concentration of 100 μg/ml. After a 1-h incubation at 25°C, 0.2-ml samples were plated in duplicate on 60-mm plates of chicken embryo cells. After adsorption for 45 min, cells were washed with warm HBSS, and plaque assays were performed as previously described (3).

For kinetic studies of neutralization and accurate determination of the persistent fraction of nonneutralized virus, AV-WT virus (5×10^6 to 1×10^7 PFU/ml) was incubated with an equal volume of ascites fluid (diluted to 50 μg/ml in HBSS). Neutralization was stopped by serial 10-fold dilution in ice-cold HBSS. With chicken embryo cells, plaque assays were performed as described above. With CHO and Vero cells, the protocol was the same, except that the overlay was minimal essential medium containing 7.5% fetal calf serum, 2% nonessential amino acids, 10 mM HEPES buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [pH 7.8]), 0.13% $NaHCO_3$, and 0.9% agar. All medium components were obtained from GIBCO Laboratories. Antibodies to the nucleocapsid protein (NP) were used as a control in all neutralization experiments. Rabbit anti-mouse immunoglobulin (RAM) was obtained from Litton Bionetics (Kensington, Md.).

RESULTS

Comparing neutralizing activities of anti-HN monoclonal antibodies. The standard procedure used by most laboratories to compare neutralizing efficacies of antibodies, monoclonal and polyclonal alike, is an endpoint dilution assay in which 100 PFU of virus is treated with twofold serial dilutions of the antibody preparation; the dilution yielding a 50% reduction in infectivity provides a measure of the antibody neutralization titer. An example of such an experiment for monoclonal antibodies to the four neutralizing sites on HN identified thus far (16) is shown in Fig. 1. The percentage of virus surviving antibody treatment is plotted against the concentration of antibody in ascites fluid (all adjusted to an initial antibody concentration of 100 μg/ml), rather than simply against the dilution of ascites fluid. This standardization to antibody concentration eliminates the possibility that observed differences in neutralization might be due to differences in antibody concentration. By this criterion, the specific neutralizing activity of antibody $HN3_a$ is only slightly greater than that of $HN4_a$, and it is four- to eightfold greater than that of $HN1_b$ and $HN2_a$. Results with nine antibodies directed to the four sites on HN suggest that the specific neutralizing activity may be site specific (Table 1).

However, further examination of Fig. 1 shows that this type of comparison, even when made as a function of antibody concentration, does not provide a true reflection of the neutralizing capabilities of these antibodies: neutraliza-

TABLE 1. Neutralizing activity of anti-HN monoclonal antibodies

Antibody	Specific neutralizing activity[a]	Persistent fraction[b] (%)
$HN1_a$	1,280	1.6 ± 0.9
$HN1_b$	640	2.7 ± 0.8
$HN1_c$	640	0.9 ± 0.6
$HN2_a$	640	5.3 ± 2.0
$HN2_b$	640	3.4 ± 1.2
$HN3_a$	2,560	6.4 ± 2.1
$HN4_a$	2,560	13.8 ± 4.7
$HN4_b$	2,560	7.6 ± 2.9
$HN4_c$	5,120	16.9 ± 2.7

[a] The reciprocal of the highest dilution of antibody (on a milligrams per milliliter basis) giving a 50% reduction in plaque number relative to anti-NP control.

[b] Values are the mean ± standard deviation of 10 to 20 determinations for each antibody.

tion obtained with increasing concentrations of each antibody is not proportional to that measured as specific neutralizing activity. Indeed, at high antibody concentrations, $HN1_b$, rather than showing one of the lowest neutralizing activities, actually causes the greatest neutralization; $HN4_a$, which appears to be one of the more potent neutralizers when measured by specific neutralizing activity, is actually the least potent as measured by the persistent fraction of virus surviving antibody treatment.

