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Monoclonal Antibodies to Newcastle Disease Virus: Delineation of Four Epitopes on the HN Glycoprotein

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Eighteen independent hybridomas producing monoclonal antibodies to Newcastle disease virus have been prepared by fusion of SP2 cells with spleen lymphocytes from a BALB/c mouse immunized with intact UV-inactivated Newcastle disease virus strain Australia-Victoria. They have been divided into three groups on the basis of radioimmunoprecipitation, infected cell surface and cytoplasmic fluorescence, and isotype. The anti-HN group is made up of nine antibodies which give surface fluorescence on infected cells and immunoprecipitate the HN glycoprotein. These antibodies bind to HN in nitrocellulose transfers of sodium dodecyl sulfate gels, but only if it has been neither reduced nor boiled. To varying degrees, all of these HN antibodies neutralize infectivity. These results suggest that they recognize exposed determinants of a conformational nature on the native HN molecule. They have been used in competition antibody-binding radioimmunoassays and additive neutralization assays, and on the basis of these studies the epitopes they recognize have been subdivided into four domains, two of which are overlapping, on the HN glycoprotein. The relatively weaker neutralizing activity observed with some of these antibodies cannot be explained by lower avidities for their epitopes because there is not an inverse correlation between estimated binding constant and neutralizing activity. The four antibodies in the second group all give a predominantly cytoplasmic fluorescence pattern, immunoprecipitate the nucleocapsid protein, and bind to nucleocapsid protein in nitrocellulose transfers of reduced and nonreduced sodium dodecyl sulfate-polyacrylamide gels. All five of the antibodies in the third group are of the immunoglobulin M class, unlike the others which are all immunoglobulin G antibodies. Members of this group show variable fluorescence patterns, but none is able to immunoprecipitate or bind to a specific viral antigen transferred to nitrocellulose paper from sodium dodecyl sulfate-polyacrylamide gels.

Newcastle disease virus (NDV) is a prototype of the paramyxovirus group of enveloped negative-stranded RNA viruses which also includes measles, mumps, respiratory syncytial, and the parainfluenza viruses. Six NDV-specific polypeptides have been identified (3, 5), including three which are associated with the RNA genome: the nucleocapsid protein (NP), the phosphoprotein (P) (25, 35), and the largest NDV polypeptide (L). There are also three membrane-associated polypeptides. Two of the latter are glycoproteins which exist as spikes protruding from the surface of the virion, a larger glycoprotein possessing both viral hemagglutinating and neuraminidase activities (HN) and a somewhat smaller one which is required for cell-fusing activity (F). The third membrane-associated viral protein (M) is a non-glycosylated structural component of the inner surface of the envelope.

Previous studies have shown for other paramyxoviruses that polyvalent antisera directed against HN inhibit hemagglutination and neuraminidase, whereas those to F inhibit hemolysis (23, 27, 28, 33); both have also been shown to neutralize infectivity (22). These studies used polyvalent sera either to virions or purified viral proteins, but the development by Köhler and Milstein (15) of hybridoma technology has made possible the production of large quantities of antibodies having specificity for individual epitopes of proteins.

Recent monoclonal antibody analyses of the surface glycoproteins of enveloped negative-stranded RNA viruses such as the HA of influenza virus, (12, 14, 42), the HN of Sendai (29)

and parainfluenza viruses (43), and the G of vesicular stomatitis virus (19) have all demonstrated the existence of four operationally nonoverlapping domains on the surface of these molecules.

We have undertaken the production of a panel of monoclonal antibodies to the proteins of NDV with special interest in the membrane-associated proteins; thus far we have 18 hybridomas, most of which produce monoclonal antibodies with specificity for either the HN or NP proteins. We have characterized these antibodies and used those to the HN glycoprotein in competitive antibody-binding radioimmunoassays (RIAs) and additive neutralization studies to delineate four determinants on this molecule, two of which are partially overlapping.

MATERIALS AND METHODS

Virus. The Australia-Victoria (AV-WT) strain of NDV was grown in the allantoic sac of 10-day-old embryonated hen's eggs at 37°C from a stock of virus one passage from cloning. After the death of the majority of the embryos, allantoic fluid was harvested, and virus was purified by differential centrifugation through sucrose density gradients as previously described (6, 40). Protein was determined by the method of Lowry et al. (21).

Cell culture. Primary and secondary chicken embryo cells were prepared and maintained in standard medium as described previously (2, 13). Chinese hamster ovary (CHO) cells were grown in Spinner culture as previously described (24) or, for fluorescence studies, on acid-cleaned cover slips at 37.5°C in modified Eagle minimal essential medium.

Immunization. Virus used as immunogen was diluted to 50 μg/ml in Dulbecco phosphate-buffered saline (PBS) (7) and inactivated by a 15-min exposure to UV irradiation from a Sylvania G15T8 germicidal lamp at a distance of 78 cm (31). A BALB/c mouse was primed intraperitoneally with 5 μg of intact NDV AV-WT in complete Freund adjuvant and boosted 1 month later with the same immunogen, but in incomplete adjuvant. One week later and 3 days before sacrificing for the fusion, it was given a second boost intravenously with intact virions in PBS.

