JOURNAL OF VIROLOGY, Feb. 1988, p. 586–593
0022-538X/88/020586-08$02.00/0

Vol. 62, No. 2

Strain Variation and Nuclear Association of Newcastle Disease Virus Matrix Protein

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Five monoclonal antibodies to the matrix (M) protein of Newcastle disease virus (NDV) Australia-Victoria (AV) strain were generated and characterized. In competitive antibody-binding assays, the antibodies fell into three discrete groups. The antigenic sites described by these antibody groups were designated M1, M2, and M3. Each antibody reacted with a panel of NDV strains in a manner unique to its group, confirming the grouping by competitive antibody binding. Only site M1 was found on all 12 of the strains tested and may be a "pan-NDV" epitope. A large portion of the M protein of strain AV was detected in the nuclei of infected cells by all five monoclonal antibodies. In addition, the antibodies only stained the nuclei of cells infected with NDV strains expressing M protein containing the corresponding antigenic site. These results confirm that the immunoreactivity in the nucleus is actually caused by the M protein and not by a cross-reacting host protein induced by viral infection.

Newcastle disease virus (NDV), an avian pathogen, is an enveloped negative-strand RNA virus belonging to the paramyxovirus family. Of the six NDV-encoded proteins, three are associated with the genomic RNA in the nucleocapsid structure, the NP (nucleoprotein), the P (phosphoprotein), and the L (large) protein. Two others are transmembranal glycoproteins, the HN (hemagglutinin-neuraminidase) and the F (fusion) protein. The sixth viral protein is the nonglycosylated M (matrix) protein.

Immunofluorescence studies of infected cells using polyvalent antiviral serum have indicated that the viral antigens are located in the infected cell cytoplasm (20, 24). For this reason and because NDV is able to replicate in cells treated with actinomycin D (2), it has been assumed that no nuclear function is required for its replication. However, Yoshida et al. (26) were able to stain the nuclei of Sendai virus-infected cells with a monospecific antiserum raised against the M protein. More recently, Hamaguchi et al. (6) and Peeples (16) have reported that monoclonal antibodies to the M protein of NDV also stain the infected cell nucleus. Only one monoclonal antibody was used in each report, and the nuclear staining was presented without comment. Although it appears from these reports that a portion of the M protein is located in the infected cell nucleus, the possibility that NDV infection stimulates production of a host cell protein which shares an antigenic site with the M protein has not been excluded.

Here, we describe the preparation and characterization of five monoclonal antibodies to the M protein of NDV Australia-Victoria (AV) strain. The antibodies represent three distinct groups which recognize discrete antigenic sites on the M protein, as determined by the ability of each antibody to compete with others for binding to the M protein. The antigenic sites corresponding to the monoclonal antibodies were found on distinct groups of NDV strains, but did not correlate with the virulence of these strains. All five monoclonal antibodies were able to stain the nuclei of strain AV-infected cells. Nuclear staining in cells infected with other strains of NDV correlated with the reactivity of the monoclonal antibodies with virions of those strains. Both of these results indicate that the NDV M protein is, indeed, associated with the infected cell nucleus.

(Presented by K. S. Faaberg in partial fulfillment of the requirements for the Ph.D. degree from the Graduate College of Rush University, Chicago, Ill., 1987.)

MATERIALS AND METHODS

Virus and cells. The AV (1932) strain of NDV (1) was propagated in the allantoic sac of 10-day-old embryonated hen eggs (SPAFAS, Inc., Norwich, Conn.) at 37°C. Virus stocks were prepared as described previously (3, 23). Stocks of other NDV strains were a gift from R. M. Iorio and M. A. Bratt. Strains B (B1-Hitchner [Blacksburg], 1948), F (New Jersey-Roakin, 1946), IM (Italy-Milan, 1945), L (L-Kansas, 1948), N (New Jersey-LaSota, 1946), RO (California-RO, 1944), IS (Iowa-Salsbury, 1949), EF (England-F [ARS], 1949), U (Ulster), and W (Wisconsin-Appleton, 1950) were all obtained from R. P. Hanson of the NDV Repository at the University of Wisconsin, Madison. Strain HP (Israel-HP, 1953) was obtained from A. Kohn of the Israel Institute for Biological Research. The protein concentration of each virus stock was determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, Calif.).

Primary and secondary chicken embryo cells were prepared and maintained in standard medium as described previously (1, 7, 17).