The greater neutralizing activity of antibody $HN1_b$ and the lower efficacy of $HN4_a$ are shown very dramatically in Fig. 2, where the kinetics of neutralization by antibodies to the four sites have been measured at a saturating (see Fig. 4) antibody concentration of 50 μg/ml. $HN4_a$ leaves a persistent fraction of ca. 15%, whereas antibody $HN1_b$ leaves a persistent fraction which is a log lower. Persistent fractions for all nine anti-HN monoclonal antibodies measured under conditions of antibody excess are also shown in Table 1. There are clear site-specific differences between the persistent fractions obtained with antibodies to each site. Antibody $HN1_c$, despite its relatively low specific neutralizing activity, is by far the most potent neutralizer of infectivity, as measured by its 0.9% persistent fraction. Antibodies $HN2_a$, $HN2_b$, and $HN3_a$ give intermediate levels of neutralization, leaving persistent fractions of 3.5 to 6.5%. Treatment of virus with antibodies $HN4_a$ or $HN4_c$ reduced infectivity to only ca. 15% of the control. Thus, comparison of the neutralizing capability of these antibodies on the basis of specific neutralizing activity is very misleading because of the limitations imposed by the use of a 50% neutralization point as a measure of antibody titer. The only exception to the site-specific level of neutralization is the slightly lower persistent fraction after treatment with antibody $HN4_b$ relative to that achieved with other antibodies to the same site (Table 1). This might suggest that antibody $HN4_b$ is directed to a different epitope within the same site as $HN4_a$ and $HN4_c$.

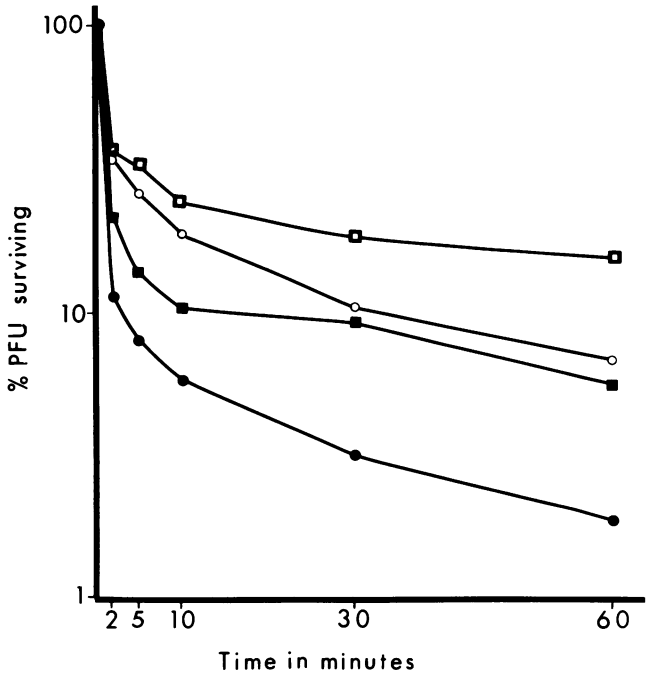

FIG. 2. Kinetics of neutralization. Monoclonal antibodies were diluted to 50 μg/ml in HBSS and mixed at 25°C with an equal volume of allantoic fluid from AV-WT-infected eggs (5×10^6 to 1×10^7 PFU/ml). Samples (0.1 ml) were removed at the time intervals shown and diluted in 0.9 ml of cold HBSS to stop the reaction. After serial dilution in cold HBSS, 0.2 ml of the appropriate dilutions were plated in duplicate, and the plaque assay was performed as described in the text. The data shown are for antibodies $HN1_b$ (●), $HN2_a$ (○), $HN3_a$ (■), and $HN4_a$ (□). Anti-NP again served as the control and was not neutralizing (data not shown).

Possible explanations for high persistent fractions in neutralization assays. A number of possible causes for the phenomenon of high persistent fractions in neutralization assays can be envisioned. These involve each of the three components in the neutralization assay system: virus, antibody, and the host cell.