Hybridoma cell lines. The preparation of hybridomas and large amounts of monoclonal antibodies were done according to the procedures, and with the assistance, of L. Edward Cannon. A suspension of spleen cells from an immunized mouse was treated with a sterile solution of 9 parts 0.16 M NH_4Cl and 1 part 0.17 M Tris-hydrochloride (pH 7.2) for 10 min at room temperature to destroy erythrocytes. The nonimmunoglobulin-producing SP2/0-Ag14 cell line (34) was maintained in complete fusion medium (Dulbecco modified Eagle medium [DMEM; with 4.9 g of glucose per liter] supplemented with 20% fetal calf serum [GIBCO Laboratories, Grand Island, N.Y.], 10% NCTC-109 [M.A. Bioproducts, Bethesda, Md.], sodium pyruvate [1 mM], nonessential amino acids [0.1 mM; GIBCO], glutamine [4 mM], and gentamicin [50 μg/ml]). After being washed in serum-free medium, spleen lymphocytes and SP2 cells were combined at a ratio of 5:1 and the mixture was centrifuged at 1,000 rpm for 10 min. The cell pellet was loosened by flicking the bottom of the tube; 0.3 ml of 30% polyethylene glycol 1000 in DMEM (adjusted to pH 7.2 with HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid]) was then added. The suspension was mixed gently and centrifuged again for 6 min. After an overall exposure to polyethylene glycol of 8 min, 10 ml of serum-free medium was added in small amounts, taking care not to disrupt the cell pellet. Cells were pelleted again and incubated in 5 ml of complete DMEM for 1 h at 37°C in a 5% CO_2 atmosphere. Cells were gently dispersed in a 40-ml total volume of complete DMEM, seeded at 2 drops per well into 96-well flat-bottom plates (Costar; M.A. Bioproducts), and incubated at 37°C. After incubation for 24 h, approximately 100 μl of hypoxanthine-aminopterin-thymidine medium (20) was added. Cells were fed with hypoxanthine-aminopterin-thymidine medium every 3 to 4 days until 14 days after the fusion, when aminopterin was omitted from the medium.

RIA. Virions were disrupted by incubation at room temperature for 15 min in PBS (7.7 mM K_2HPO_4, 2.35 mM KH_2PO_4, 8.5% NaCl) supplemented with 1 M KCl and 0.5% Triton X-100 and were then diluted with 9 volumes of PBS. To each well of a microtiter plate (Dynatech Laboratories, Alexandria, Va.) was added 1 μg of disrupted virion protein, using a Titertek 12-channel Multipette (Flow Laboratories, McLean, Va.). After being allowed to dry overnight, plates were washed and blocked by a 1-h incubation with 2% agamma horse serum (AgHS) (GIBCO). Wells were adsorbed with 25 μl of hybridoma supernatant for 2 h at 37°C. After being washed, wells were adsorbed with ^{125}I-labeled rabbit anti-mouse kappa antibody (kindly provided by Robert T. Woodland) for 3 h at 37°C, washed vigorously with tap water, and dried. Wells were cut off and placed in carrier tubes for counting in a gamma counter.

Fluorescence. For assay of membrane fluorescence, CHO cells in Spinner culture were infected at a multiplicity of infection of 5. At 6 h postinfection, 5×10^5 cells were incubated with hybridoma supernatant followed by a 1/40 dilution of fluorescein-conjugated goat anti-mouse immunoglobulin G (IgG), IgM, and IgA (Cappel Laboratories, Cochranville, Pa.) (26).

For cytoplasmic fluorescence, CHO cells seeded on cover slips were infected as above, fixed with ether-ethanol, and stained (41).

Cloning hybridomas. Hybridomas were cloned in Sea Plaque semisolid agarose (FMC Corp., Marine Colloids, Rockland, Maine). Briefly, a bottom layer of 0.5% agarose in DMEM supplemented with 20% newborn calf serum was overlaid with a suspension of cells in 0.38% agarose in complete DMEM. Clones (apparent after 2 weeks) were assayed for antibody production and expanded.

Immunoprecipitation. For the preparation of NDV-infected cell lysates, cells were labeled with [^{35}S]methionine at 5 h postinfection for 1 h, and 2×10^6 cells were lysed in 0.3 ml of NET buffer containing 0.5% Nonidet P-40 (4). [^{35}S]methionine-labeled, infected cell lysates were incubated with hybridoma supernatant, and immune complexes were precipitated with rabbit anti-mouse immunoglobulin beads (Bio-Rad Laboratories, Rockville Centre, N.Y.).

Polyacrylamide gel electrophoresis. Virion proteins and immunoprecipitates were resolved by electropho-

resis in 10% sodium dodecyl sulfate (SDS)-polyacrylamide slab gels (16) under either reducing or nonreducing conditions. The gels were dried and exposed to Kodak Royal X-Omat film.

Gel transfers to nitrocellulose. Virion proteins (25 μg per track) were electrophoresed in SDS-polyacrylamide gels as described above. After presoaking for 2 h in 25 mM Tris (pH 8.3)–192 mM glycine–50 mM NaCl–7 M urea, proteins were transferred electrophoretically to nitrocellulose filters (0.45-μM pore size; Millipore Corp., Bedford, Mass.) for 18 h at 250 mA and 6 V in a transfer buffer composed of 25 mM Tris (pH 8.3), 192 mM glycine, and 150 mM NaCl (38). Additional protein-binding sites were blocked with 20% AgHS, and strips were incubated overnight at 4°C with 15 to 20 μg of antibody per ml. After three washes with AgHS, strips were reacted for 2 h with 10^6 cpm of ^{125}I-labeled rabbit anti-mouse kappa antibody per ml, washed repeatedly with PBS containing 2% AgHS and 0.5% Nonidet P-40, dried, and exposed overnight at −70°C to Kodak X-Omat film, using an intensifying screen to detect bound ^{125}I-labeled rabbit anti-mouse kappa antibody (L. E. Cannon, personal communication).

Isotype determinations. Monoclonal antibody isotypes were determined by Ouchterlony double immunodiffusion gels, using isotype-specific antisera (Litton Bionetics, Kensington, Md.), and a cell lysate was made from approximately 10^7 hybridoma cells in 0.2 ml of 0.5% Nonidet P-40 in PBS. Wherever possible, the determinations were verified by a solid-phase RIA, using isotype-specific reagents (kindly provided by R. T. Woodland).

Ascites fluids. For the induction of ascites tumors, BALB/c mice were primed with an 0.5-ml intraperitoneal injection of pristane followed by a second injection 10 days later. After 3 to 5 days, mice were injected with 5×10^6 to 1×10^7 hybridoma cells, and fluid was collected beginning 7 to 10 days later. Ascites fluids were clarified by sodium dextran sulfate and ammonium sulfate precipitations (39) and stored at −70°C in small amounts.