Monoclonal antibody isolation. Some mice were immunized with purified M protein from virions of strain AV, prepared by the method of Scheid and Choppin (18). Briefly, virions were disrupted in Triton X-100–1 M KCl, ribonucleoprotein (RNP) complexes were removed by centrifugation, and purified M protein was precipitated under low-ionic-strength conditions. The precipitate was collected by centrifugation in an Eppendorf 5412 centrifuge and suspended in phosphate-buffered saline (PBS). The precipitate was esti-

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TABLE 1. Isolation of NDV-reactive hybridomas and their specificities

Isolation attempt	Immunogen	Mouse strain	No. of hybridomas producing antibodies to viral antigen[a]:					
			M	F	HN	P	NP	Unknown[b]
A	M[c]	CAF_1	0	0	0	0	2	3
B	M	CAF_1	2	2	5	4	1	5
C	RNP-M[d]	CAF_1	1	0	14	1	0	8
D	RNP-M	C57BL/6	1	0	0	0	0	0

[a] Determined by immunoprecipitation.
[b] No proteins immunoprecipitated.
[c] Isolated by the method of Scheid and Choppin (18).
[d] Isolated as described in Materials and Methods.

mated to be >90% M protein by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and Coomassie brilliant blue staining. Other mice were immunized with RNP-M complexes. These complexes were isolated by disrupting virions in Triton X-100 without 1 M KCl. The resulting RNP-M complexes were pelleted as described above and suspended in PBS.

Four attempts to isolate monoclonal antibody-producing hybridomas (fusions A to D) are described (Table 1). Mice (Jackson Laboratory, Bar Harbor, Maine) were immunized intraperitoneally with 50 μg of purified M protein or with 100 μg of RNP-M emulsified in complete Freund adjuvant (Difco Laboratories, Detroit, Mich.). This injection was repeated 4 weeks later in incomplete Freund adjuvant (Difco). One week later, the mice were injected intravenously with the M protein in PBS. The only deviation from this routine was in fusion A; 100 μg of purified M protein was injected on each of the three occasions, but no adjuvant was added.

At 3 days after the intravenous injection, the mice were sacrificed and their spleen cells were fused with SP2/0-Ag14 myeloma cells, as described by Iorio and Bratt (8). Cells in 96-well plates which survived hypoxanthine-aminopterin-thymidine selection were screened for production of antibodies reacting with viral proteins in a radioimmunoassay (8). Cells in positive wells were cloned in soft agar (8). The antigen recognized by each monoclonal antibody was determined by immunoprecipitation.

Ascites fluids. Ascites tumors were induced in CAF_1 mice as described previously (8). Intraperitoneal fluids were collected and clarified by ammonium sulfate precipitation (8, 22) and stored at −70°C in small portions. For competition antibody binding experiments, 10 mg of each clarified ascites was further purified by affinity to MAPS resin (Bio-Rad). Specifically eluted antibodies were concentrated to 2 ml in an Amicon concentrator with a PM10 membrane (Amicon Corp., Lexington, Mass.).

One milliliter of each of these purified antibody preparations was covalently linked to horseradish peroxidase (P-8375; Sigma Chemical Co., St. Louis, Mo.) by the method of Wilson and Nakane (25), except that (i) the peroxidase solution was dialyzed against 0.01 M carbonate buffer (pH 9.5) and (ii) the labeled antibody was separated from free peroxidase by two precipitations with 50% saturated ammonium sulfate, followed by extensive dialysis against PBS. The solution was filtered through a 0.22-μm-pore-size filter and stored at −20°C until use.

Immunoprecipitation. Secondary chicken embryo cells were infected with NDV AV strain at a multiplicity of infection of 5 and incubated at 37°C in 5% CO_2. At 6 h postinfection, cells were incubated with 50 μCi of [^{35}S]methionine (Amersham Corp., Arlington Heights, Ill.) per ml in medium lacking methionine and supplemented with 2.5% dialyzed fetal bovine serum. After 1 h, cells were rinsed with PBS and lysed in 1 ml of PBS with 1% Triton X-100. The lysate was vortexed, and insoluble material was removed by centrifugation.

Strain AV virions were metabolically labeled with [^{35}S]methionine during replication in chicken embryo cells and purified as described previously (17). Virions collected from the step sucrose gradient were disrupted in RIPA buffer (10 mM Tris [pH 7.2]–150 mM sodium chloride–1% sodium deoxycholate–1% Triton X-100–0.1% SDS) before immunoprecipitation.

The labeled cell lysate or disrupted AV virion preparation was mixed with a dilution (usually 20 μl of a 1:1,000 dilution) of a monoclonal antibody-containing ascites preparation followed by anti-mouse immunoglobulin beads (Bio-Rad) as described by Morrison et al. (13).