(i) Variation in the virus stocks? Although our virus stocks are only two passages from cloning (3), it is possible that high persistent fractions left after treatment of virus with anti-HN monoclonal antibodies are due to the presence of a high percentage of variants which can escape neutralization. To explore this possibility, virus was picked from those plaques which were formed after treatment individually with an antibody to each of the four sites. These were then grown in embryonated eggs and retested for neutralizability. In every instance, treatment of this virus preparation with the same antibody resulted in neutralization at the same rate and with the same persistent fraction as the original virus (data not shown). Thus, as one might have expected in light of its magnitude, the persistent fraction is not due solely to genetically stable nonneutralizable variants present in our cloned virus stocks. Of course, this experiment does not rule out the existence of phenotypically variant particles in our stocks.

(ii) Aggregation in the virus stock? It is also possible that persistent fractions can be attributed to the presence of aggregates in our virus stocks. The presence of preexisting aggregates in our virus stock was ruled out by the use of allantoic fluid preparations routinely filtered through 0.2-μm filters before treatment with antibody as described above. Virus capable of passing through these filters showed identical neutralization kinetics and persistent fractions as unfiltered virus (data not shown).

(iii) Virus with higher affinity for host cell receptor than antibody (i.e., neutralizing antibody of low affinity)? We have previously shown that the avidities of these antibodies differ very little (16). However, this analysis was performed in a binary system measuring the avidity of antibody binding to virus in a solid-phase radioimmunoassay (16). We had not determined the effect of introducing a third variable, the host cell, into the system. If virus had a higher affinity for host cell receptors than for antibody and if the virus-antibody binary complex were constantly dissociating and reassociating, one might expect the persistent fraction of virus surviving treatment with antibody to increase gradually with adsorption time on the host cell monolayer. We therefore compared the kinetics of infection by virus treated with anti-HN and anti-NP (control) antibodies. The results of this experiment are shown in Fig. 3A; PFU are plotted as a function of increasing adsorption time for virus which has been treated with antibody $HN4_b$ or the control antibody. In both cases, nearly maximal infection is found within 10 min of exposure to the host cell. Moreover, the percentage of infectivity remaining after neutralization relative to the non-neutralized control hovers around 20% at all adsorption times tested, especially near 30 min, which is the normal protocol for our experiments (Fig. 3B). This provides a strong argument against either dissociation or avidity as the sole cause of the persistent fraction.