The monoclonal antibody concentration in each ascites preparation was estimated by comparing the amount of ^{125}I-labeled second antibody bound in the RIA to that bound by an affinity-purified preparation (8) of one of the anti-HN monoclonal antibodies (43).

Neutralization assays. NDV AV-WT (10^3 PFU/ml in Hanks balanced salt solution) was mixed with an equal volume of hybridoma supernatant. After a 1-h incubation at 37.5°C, 0.2-ml aliquots were plated in duplicate on 60-mm plates of chicken embryo cells. After adsorption for 45 min, cells were washed with warm Hanks balanced salt solution and plaque assays were performed as described previously (2).

Additive neutralization assays. For additive neutralization assays, NDV AV-WT (5×10^6 PFU/ml) was incubated for 90 min at 25°C with an equal volume of one monoclonal antibody (50 μg/ml in Hanks balanced salt solution). Neutralization was stopped by serial 10-fold dilution of the mixture in ice-cold Hanks balanced salt solution. This virus-antibody mixture was then incubated under the same conditions with a second antibody. Plaque assays were performed as described above.

Radioiodination of monoclonal antibodies. Monoclonal antibodies were purified from ascites fluids by a batchwise method, using protein A-Sepharose (A. Portner, personal communication). The immunoglobulin-protein A-Sepharose complex in 0.1 M phosphate (pH 8.1) was cleared by centrifugation, and bound immunoglobulin was eluted by suspension in 3 bed volumes of 0.5 M glycine–0.14 M NaCl, pH 3.0. After adjustment of the pH to neutrality with 1 M NaOH, immunoglobulins were dialyzed overnight against PBS.

For iodination of immunoglobulins, 50 μg of affinity-purified antibody was reacted for 5 min with 1 mCi of Na^{125}I (Amersham Corp., Arlington Heights, Ill.) and two IODO-BEADS (Pierce Chemical Co., Rockford, Ill.) in 0.2 M PO_4 buffer (pH 7.2). Unreacted ^{125}I was removed by passage of the mixture through a 1.5-ml packed column of Ag-X-8 resin. The column was rinsed with 20% AgHS, and the final volume was made up to 10 ml with the same.

Competition antibody-binding assay. Microtiter wells coated with 1 μg of intact virions were reacted for 2 h at 37°C with 50 μl of serial 10-fold dilutions of a 1-mg/ml preparation of competing antibody. This was followed by 50 μl of ^{125}I-labeled antibody at a concentration of 2 μg/μl (40,000 to 80,000 cpm) for 3 h at 37°C. Wells were processed as described previously.

RESULTS

Immunoprecipitation, fluorescence, and isotypes. Spleen lymphocytes from a BALB/c mouse immunized with intact, UV-inactivated, egg-grown NDV were fused with SP2 cells. After selection with hypoxanthine-aminopterin-thymidine medium, foci were screened in the solid-phase RIA. Subsequently, RIA-positive cultures were screened by immunofluorescence on live and fixed NDV-infected CHO cells, resulting in the isolation of 18 hybridomas specific for NDV. Figure 1 shows an autoradiogram of a reduced SDS-polyacrylamide gel of the proteins immunoprecipitated by these antibodies from [^{35}S]methionine-labeled, infected CHO cell lysates. Each of the immunoprecipitation-positive antibodies can be seen to precipitate a single band of NDV protein. On the basis of these experiments the antibodies have been divided into three groups (Table 1): anti-HN antibodies (9), anti-NP antibodies (4), and IgM antibodies of undetermined specificity (5).

The anti-HN antibodies have been grouped on the basis of their specificity for the HN glycoprotein as determined by their ability to immunoprecipitate HN from infected cell lysates (Fig. 1). (The immunoprecipitation of HN by antibody $HN4_b$ in Fig. 1 [lane 10] was rather weak, which was not usually the case.) As expected, these antibodies give strong surface fluorescence on live infected cells, as well as, to a lesser degree, cytoplasmic fluorescence on fixed cells. Moreover, each is a member of the IgG class of antibodies with a marked predominance of the IgG2a subclass (Table 1).

The anti-NP antibodies precipitate only the NP protein, consistent with their predominantly

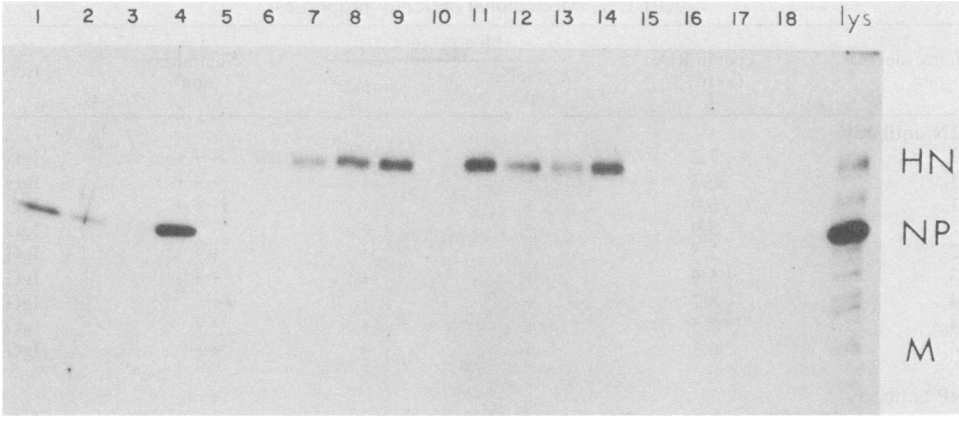

FIG. 1. Immunoprecipitation of NDV proteins by hybridoma supernatants. [^{35}S]methionine-labeled, infected CHO cell lysates were subjected to immunoprecipitation with hybridoma supernatants sampled 3 days after feeding. The immune complexes were collected on rabbit anti-mouse immunoglobulin beads (Bio-Rad Laboratories, Richmond, Calif.), electrophoresed, and autoradiographed as described in the text. Supernatants were sampled from hybridomas, and their immunoprecipitates were electrophoresed in lanes numbered as follows: NP1 (lane 1); NP3 (lane 2); NP4 (lane 3); NP2 (lane 4); IgM1 (lane 5); $HN2_a$ (lane 6); $HN1_b$ (lane 7); $HN1_c$ (lane 8); $HN4_a$ (lane 9); $HN4_b$ (lane 10); $HN2_b$ (lane 11); $HN4_c$ (lane 12); $HN3_a$ (lane 13); $HN1_b$ (lane 14); IgM2 (lane 15); IgM3 (lane 16); IgM4 (lane 17); IgM5 (lane 18); cell lysate (lys).