Enzyme immunoassay (EIA). Disrupted virion-coated 96-well polyvinyl assay plates (Costar, Cambridge, Mass.) (0.5 μg per well) were prepared as described previously (8). Nonspecific binding was blocked with 5% bovine serum albumin (BSA; A-4503; Sigma) for 2 h at room temperature, and the plates were emptied and stored at −20°C until use. Plates were rinsed with PBS between each subsequent step, and all incubations were at 37°C for 1 h unless otherwise noted. Dilutions of monoclonal antibodies in 1% BSA in PBS were added to each well, followed by 50 μl of peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.) in 1% BSA, followed by the addition of 100 μl of 1.9 mM 2′,2′azinobis-(3-ethylbenzothiazolin sulfonate) [ABTS] in 0.1 M citrate glycine buffer (pH 4) with freshly added 2.4 mM H_2O_2 to each well. Each plate was incubated for 15 min at 37°C, and the intensity of the resulting green color was quantified by a Titertek (Flow Laboratories, Inc., McLean, Va.) optical plate reader at a wavelength of 414 nm.

The concentration of each purified monoclonal antibody was determined by comparing its binding activity with that of antibody M2a by EIA by using a peroxidase-conjugated goat anti-mouse antibody (Kirkegaard and Perry) to detect monoclonal antibody bound to antigen-coated wells. A known protein concentration (determined by optical density [OD] at 280 nm [OD_{280}]) of affinity-purified M2a was diluted serially twofold and used to construct a standard curve. Dilutions of each test antibody which gave OD_{414} EIA readings in the linear portion of the M2a curve were extrapolated to determine the effective protein concentration. The results of three antibody concentration determinations were averaged.

For competitive antibody-binding experiments, serial dilutions of unlabeled purified monoclonal antibody were first attached to the antigen-coated wells. After being rinsed with PBS, equal amounts of peroxidase-conjugated monoclonal antibody (described in the previous section) were deposited in each well. After being rinsed, substrate was added and the color was developed. The percent competition at each concentration of competing unlabeled antibody was calculated by first subtracting the background OD_{414} of the peroxidase-conjugated antibody on BSA-treated polyvinyl wells lacking antigen and then by using the formula: percent competition = (OD without competitor − OD with competitor) × 100/(OD without competitor).

PAGE. Viral proteins were reduced with 2-mercaptoethanol in sample buffer (0.125 M Tris hydrochloride [pH 6.8], 20% glycerol, 6% SDS, 0.0001% bromphenol blue), boiled,

and resolved by electrophoresis on 10% polyacrylamide slab gels (10). Gels with radioactive proteins were treated with En^{3}Hance (New England Nuclear Corp., Boston, Mass.), dried, and exposed to preflashed (11) film (X-Omat AR; Eastman Kodak Co., Rochester, N.Y.).

Immunoperoxidase staining. A full description of the immunostaining procedure is presented elsewhere (M. E. Peeples, Virology, in press). Briefly, secondary chicken embryo cells uninfected or infected at a multiplicity of infection of 5 were grown on wells of a glass microscope slide. Cells were fixed in 3% paraformaldehyde (12), permeabilized with 0.05% Triton X-100, and treated successively with normal goat serum, test antibody, biotinylated goat anti-mouse IgG, peroxidase-linked streptavidin, and diaminobenzidine (0.5 mg/ml in 1% BSA in PBS) with 0.02% H_2O_2. Glycerol-PBS (9:1) was added to each slide followed by a large cover slip. Slides were observed and photographed on a fluorescence microscope (Laborlux; Leitz/Opto-Metric Div. of E. Leitz, Inc., Rockleigh, N.J.) by using 35-mm Tri-X film (Kodak).

The monoclonal antibodies against the M protein are characterized in this report. Monoclonal antibodies NP3 against the NP protein and HN1a against the HN protein have been described previously (8) and were a gift from R. M. Iorio and M. A. Bratt. Two monoclonal antibodies generated during the present study, Fu1a, against the F protein and P1a against the P protein have been described previously (14, 16).

RESULTS

Isolation of hybridomas. Mice were immunized with M protein or RNP-M complexes from NDV strain AV virions. Four hybridomas produced antibodies which immunoprecipitated the M protein (Fig. 1). The antibodies were designated M1a, M2a, M3a, and Mz for reasons which will become clear later. The fifth antibody, M2b, given to us by R. M. Iorio and M. A. Bratt, also immunoprecipitated M protein (Fig. 1). All the antibodies were isolated from separate fusion attempts, except M1a and Mz, both of which resulted from fusion B, as shown in Table 2. It is interesting that in fusion processes in which 90% pure M protein had been used to immunize the mice, more hybridomas were generated against other viral proteins than were generated against the M protein (Table 1).