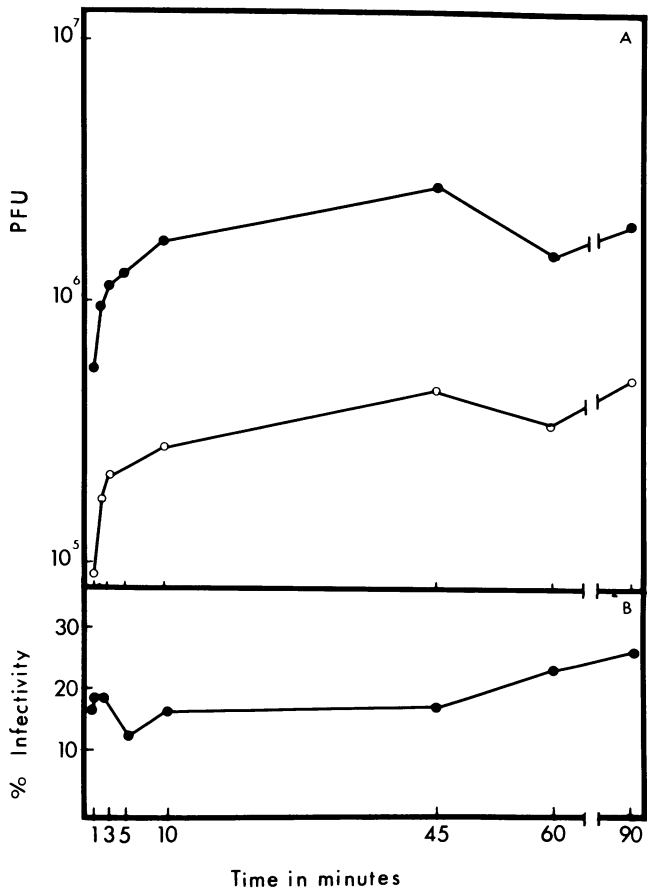

FIG. 3. Infectivity of virus treated with anti-HN and anti-NP (control) monoclonal antibodies as a function of adsorption time. Virus and antibody, at the same concentrations as those shown in the legend to Fig. 2, were incubated for 60 min at 25°C. These mixtures were then serially diluted, and 0.2-ml samples were plated in duplicate as before. After various adsorption periods, the plates were washed with 5 ml of warm HBSS and immediately overlaid. (A) Infectivity as a function of adsorption time after treatment with antibody NP2 (●) or $HN4_a$ (○). (B) Percent infectivity of the $HN4_a$-treated virus relative to the NP2-treated control.

Another consideration here is the host cell itself. Perhaps the phenomenon we are seeing is peculiar to the chicken cell as viral host in the plaque assay, rather than being a property of the virus, antibody, or virus-antibody complex. A possible complication might, for instance, be the presence of Fc receptors on chicken cells which could act as receptors for the virus-antibody complex, resulting in enhancement of infectivity. In fact, Kliks and Halstead (18) have demonstrated that chicken embryo monolayers are composed of ca. 2% functionally active mononuclear phagocytes, which can engulf immune complexes and become infected. To examine this possibility, we compared the infectivity of virus neutralized by each of our antibodies on different cell types. The persistent fraction of nonneutralized virus assayed on CHO and Vero cells compared with that on chicken cells is shown in Table 2. In no instance is the persistent fraction significantly lower with either CHO or Vero cells than it is with chicken cells, and with some antibodies, especially those to site 1, it is even higher on Vero cells. Thus, both untreated and antibody-treated virus is at least as infectious on two other cell types as it is on chicken cells. This suggests that the high persistent fractions cannot be attributed to the use of chicken cells as host, at least to the extent that similar results are obtained with cells from different organisms. (Vero cells are derived from monkey kidneys and CHO cells from hamster ovaries.) Moreover, monoclonal antibodies to Sindbis (4) and West Nile (25) virus enhance infectivity in an Fc receptor-bearing cell line but not in Vero cells, strongly suggesting that Vero cells lack Fc receptors.

(iv) Antibody sufficiency? Another potential cause of high persistent fractions is the use of limiting antibody concentrations in neutralization assays (29). To test this possibility, virus and antibody were mixed and incubated and then assayed for infectivity at various times. Additional antibody or additional virus was added after the persistent fraction of nonneutralized virus had been established. Examples of this for one of the weakest ($HN4_c$) and one of the strongest ($HN1_c$) neutralizing antibodies are shown in Fig. 4. The addition of more antibody did not reduce infectivity any further. In contrast, virus added in a second round was neutralized to the same extent as virus added at the outset. Both of these results indicate that antibody was present in excess.

(v) Lack of an important neutralizing antibody? We have presented evidence that antibodies to four different sites on the surface of the HN molecule neutralize the virus but in the process leave high persistent fractions. It is by no means certain that these are the only neutralizing sites on the HN glycoprotein. Since we have previously demonstrated the additivity of antibodies to pairs of sites, an indirect approach to this question involves the comparison of the neutralization achieved by a mixture of antibodies to these four sites with that achieved with polyvalent mouse anti-NDV serum. Filtered allantoic fluid (300 μl) from AV-WT-infected eggs was mixed with an equal volume of 1/10-diluted serum or a mixture of monoclonal antibodies ($HN1_b$ plus $HN2_b$ plus $HN3_a$ plus $HN4_c$) to four different sites on the HN glycoprotein. Incubation was for 60 min at 25°C before plating on chicken cells in the plaque assay. Values are relative to those for a sample incubated with HBSS. The percent PFU surviving after treatment with mouse anti-NDV sera was 0.076 ± 0.055 (mean ± standard deviation for serum samples from five animals); the percent PFU surviving after treatment with the mixture of antibodies was 0.091. These similar persistent fractions suggest that our panel of neutralizing anti-HN monoclonal antibodies encompasses all those present in the polyvalent mouse sera. However, we must stress that this does not speak to the role of anti-F antibodies in the neutralization of NDV since we cannot demonstrate the