cytoplasmic fluorescence pattern on NDV-infected cells (Table 1). These assignments have been verified by electrophoresis under nonreducing conditions (data not shown) where the P phosphoprotein and the F glycoprotein (which are not resolved from NP in reduced gels) migrate as a multimer near the top of the gel and above NP, respectively (25, 35). Also, all of these antibodies are of the IgG class (Table 1).

The third group of antibodies, no member of which has yet been shown to precipitate an NDV protein, is more heterogeneous than the first two. The different fluorescence patterns they present are probably indicative of differing specificities. We have designated this as the IgM group of antibodies on the basis of the fact that, unlike any of the other antibodies, they are all of the IgM class. Moreover, they all fail to immunoprecipitate any protein.

Gel transfers. The binding of monoclonal antibodies to NDV proteins separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose strips was investigated as an alternate method for determining the protein specificity of the antibodies in the IgM group and to verify the protein assignments of the anti-HN antibodies. Figure 2 shows the binding of monoclonal antibodies to virion proteins (boiled and electrophoresed under reducing conditions) as detected by an indirect technique, using ^{125}I-labeled anti-kappa as a second antibody. Each of the three anti-NP antibodies assayed in this manner (NP1, NP2, and NP4) could still detect their determinants on the NP molecule even after boiling and reduction (Fig. 2, strips 2, 3, and 6). On the other hand, the determinants of the HN glycoprotein appeared to be much more sensitive to boiling and reduction than those of NP. When six of the anti-HN antibodies were reacted with NDV proteins which had been boiled in reducing sample buffer before electrophoresis in an SDS slab gel and transfer to nitrocellulose, none was able to detect its antigenic determinant (two of these are shown in Fig. 2, strips 1 and 4). Similar results were obtained when samples were either reduced and not boiled (data not shown) or boiled under nonreducing conditions (Fig. 3, strips 2 to 4). Only when the NDV proteins were electrophoresed in a nonreducing gel buffer without prior boiling could we demonstrate the binding of anti-HN monoclonal antibodies to the nitrocellulose strip (Fig. 3, strips 6 to 8). This is clearly not a general phenomenon of NDV proteins since antibody NP4 (Fig. 3, strips 1 and 5) and others (not shown) can detect NP under any of these conditions. Under nonreducing conditions, the HN glycoprotein migrates as a dimer near the top of the gel. The heavy band above HN is the interface of the stacking and resolving gel and is probably due to a major portion of the unboiled HN glycoprotein not entering the resolving gel because of incomplete disruption of virions or the formation of aggregates.

We have also investigated the binding of some of the IgM antibodies to blots of NDV proteins separated under reducing and nonreducing conditions. Antibodies IgM3 and IgM4 give anomalous results in that under both reducing (Fig. 2, strips 7 and 8) and nonreducing (data not shown)

TABLE 1. Monoclonal antibody properties

Hybridoma supernatant	cpm in RIA ($\times 10^{-3}$)[a]	Fluorescence		Neutralization[b]	Isotype
		Cytoplasmic[c]	Surface[d]		
Anti-HN antibody					
$HN1_a$	7.2	+	+	+++	IgG2b
$HN1_b$	6.3	+	+	+++	IgG1
$HN1_c$	6.0	+	+	+++	IgG2b
$HN2_a$	5.9	+	+	−[e]	IgG2a
$HN2_b$	7.5	+	+	+++	IgG2a
$HN3_a$	5.9	+	+	++	IgG2a
$HN4_a$	8.7	+	+	+	IgG2a
$HN4_b$	5.5	+	+	++	IgG2a
$HN4_c$	6.3	+	−	++	IgG2a
Anti-NP antibody					
NP1	8.6	+	±	−	IgG2a
NP2	7.9	+	−	−	IgG2a
NP3	8.7	+	±	−	IgG1
NP4	5.3	+	±	−	IgG1
IgM antibody					
IgM1	6.6	+	−	−	IgM
IgM2	6.4	−	±	−	IgM
IgM3	7.7	−	±	+	IgM
IgM4	6.4	−	−	+	IgM
IgM5[f]	9.1	±	+	+++	IgM

[a] Total amounts of ^{125}I-labeled anti-kappa = 43,295 cpm; growth medium = 751 cpm; mouse anti-NDV = 11,328 cpm.

[b] Hybridomas were graded on the basis of the percent PFU remaining after treatment with hybridoma supernatant: −, >90%; +, 25 to 90%; ++, 5 to 25%; +++, <5%.

[c] On fixed cells: −, none detected; ±, weak; +, strong.

[d] On live cells: −, none detected; ±, weak; +, strong.

[e] Ascites preparations of this hybridoma are neutralizing.

[f] Unstable.

conditions they seem to bind to several proteins in the nitrocellulose strip. To determine whether this binding is specific for NDV proteins, their ability to recognize proteins in infected and uninfected cells was compared. The binding of these antibodies to many bands in infected and in several, though fewer, bands in uninfected chick and CHO cell lysates (data not shown) suggests their specificity for a determinant common to several cellular proteins which is also present on one or more NDV proteins. This is in sharp contrast to the gel transfer binding specificities of the other IgM antibodies. Antibodies IgM1 (Fig. 2, lane 5) and IgM2 (not shown) bind only to viral moieties which do not enter the resolving gel, suggesting the possibility that their antigenic determinants might be defined by an aggregate of more than one viral protein.