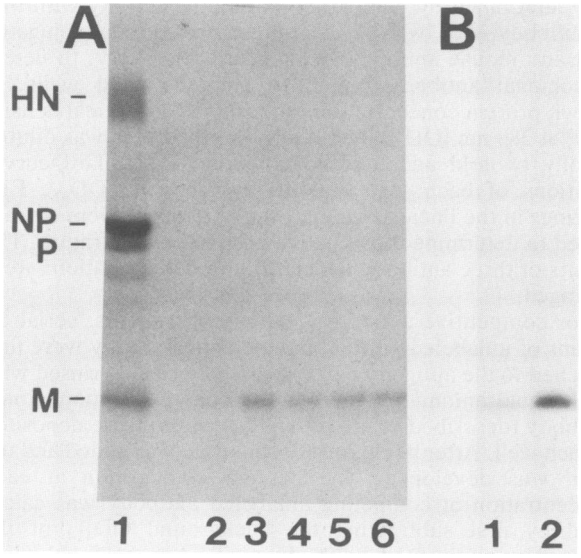

FIG. 1. Immunoprecipitation of strain AV M protein by monoclonal antibodies. (A) [^{35}S]methionine-labeled virions (lane 1) were disrupted and subjected to immunoprecipitation with PBS (lane 2) or diluted ascites fluids containing M1a (lane 3), M3a (lane 4), M2a (lane 5), or M2b (lane 6). (B) Immunoprecipitation of [^{35}S]methionine-labeled strain AV-infected chicken embryo cell lysate with medium (lane 1) or Mz hybridoma supernatant (lane 2).

TABLE 2. Monoclonal antibody subclass and equilibrium binding constants

Monoclonal antibody	Fusion designation[a]	Heavy chain[b]	Light chain[b]	Equilibrium binding constant
M1a	B	IgG1	K	1.2×10^9 M^{-1}
M2a	D	IgG2b	K	4.0×10^8 M^{-1}
M2b	Gift[c]	IgG1	K	2.7×10^9 M^{-1}
M3a	C	IgG1	K	1.3×10^9 M^{-1}
Mz	B	IgG1	K	7.5×10^6 M^{-1}[d]

[a] Fusion designations are described in Table 1.
[b] Determined with a kit from Miles Laboratories, Inc., Elkhart, Ind. K, Kappa.
[c] Monoclonal antibody M2b, derived from BALB/c mouse, was a gift from R. M. Iorio and M. A. Bratt.
[d] Approximate value for this equilibrium constant.

The subclass of each antibody, heavy and light chain, and the estimated avidity of each antibody are shown in Table 2. Four of the five antibodies have an IgG1 heavy chain. Only M2a has an IgG2b heavy chain. All five antibodies have Kappa light chains. The avidities (equilibrium binding constants) of the monoclonal antibodies for the M protein were determined by the method of Frankel and Gerhard (5). Antibodies M1a, M2a, M2b, and M3a exhibited high avidity binding to the M protein, with equilibrium binding constants between 4.0×10^8 M^{-1} and 2.7×10^9 M^{-1}. Mz displayed an equilibrium binding constant that was much lower and that was difficult to measure accurately by this procedure.

Grouping the antibodies. The ability of one antibody to compete with a peroxidase-conjugated antibody for binding to the M protein was determined by incubating disrupted virion-coated assay wells with serial dilutions of unlabeled, affinity-purified antibody followed by the peroxidase-conjugated antibody. Affinity-purified Fu1a, which is directed against the NDV F protein (14), did not prevent any of the labeled antibodies from binding to the M protein, indicating that nonrelated antibodies do not interfere (Fig. 2).

Competitive antibody-binding experiments between all pairs of antibodies to the M protein, in both directions, was performed. No other antibody, besides homologous M1a, inhibited the binding of peroxidase-conjugated M1a (Fig. 2A). The site on the M protein recognized by this antibody was designated M1, and the first antibody which bound to site M1 was designated M1a. Likewise, M3a was the only antibody which prevented itself from binding to site M3 (Fig. 2B). Antibodies M2a and M2b inhibited the binding of each other in a reciprocal manner, indicating that they bind to the same or overlapping determinants, designated M2, on the M protein (Fig. 2C and D).

One monoclonal antibody, Mz, whose equilibrium binding constant was very low (Table 2), exhibited unclear results. Mz would not inhibit the binding of any of the other antibodies (data not shown). Antibodies M2a, M2b, M3a, the control antibody Fu1a, and the homologous antibody Mz displayed a high background inhibition of peroxidase-conjugated Mz binding (Fig. 3). Only antibody M1a reproducibly inhibited Mz binding in a concentration-dependent manner. This result suggests that M1a binds to the same site on the M protein as Mz.