TABLE 2. Persistent fractions with CHO and Vero cells

Antibody	Persistent fraction (%) in:		
	Chicken cells	CHO cells	Vero cells
$HN1_a$	0.3	0.6	5.4
$HN1_b$	2.4	3.7	13.7
$HN1_c$	0.4	3.1	6.7
$HN2_a$	2.7	5.7	7.1
$HN2_b$	2.8	9.3	5.6
$HN3_a$	7.8	4.9	14.9
$HN4_a$	15.1	13.5	16.1
$HN4_b$	9.5	12.0	10.8
$HN4_c$	18.9	15.2	17.2

presence of these antibodies in BALB/c mouse serum by immunoprecipitation (data not shown).

Nonneutralized virus has bound antibody. Having demonstrated that there is a high level of virus surviving treatment with a saturating amount of a neutralizing antibody, we next wanted to ascertain whether virus in the persistent fraction has bound antibody. The addition of a second antibody specific for a nonneutralizing first antibody has long been known to bring about an enhanced level of neutralization (1, 13). We therefore used further neutralization by RAM as an assay for the presence of bound antibody on the virus in the persistent fraction. Figure 5 shows an example of such an experiment, in which the persistent fraction of virus surviving neutralization by antibody $HN4_c$ was diluted and treated with RAM. The addition of RAM gave a further reduction in the persistent fraction, the magnitude of which depended on the concentration of second antibody. Similar treatment of the persistent fractions left by other antibodies with RAM also gave greatly enhanced neutralization, ranging from two to three logs, depending on the antibody (Table 3), whereas normal rabbit serum did not neutralize (data not shown). As before, the addition of more of the initial antibody also was not neutralizing. Similar results were obtained with complement, except that, unlike RAM, it was able to neutralize virus without prior addition of antibody (data not shown), probably due to the virucidal effect described by Welsh (31). Thus, a very large percentage of the original virus is able to bind otherwise neutralizing antibody and still retain its infectivity.

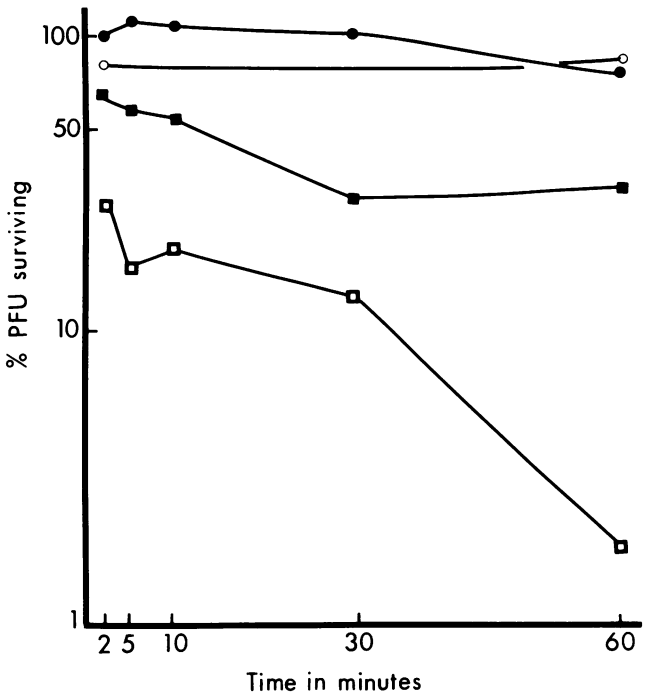

FIG. 5. Addition of RAM to the persistent fraction left after treatment with antibody $HN4_c$. The persistent fraction of $HN4_c$-treated virus was established as described in the legend to Fig. 2 and diluted 1/50 in HBSS. This was then incubated at 25°C with more $HN4_c$ (●), normal rabbit serum (○), or RAM diluted by a factor of 1/500 (■) or 1/100 (□). Samples (0.1 ml) were removed at the time intervals shown and plated at the appropriate dilution (or undiluted) in duplicate.