Neutralization studies. In an effort to further characterize these monoclonal antibodies, we determined the ability of each hybridoma supernatant to neutralize the infectivity of NDV AV-WT. Table 1 shows the relative neutralizing activity of hybridoma supernatants in each group. As one would expect, treatment with the anti-NP hybridoma supernatants did not significantly reduce the number of plaques. On the other hand, treatment with four of the nine anti-HN hybridoma supernatants gave a 90% or greater reduction in plaque number, indicating that these antibodies are directed to functionally important areas of the HN molecule on the surface of the virion. Whereas antibodies $HN3_a$, $HN4_a$, $HN4_b$, and $HN4_c$ were also neutralizing, they were less so than the others. Also, although supernatants from $HN2_a$ did not appear to neutralize NDV, we have since made ascites preparations of this antibody and shown them to have a significant neutralizing titer.

As expected from the variation in fluorescence patterns observed in Table 1, we found differences in the neutralizing activities of the IgM antibodies. Hybridoma IgM1, which gave cytoplasmic fluorescence on fixed cells, and hybridoma IgM2, despite its weak surface fluorescence, were non-neutralizing. Hybridomas IgM3 and IgM4, only one of which gave weak surface fluorescence on live cells, both gave an intermediate level of neutralization. Although it is possible that the amount of neutralization

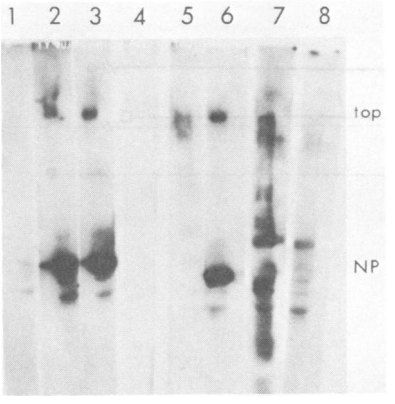

FIG. 2. Binding of monoclonal antibodies to reduced NDV proteins. Virion proteins (25 μg per track) were boiled in reducing gel buffer and electrophoresed in SDS-polyacrylamide gels as described in the text. Separated proteins were then transferred to nitrocellulose paper, which was cut into strips and reacted with ascites fluids as follows: $HN4_c$ (strip 1); NP1 (strip 2); NP2 (strip 3); $HN1_b$ (strip 4); IgM1 (strip 5); NP4 (strip 6); IgM3 (strip 7); IgM4 (strip 8). Bound antibodies were detected by reaction with ^{125}I-labeled rabbit anti-mouse kappa, and autoradiography was performed as described in the text.

obtained with these antibodies is related to their IgM isotype, there is not an absolute causal relationship between the two, since hybridoma IgM5, which later proved to be unstable, produced an IgM antibody which reduced infectivity by 99%.

Binding constants. After cloning in semisolid agarose, hybridomas were used to induce ascites tumors in mice for the production of large quantities of antibody. The concentration of monoclonal antibody in each ascites preparation was determined as described in Materials and Methods. Since it is our intention to use the anti-HN monoclonal antibodies in functional inhibition studies, we deemed it important to be certain that we would not be comparing antibodies with grossly different binding constants. We therefore used the solid-phase RIA to estimate the binding constant of each anti-HN antibody by the method of Frankel and Gerhard (9) as modified by Stone and Nowinski (36). The titration curves for several of the anti-HN antibodies are shown in Fig. 4. By comparing the maximum amount of antibody bound and the amount required to saturate the antigen, one can estimate the relative binding constants of the group of antibodies for their epitopes. In each case, antibody excess was observed at approximately 10 to 20 μg of antibody per well, and the maximum level of bound antibody was similar. Moreover, all of the other anti-HN antibodies gave binding curves within the two extremes shown here.

Competition antibody-binding assays of anti-HN monoclonal antibodies. The nine monoclonal antibodies specific for the HN glycoprotein were purified from ascites fluids, radioiodinated, and used in competition antibody-binding RIAs. An ascites preparation of each monoclonal antibody was assayed for its ability to compete for the binding of each ^{125}I-labeled antibody to microtiter wells coated with intact virions. Figure 5 shows the competition for the binding of four of the nine ^{125}I-labeled monoclonal antibodies by several (including homologous) unlabeled antibodies as a function of increasing unlabeled antibody concentration. Figure 5A shows that the binding of ^{125}I-labeled $HN1_a$ was competed for by the homologous antibody as well as by antibodies $HN1_b$ and $HN1_c$, indicating their specificity for topologically overlapping epitopes on the HN molecule. However, the binding of antibody $HN1_a$ is clearly not competed for by any of the other antibodies, indicating their specificity for other nonoverlapping determinants.

Similarly, Fig. 5D shows the competition for the binding of ^{125}I-labeled $HN4_c$ by the homologous antibody as well as antibodies $HN4_a$ and $HN4_b$. This indicates that these three antibodies bind to overlapping epitopes which are, however, distinct from those recognized by antibodies $HN1_a$, $HN1_b$, and $HN1_c$. All other antibodies shown fail to compete for the binding of the labeled antibody even at the highest concentration.

Figure 5B and C shows the percent competition for the binding of ^{125}I-labeled antibodies $HN2_b$ and $HN3_a$ by several unlabeled antibodies. Whereas the binding of ^{125}I-labeled $HN2_b$ is competed for by the homologous antibody and

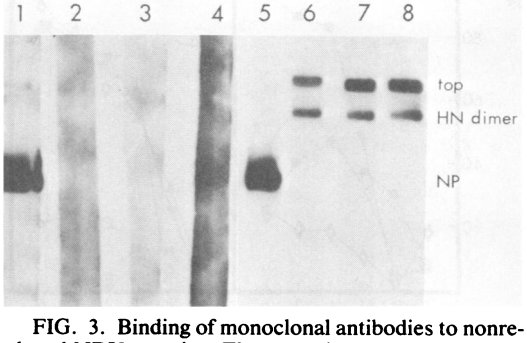

FIG. 3. Binding of monoclonal antibodies to nonreduced NDV proteins. The procedure was the same as for Fig. 2 except that 2-mercaptoethanol was omitted from the gel buffer, strips 1 to 4 contained boiled proteins, and strips 5 to 8 contained nonboiled proteins. The strips were reacted with ascites fluids as follows: NP4 (strips 1 and 5); $HN4_c$ (strip 2); $HN1_c$ (strip 3); $HN2_a$ (strips 4 and 7); $HN1_b$ (strip 6) and $HN3_a$ (strip 8).