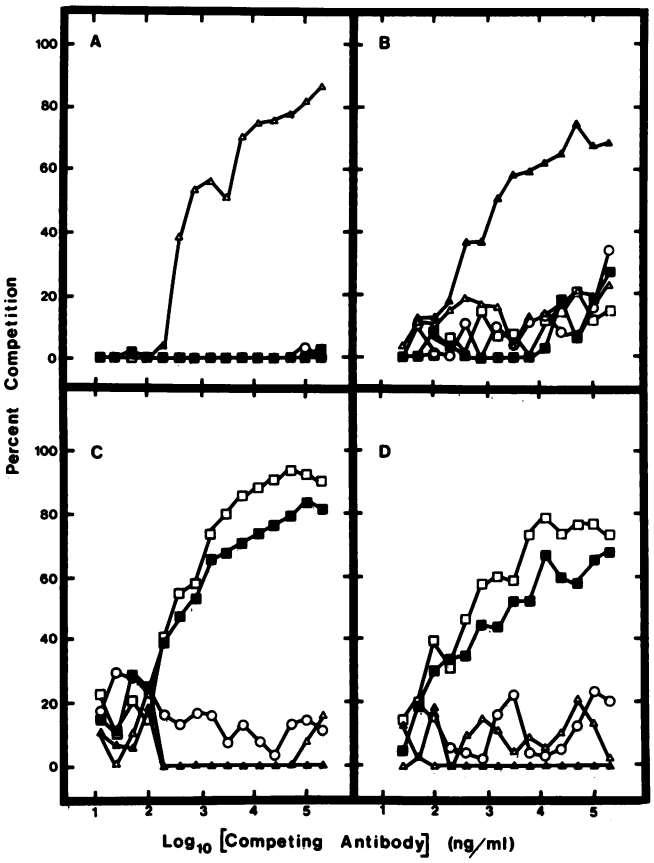

FIG. 2. Competition among the monoclonal antibodies for binding to M protein. Microtiter wells coated with disrupted virions were incubated sequentially with (i) serial dilutions of a purified monoclonal antibody (Mla, △; M2a, □; M2b, ■; M3a, ▲), (ii) peroxidase-conjugated monoclonal antibody M1a (panel A), M3a (panel B), M2a (panel C), or M2b (panel D), and (iii) the ABTS-H_2O_2 peroxidase substrate. An irrelevant monoclonal antibody (Fula, ○) against another viral protein, the fusion protein, was included as a control.

Monoclonal antibody reactivities with NDV strains. The five monoclonal antibodies to the strain AV M protein were tested for binding to 11 other NDV strains. The results are summarized in Table 3. The numbers in the table represent the binding of one antibody to a particular strain compared with the same antibody binding to strain AV. In most cases, the antibodies bound efficiently (29 to 120%) or did not bind. However, in some cases, such as M1a binding to strain IM or M2a binding to strain RO, reactivities were low but detectable.

Before drawing firm conclusions about the reactivities of these particular strain and antibody combinations, purified monoclonal antibodies M1a and M2a were titrated against fixed amounts of disrupted AV, RO, HP, and IM virions. M1a reacted with all four test strains, including low but definite binding to strain IM (Fig. 4). M2a reacted with strain AV but not with strains RO, HP, or IM. Their low reactivities, 1.9 to 12% (Table 3), were not verified and were probably caused by variation at the lower limits of this assay.

The reactivity patterns of these five monoclonal antibodies with the 12 NDV strains are emphasized in Table 3 by boxes drawn around the strains reacting with each antibody. Antibody M1a bound to all 12 strains, with only strain IM

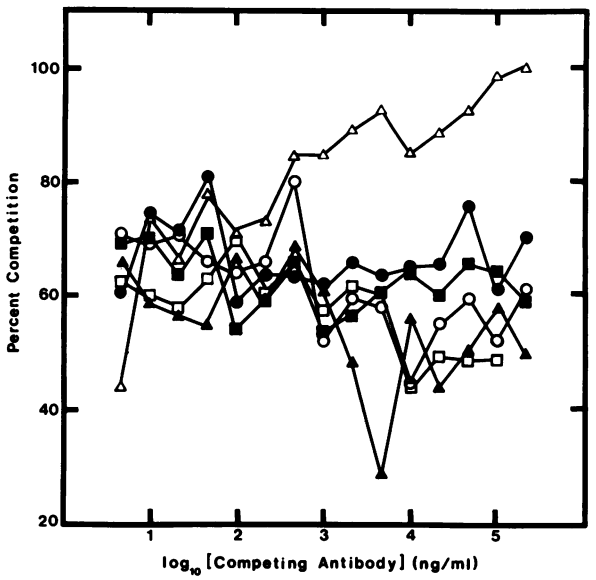

FIG. 3. Competition among monoclonal antibodies and peroxidase-conjugated monoclonal antibody Mz for binding to M protein. Unlabeled monoclonal antibodies M1a (△), M2a (□), M2b (■), M3a (▲), Mz (●), and Fula (○) were bound, followed by peroxidase-conjugated monoclonal antibody Mz.

showing low but detectable reactivity. Mz displayed a similar reactivity pattern, including low reactivity to strain IM, again suggesting that Mz binds to the same site as M1a. Antibodies M2a and M2b bound to the same set of nine strains, confirming that they recognize the same or nearly the same epitope on the M protein. M3a was the most selective antibody, binding to only strains AV, HP, and IM.