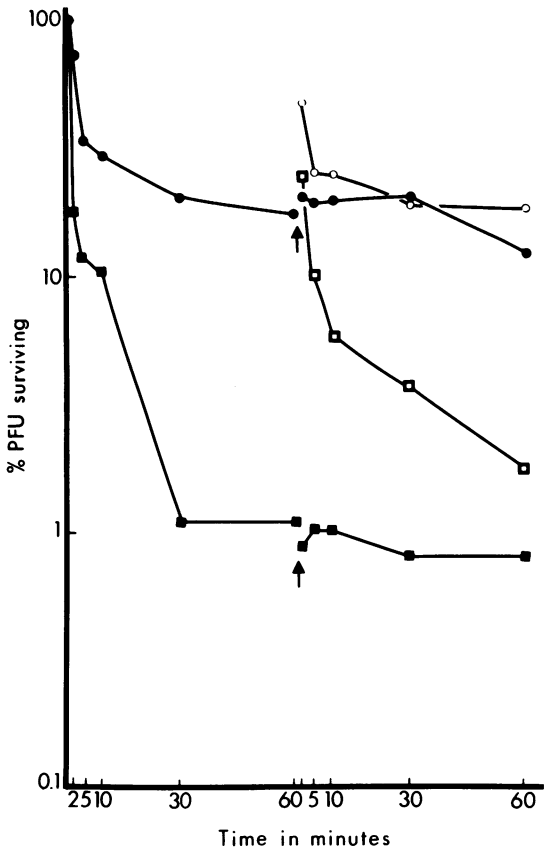

FIG. 4. Addition of more virus or more antibody to $HN4_c$ and $HN1_c$ persistent fractions. The persistent fractions after treatment with antibodies $HN4_c$ (●) and $HN1_c$ (■) were established (after 60 min) as described in the legend to Fig. 2. The $HN4_c$-virus mixture was mixed (indicated by arrows) with an equal volume of $HN4_c$ (●) or more virus (○) at the same concentrations added initially, and samples were taken at the time intervals shown. The data shown are corrected for the twofold dilution factor resulting from the second addition of antibody or virus. The $HN1_c$-virus mixture was treated in the same way with either more $HN1_c$ (■) or more virus (□).

DISCUSSION

We have presented data that raise several important questions about the mechanism of neutralization of NDV. Monoclonal antibodies to any of four previously identified antigenic sites on the HN glycoprotein of NDV are able to neutralize virus only to an apparently site-specific extent. These persistent fractions of nonneutralized virus range from ca. 1% (site 1) to as high as 17% (site 4) compared with the less than 0.1% persistent fraction left by polyclonal mouse serum. We have eliminated several potentially artifactual explanations for failure to neutralize virus completely: (i) the presence of a high level of variants, (ii) preexisting aggregates in the virus stock, (iii) low antibody avidity, (iv) the presence of Fc receptors on the chicken embryo fibroblast used as the host cell in the plaque assay, and (v) the use of insufficient antibody. We also feel that we have isolated monoclonal antibodies to all the important neutralizing sites on the HN spike recognized by BALB/c mice because treatment with a mixture of antibodies to all four sites gives a level of neutralization comparable with that obtained with polyclonal BALB/c mouse anti-NDV serum. This strongly suggests that our panel of neutralizing antibodies encom-

TABLE 3. Neutralization of persistent fractions with antiimmunoglobulin[a]

Antibody	% Survival	
	With more of same antibody	With RAM
$HN1_a$	47.8	0.7
$HN1_b$	91.8	1.8
$HN1_c$	45.2	1.7
$HN2_a$	72.5	0.2
$HN2_b$	68.0	0.2
$HN3_a$	75.3	0.1
$HN4_a$	70.4	0.1
$HN4_b$	42.5	0.1
$HN4_c$	95.0	0.1