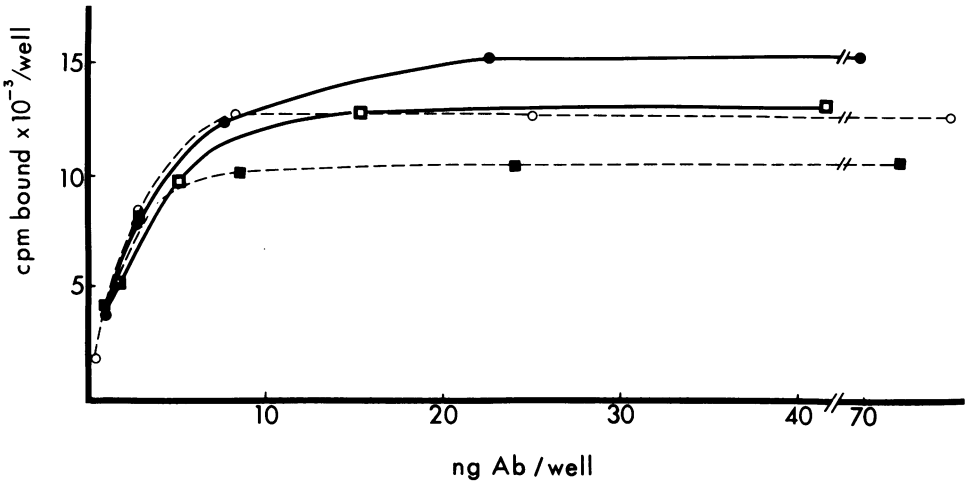

FIG. 4. Estimation of the binding constant of monoclonal antibodies in ascites fluid preparations. The concentration of monoclonal antibody (Ab) in each ascites preparation was determined as described in the text. A constant antigen concentration (1 μg per well) was titrated with an increasing amount of monoclonal antibody as described in the text for the RIA: ●, $HN2_a$; ○, $HN1_b$; ■, $HN1_c$; □, $HN4_a$. Total amount of rabbit anti-mouse kappa = 41,800 cpm.

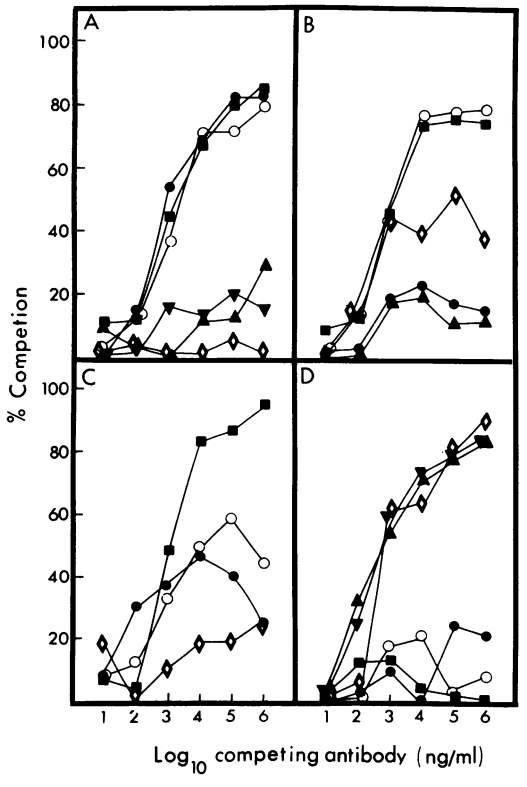

FIG. 5. Competition antibody-binding assays with anti-HN hybridomas. Microtiter wells coated with intact virions were incubated with serial 10-fold dilutions of an ascites preparation of each anti-HN hybridoma (adjusted to an initial antibody concentration of 1 mg/ml). This was followed by the appropriate ^{125}I-labeled anti-HN antibody. The data represent the percent competition by increasing concentrations of unlabeled antibodies for the binding of ^{125}I-labeled antibody relative to the control (NP4 antibody-blocked, antigen-coated wells). (A) Inhibition of ^{125}I-labeled $HN1_a$ binding by nonlabeled antibodies: ●, $HN1_a$; ○, $HN1_b$; ■, $HN1_c$; ◇, $HN2_a$; ▲, $HN3_a$; ▼, $HN4_c$. (B) Inhibition of ^{125}I-labeled $HN2_b$ binding by nonlabeled antibodies: ●, $HN1_a$; ○, $HN2_a$; ■, $HN2_b$; ◇, $HN3_a$; ▲, $HN4_a$. (C) Inhibition of ^{125}I-labeled $HN3_a$ binding by nonlabeled antibodies: ●, $HN1_b$; ○, $HN2_a$; ■, $HN3_a$; ◇, $HN4_c$. (D) Inhibition of ^{125}I-labeled $HN4_c$ binding by nonlabeled antibodies: ●, $HN1_b$; ○, $HN2_a$; ■, $HN3_a$; ◇, $HN4_a$; ▲, $HN4_b$; ▼, $HN4_c$.

antibody $HN2_a$ with similar affinity, competition to a somewhat lesser extent is also evident with antibody $HN3_a$. The converse is true with ^{125}I-labeled $HN3_a$ (Fig. 5C), whose binding is completely blocked by only the homologous antibody, whereas antibody $HN2_a$ (and to some extent $HN1_b$) gave a somewhat lower level of competition. In these studies, antibodies that compete for the binding of the ^{125}I-labeled antibody with an affinity similar to that obtained with the homologous antibody are assumed to be recognizing overlapping antigenic determinants on the HN molecule. Thus, these studies delineate four topological epitopes on the surface of the HN molecule, although epitopes 2 and 3 are apparently partially overlapping (Table 2).