A monoclonal antibody, NP3 (8), directed against the nucleoprotein was included as a control in these experiments. NP3 reacted similarly with all strains, indicating that

TABLE 3. Reactivities of monoclonal antibodies with NDV strains[a]

Virus strains	% Reactivity[b] with monoclonal antibody:					
	M1a	M2a	M2b	M3a	Mz	NP3
Virulent						
AV	100	100	100	100	100	100
HP	68	3.4	5.0	64	86	81
IM	18	1.9	5.7	98	34	116
RO	106	12	9.5	3.3	124	106
F	67	58	38	1.7	73	111
IS	88	57	38	3.1	115	101
L	74	48	29	0.8	113	78
Avirulent						
N	74	45	30	2.6	86	97
W	103	103	89	1.4	116	102
U	136	195	190	6.3	158	96
EF	78	41	34	3.1	109	93
B	71	51	31	3.4	117	102

[a] Wells are coated with 0.5 μg of purified, disrupted virus from each NDV strain. EIA results are expressed as the percent reactivity in an EIA compared with strain AV. Culture supernatants were used as the sources of antibodies, with the exception of antibodies Mz and NP3, for which diluted ascites fluids were used. Values represent the average of three separate experiments.

[b] Reactivity patterns of the monoclonal antibodies with the NDV strains are shown by the boxes.

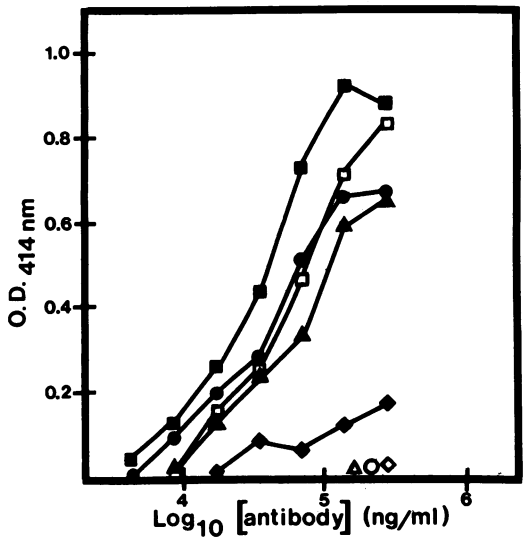

FIG. 4. Titration of two monoclonal antibodies against four strains of NDV. Microtiter wells coated with disrupted virions of strains AV (■, □), RO (●, ○), HP (▲, △), or IM (◆, ◇) were incubated sequentially with (i) dilutions of purified M1a (closed figures) or M2a (open figures), (ii) peroxidase-conjugated goat anti-mouse immunoglobulin, and (iii) ABTS-H_2O_2 substrate.

this epitope of NP is highly conserved among these strains of NDV. It also verifies that the amounts of viral particles used for each strain were roughly equivalent and that differences in antibody binding among strains were not due to gross differences in viral protein quantity.

Immunostaining of infected cells. To determine the intracellular location of the NDV proteins, strain AV-infected cells were stained with one monoclonal antibody to each of the viral antigens, with the exception of the L protein. The results are presented in Fig. 5. Antibodies to NP and to P densely stained the perinuclear regions of infected cells (Figure 5A and C). Antibodies to HN and F stained infected cells in a diffuse speckled manner (Fig. 5G and I). An antibody to M (M1a) diffusely stained the infected cell cytoplasm but strongly stained the nucleus (Fig. 5E).

None of the antibodies stained uninfected cells, indicating that staining was related to virus infection. However, NDV infection has been shown to stimulate the production of some host cell proteins (4). Since the nuclear staining shown in Fig. 5E, as well as that in previous reports (6, 16), has depended on the reactivity of single monoclonal antibodies to the M protein, it is possible that infection stimulates cells to express a nuclear protein which fortuitously shares an antigenic site with the M protein. However, it is unlikely that a cross-reacting, virus-stimulated host protein would share more than one epitope with the M protein. To test this possibility, infected cells were stained with each of the five monoclonal antibodies against M. As shown in Fig. 6, all five antibodies stained the nucleus of infected cells, strengthening the suggestion that the nucleus-associated antigen is indeed the M protein.

Cells infected with other NDV strains displayed nuclear staining by M protein-specific antibodies which correlated well with the antibody reactivity to virions of those strains. An example of M protein-specific monoclonal antibody staining of cells infected with the L strain of NDV is shown (Fig. 7); the nucleus was stained with antibody M1a but not with antibody M3a. This pattern was expected because M1a reacts with strain L virions but M3a does not (Table 3). If these antibodies were reacting with a virus-induced host protein, the nucleus would have been stained with both antibodies in strain L-infected cells, as was the case in strain AV-infected cells (Fig. 5). Since M1a stains the nucleus of the strain L-infected cells but M3a does not, the staining must be due to the NDV M protein and not to a cross-reactive, virus-induced host protein.