[a] Virus (5×10^6 to 5×10^7 PFU/ml) was treated with the antibodies shown (50 μg/ml) for 60 min at 25°C. After a 1/50 dilution, the mixture was incubated as before with a 1/20 dilution of either RAM or more of the same antibody. Infectivity was assayed in the plaque assay. The percent survival is relative to that after treatment with an anti-NP monoclonal antibody in the second round. Antibodies to the NP protein, RAM, and normal rabbit serum were not neutralizing by themselves.

passes all those present in polyclonal mouse serum. Whether BALB/c mouse anti-NDV serum recognizes all of the neutralizing sites on the F glycoprotein, as well as the HN glycoprotein, that are recognized by genetically different mice is presently under investigation.

Treatment of the persistent fraction left by any of our antibodies with RAM gives a further two- to three-log reduction in infectivity in every case. Thus, our task is to explain the apparent anomaly that all the viral particles bind monoclonal antibody, yet as many as 1 to 15% of them, depending on the site specificity of the antibody, retain their infectivity and the fact that antibodies to four antigenic sites are required for complete neutralization.

The evidence for a single-hit model for neutralization of enveloped animal viruses is based primarily on kinetic observations (12). The initial phase of the neutralization curve shows an immediate exponential inactivation of viral infectivity. Although our data also show this (Fig. 2), our first sample was taken 2 min after the addition of antibody to virus. Whether our findings are really supportive of single-hit kinetics or simply reflect the limitations of our ability to measure the rapid kinetics of the system is unclear. Lafferty (20) has demonstrated a lag in the neutralization of influenza virus with polyvalent serum when the neutralization reaction is run at low temperature.

Evidence for a multiple-antibody mechanism of neutralization has been gathered in several systems with both polyclonal and monoclonal antibodies (reviewed by Della-Porta and Westaway [7]). Granoff (11, 12) showed that phenotypically mixed particles of two strains of NDV leave high persistent fractions of nonneutralized virus after treatment with antisera to either strain, but neutralization is essentially complete if antisera to both strains are used in tandem. Bradish et al. (2) found with foot-and-mouth-disease virus that the infectivity surviving in equilibrated virus-antibody mixtures is due to complexes of virus and antibody. These infectious virus-antibody complexes—"sensitized" virus—were further characterized by the demonstration that the addition of anti-gamma globulin can neutralize them (1, 13) to the point that one can now use this system as an assay for bound antibody. More recently, monoclonal antibodies to Sindbis virus glycoprotein E1 have been shown to both neutralize and enhance virus infectivity, depending on the antibody concentration (4). The enhancement of infectivity is mediated by the presence of Fc receptors on the host cell. We feel that enhancement of infectivity does not apply in the NDV-chicken cell system because in fluorescence assays with uninfected cells, antibody added to the cells is not detected either at the surface or inside the cell (data not shown) and because persistent fractions were found on other cell types (Table 2), including Vero cells, which do not bear Fc receptors (4, 25).

Although we are aware of the pitfalls inherent in comparing neutralization mechanisms in nonenveloped and enveloped viruses, we feel that our data is consistent with that recently obtained with nonenveloped viruses. Perhaps the most convincing evidence for a multihit model for neutralization comes from studies with a monoclonal antibody to poliovirus which gives first-order neutralization kinetics but which requires an average of four bound antibodies per virion to neutralize the virus (15). This contrasts with the antibody-induced cooperative transition in the poliovirus capsid proposed by Mandel (21) to explain single-hit kinetics.

In this report, we have presented evidence supporting a multihit model for the neutralization of an enveloped virus, NDV. A site-specific percentage of virus retains its infectivity despite having antibody bound to it. It may be that although all viral particles can indeed bind antibody, only a certain percentage of it is bound bivalently and only then does it cause neutralization. One may even invoke the existence of phenotypic variants in our virus stock having different amounts of the HN glycoprotein. We are presently exploring these theories through the isolation and use of site-specific variants which escape neutralization and the use of monovalent Fab fragments in neutralization assays.

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