Additive neutralization assays. Although all of the anti-HN monoclonal antibodies are neutralizing, each leaves a persistent fraction of non-neutralized virus characteristic of antibodies to that epitope. The comparatively high persistent

TABLE 2. Epitope assignments of anti-HN monoclonal antibodies

^{125}I-labeled antibody	Competing antibody[a]									Epitope
	$HN1_a$	$HN1_b$	$HN1_c$	$HN2_a$	$HN2_b$	$HN3_a$	$HN4_a$	$HN4_b$	$HN4_c$	
$HN1_a$	+	+	+	−	−	−	−	−	−	1
$HN1_b$	+	+	+	−	−	−	−	−	−	1
$HN1_c$	+	+	+	−	−	−	−	−	−	1
$HN2_a$	−	−	−	+	+	±	−	−	−	2
$HN2_b$	−	−	−	+	+	±	−	−	−	2
$HN3_a$	−	−	−	±	±	+	−	−	−	3
$HN4_a$	−	−	−	−	−	−	+	+	+	4
$HN4_b$	−	−	−	−	−	−	+	+	+	4
$HN4_c$	−	−	−	−	−	−	+	+	+	4

[a] +, >60% competition; ±, 50 to 60% competition; −, <50% competition.

fractions (0.4 to 13.7%) remaining after neutralization with these antibodies allows one to use additive neutralization assays to construct an operational map of the neutralizing epitopes of the HN molecule. These assays involve the neutralization of virus with a saturating amount of antibody which results in a non-neutralized persistent fraction characteristic of antibodies to that epitope followed by a second round of neutralization with another antibody. An example of the neutralization of the persistent fraction of antibody $HN4_a$ is shown in Fig. 6. As expected with a saturating amount of antibody $HN4_a$ added in the first round of neutralization, the addition of more homologous antibody gives no further neutralization. Similarly, antibody $HN4_c$ is also unable to reduce the persistent fraction of $HN4_a$. Thus, in light of this and the RIA data, these two antibodies recognize epitopes that are both topologically and operationally overlapping. The additive neutralization given in this system upon the addition of antibodies such as $HN1_c$, $HN2_b$, and $HN3_a$ indicates that they recognize epitopes which are operationally nonoverlapping with that recognized by antibody $HN2_a$.

Experiments similar to this were performed with all possible combinations of antibodies and the data are summarized in Table 3. NDV AV-WT was neutralized with the antibody shown on the left, and the persistent fraction of non-neutralized virus was subjected to another round of neutralization with each of the antibodies. The data are entirely consistent with the conclusion made from the competition antibody-binding assays in that the addition of antibody to the homologous epitope gives no significant reduction in infectivity in any case, whereas the addition of antibody specific for a different epitope does give further neutralization, indicating that the two antibodies recognize operationally nonoverlapping epitopes.

The competitive RIA demonstrated that epitopes 2 and 3 are, at least partially, topologically overlapping. Similarly, treatment of the $HN2_a$ or $HN2_b$ persistent fraction with antibody $HN3_a$ gives only a slight reduction in infectivity, whereas antibodies to epitope 2, although themselves strongly neutralizing, are able to neutralize only about 50% of the $HN3_a$ persistent fraction. Thus, these antibodies bind to epitopes that are partially overlapping in an operational sense as well.

DISCUSSION

We have described the preparation of a panel of hybrid cell lines which secrete NDV-specific monoclonal antibodies. The specificity of the antibodies, as determined by immunoprecipitation and binding to nitrocellulose blots of SDS gels, agrees well with the patterns of intracellular and membrane fluorescence obtained with these antibodies on fixed and unfixed NDV-infected CHO cells, respectively.

We have used our panel of nine anti-HN monoclonal antibodies in competition antibody RIAs and additive neutralization assays to delineate four epitopes on the surface of the HN molecule. Combining these two procedures affords a means of constructing both a topological and an operational map of HN. The binding of any of the radioiodinated antibodies to epitope 1 is competed for only by antibodies to that epitope (Fig. 5A). A similar conclusion can be drawn regarding epitope 4 (Fig. 5D), indicating that these represent topologically distinct epitopes on the surface of the HN molecule. That these antibodies give further neutralization upon addition to persistent fractions of antibodies to heterologous epitopes indicates that they are operationally nonoverlapping as well.

Epitopes 2 and 3, although nonoverlapping with respect to epitopes 1 and 4 in both competi-

tion antibody-binding and additive neutralization assays, are apparently mutually slightly overlapping in both a topological and an operational sense. This may mean that the binding of an antibody to either of these epitopes results in a slight steric hindrance to the binding of an antibody to the second epitope which is closely proximated to the first. Alternatively, the two epitopes are widely separated, but the binding of an antibody to one induces a conformational change in the second epitope such that antibodies to it do not recognize it as well. The presence of four antibody-binding sites, two of which are partially overlapping topologically, has also been demonstrated for Sendai (29) and parainfluenza 1 (43) viruses.

The predominance of hybridomas (at least 9 of 18) specific for surface glycoproteins has been seen by other investigators with viruses such as influenza (10), vesicular stomatitis (19), and measles (1, 12, 37) after immunization with intact virions. Our immunization procedure may have restricted the variety of monoclonal antibody-producing hybridomas even further; we used UV-inactivated virions as antigen, which certainly reduced the effective immunogen concentration. It appears that only when virions are modified with heat or detergent (1), the route of immunization or source of lymphocytes is varied (10), or infectious virus (19) or individual purified proteins (39) are used as immunogen is the diversity of hybridomas significantly increased.

Our difficulty, thus far, in isolating a monoclonal antibody to the F glycoprotein of NDV was not unexpected in light of the poor response to this protein found in polyvalent sera (unpublished data). Similarly, Formalin-treated paramyxovirus vaccines have failed to elicit an appreciable response to the F glycoprotein (22). Perhaps release of F and HN from the surface of the virion by detergent (such as β-D-octylglucoside) (39) and removal of HN by immunoprecipitation will render F more immunogenic.