DISCUSSION

Monoclonal antibodies directed against the M protein of NDV AV strain were generated and characterized by competition antibody-binding assays and by reactivity with 12 strains of NDV. Four of the five monoclonal antibodies were of sufficient avidity to allow unambiguous topological mapping. The antibody binding studies revealed that these four

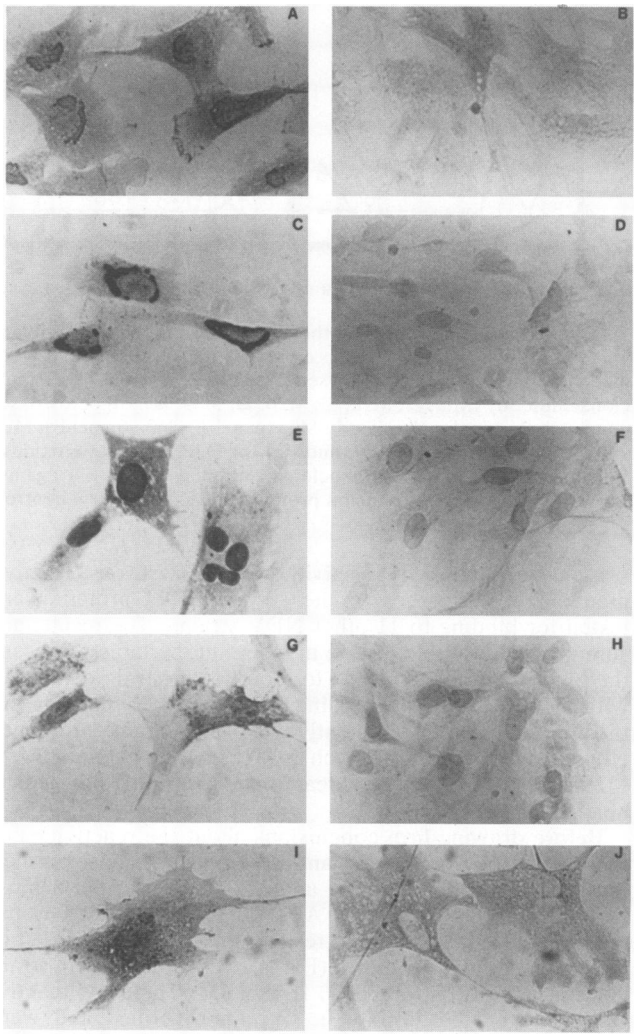

FIG. 5. Immunoperoxidase localization of NDV antigens in infected chicken embryo cells. Infected (A, C, E, G, and I) and uninfected (B, D, F, H, and J) cells were stained with monoclonal antibodies against the NP protein (A and B), the P protein (C and D), the M protein (antibody M1a) (E and F), the HN glycoprotein (G and H), or the F glycoprotein (I and J).

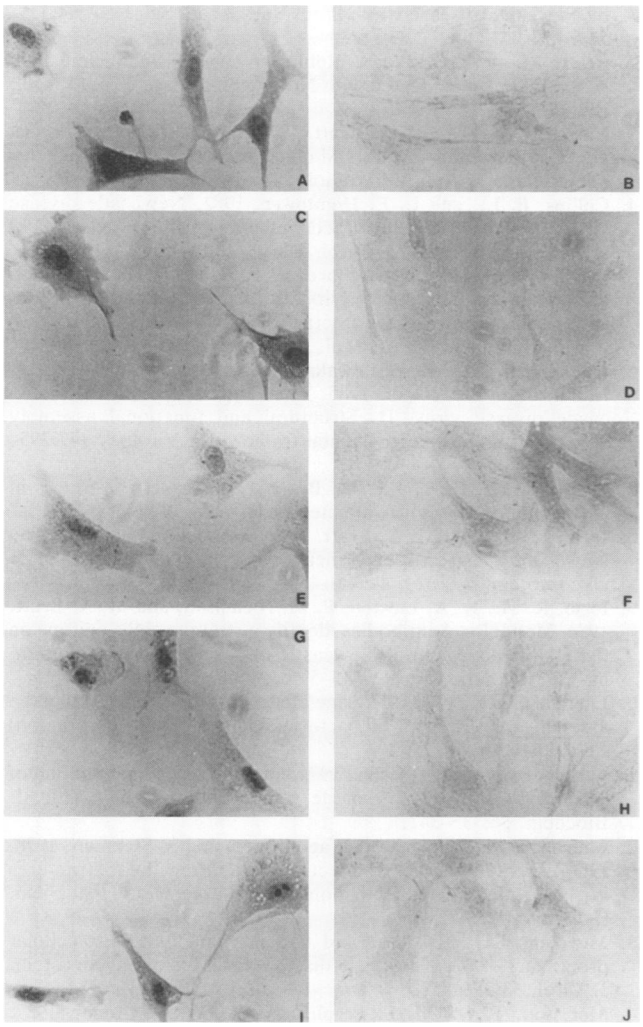

FIG. 6. Immunoperoxidase localization of the NDV M protein in infected chicken embryo cells. Infected (A, C, E, G, and I) or uninfected (B, D, F, H, and J) cells were stained with monoclonal antibodies M1a (A and B), M2a (C and D), M2b (E and F), M3a (G and H), or Mz (I and J).