The failure of monoclonal antibodies to immunoprecipitate any viral protein or to detect individual viral proteins transferred from an SDS slab gel to nitrocellulose has also been seen with other viruses such as measles (12) and influenza (43). Perhaps the antibody is unable to recognize its determinant once the protein on which it resides is separated from the other proteins in the virion. This is apparently the case for IgM1 and IgM2 antibodies which are able to bind only to proteins which remain at the top of the resolving gel. Precedents for this are the isolation of an antibody specific for intact Sindbis virus with no demonstrable activity against the purified viral proteins (32), an antibody whose antigenic site includes sequences of both the u1/u1c and three proteins of reovirus (18), and several neutralizing anti-poliovirus antibodies which do not recognize any separated proteins either in immunoprecipitation or gel transfers (G. Schild, personal communication).

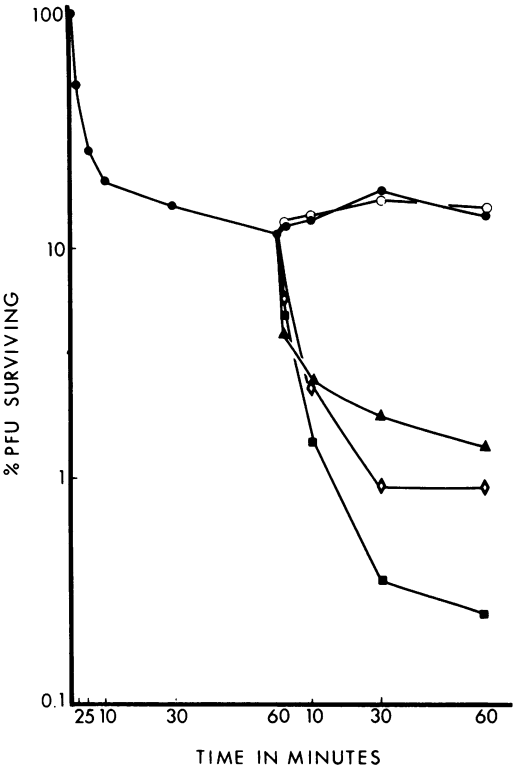

FIG. 6. Additive neutralization assay of the $HN4_a$ persistent fraction. NDV AV-WT (5×10^6 PFU/ml) was mixed with an equal volume of $HN4_a$ antibody at 50 μg/ml in Hanks balanced salt solution. Samples were taken over a 60-min period of incubation at 25°C and diluted into ice-cold Hanks balanced salt solution before plating. The virus-antibody mixture was then added to an equal volume of second antibody at the same concentration, and samples were again taken during a 60-min incubation period. The data represent the percent survival relative to antibody NP4 as a control, with the second round figures corrected for dilution. Symbols: ●, $HN4_a$; ○, $HN4_c$; ■, $HN1_c$; ◇, $HN2_b$; ▲, $HN3_a$.

The binding of antibodies IgM3 and IgM4 to several bands in nitrocellulose blots of NDV proteins as well as those of both uninfected and infected chick cell lysates is probably indicative of their specificity for common determinants on all of these proteins. With the advent of monoclonal antibody technology, such demonstrations of homologous cross-reacting determinants on unrelated antigens have become commonplace (17, 32). However, the recognition by these antibodies of several proteins even in

TABLE 3. Additive neutralization assays

1st antibody	% Infectivity remaining after second antibody[a]								
	$HN1_a$	$HN1_b$	$HN1_c$	$HN2_a$	$HN2_b$	$HN3_a$	$HN4_a$	$HN4_b$	$HN4_c$
$HN1_a$	91.0	99.5	109.0	14.5	24.8	22.4	12.1	9.5	33.3
$HN1_b$	92.6	79.4	76.5	11.5	9.1	13.2	52.9	11.3	17.1
$HN1_c$	69.4	66.7	94.4	6.4	5.5	8.1	17.5	3.9	6.4
$HN2_a$	6.3	5.2	8.0	100.0	123.0	72.6	16.0	13.5	17.1
$HN2_b$	10.7	5.3	8.4	88.4	69.5	65.3	7.6	6.9	8.3
$HN3_a$	1.1	7.7	0.9	50.4	59.8	102.4	53.5	32.3	19.7
$HN4_a$	2.6	9.8	1.5	8.9	5.1	8.0	77.8	46.7	82.2
$HN4_b$	4.1	0.7	0.8	3.8	3.2	6.8	88.1	91.9	71.9
$HN4_c$	3.2	2.6	1.2	6.2	6.8	15.6	88.7	79.6	109.0

[a] Data are relative to those with a heterologous anti-NP (NP2) antibody as second antibody.

uninfected CHO cell lysates suggests specificity for a rather ubiquitous determinant and potentially limits their usefulness as probes of NDV structure and function.

The evidence from our studies of the binding of HN-immunoprecipitating monoclonal antibodies to the HN glycoprotein in nitrocellulose blots suggests a dependence of antigenicity on disulfide bonding. This probably means that all of the HN antibodies are directed against determinants of a conformational nature rather than primary amino acid sequences. A logical extension of these two findings would appear to be that disulfide bonding is also crucial to the integrity of the functional areas of the HN molecule. The importance of interpeptide disulfide bonds for the expression of the biological activities of the Sendai virus HN glycoprotein is supportive of this caveat (30). Although it is true that one-third of these antibodies have relatively low neutralizing activities, it is also clear from the binding curves in Fig. 4 that this is not due to differences in the avidities of the antibodies; i.e., hybridoma antibody $HN1_c$, although having potent neutralizing activity, has perhaps the weakest avidity curve.

We are presently using our panel of anti-HN monoclonal antibodies to establish the functional relevance of the four epitopes they delineate and to understand the nature of the high persistent fractions of non-neutralized virus remaining after treatment of the virus with saturating amounts of these antibodies. This may have a great deal of relevance to the mechanism of neutralization of NDV.

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