high-affinity monoclonal antibodies could distinguish a total of three antigenic sites (M1, M2, and M3) on the M protein. Since the inhibition between these antibodies was completely reciprocal, these three sites must be physically separate.

The distinct pattern of binding to different strains of NDV exhibited by each set of antibodies supports the data indicating three distinct antigenic sites on the M protein. Site M1 is found on all 12 of the NDV strains tested; it may represent a "pan-NDV" epitope. Site M2 is the second most common antigenic site and appears on 9 of the 12 strains. Both antibodies M2a and M2b bound in this pattern, confirming their assignment to the same site. Site M3 is the least common site, appearing on only three strains. Only antibody M3a bound in this pattern, confirming that it recognizes a different epitope from that recognized by the others.

Monoclonal antibodies prepared against the orthomyxovirus influenza A virus and the rhabdovirus vesicular stomatitis virus (VSV) have been used to topologically map the M proteins of these viruses. Three separate sites have been identified on the M protein of influenza A virus (21). Four sites, two of which overlap, have been identified on the M protein of VSV (15). Since the M proteins of influenza A virus and VSV are smaller (28 and 26 kilodaltons, respectively) than the M protein of NDV (41 kilodaltons), it is possible that there are additional sites on the NDV M protein not represented by the panel of monoclonal antibodies presented in this report. In support of this, monoclonal antibodies prepared against the M protein (36 kilodaltons) of another paramyxovirus, measles virus, have been separated into six reactive groups by their abilities to immunoprecipitate the M protein of a series of measles virus isolates (19).

Strains other than the immunogen strain AV fall into two groups with regard to reactivity with these antibodies. The larger group contains site M2 but not M3. The smaller group, consisting of strains HP and IM, contains site M3 but not M2. The only exception to this generality is strain RO which contains neither site M2 nor site M3 and therefore appears to be the most different from strain AV.

The results of Iorio et al. (9) suggest that monoclonal antibodies to sites HN1 and HN2 may distinguish between virulent and avirulent strains of NDV. In the present report, all of the strains containing site M3 are virulent. However,

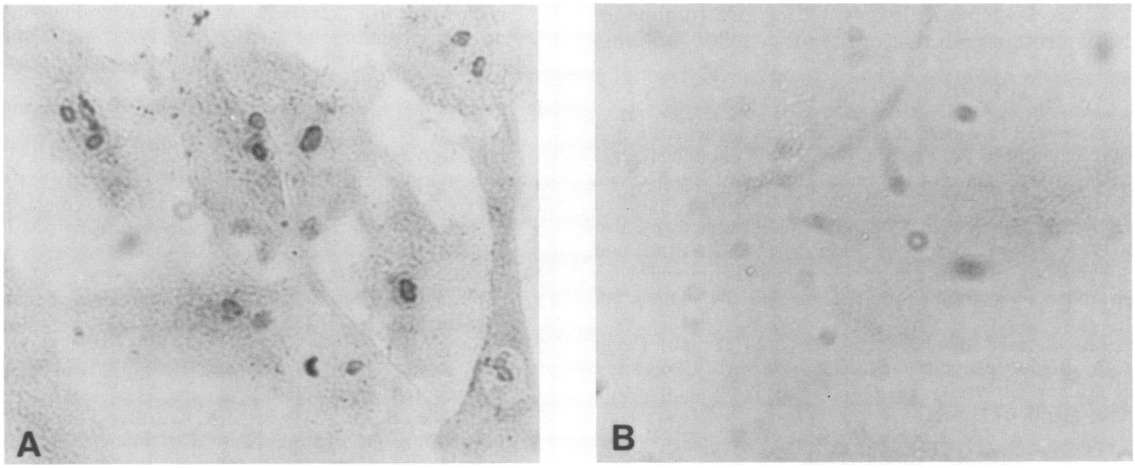

FIG. 7. Immunoperoxidase staining of chicken embryo cells infected with NDV strain L. Cells were stained with monoclonal antibody M1a (A) which reacts with strain L virions (Table 3) or with monoclonal antibody M3a (B) which does not react with strain L virions (Table 3).

the strains lacking site M3 include both virulent and avirulent strains. Although all avirulent strains contain site M2, so do half the virulent strains. The virulence of these NDV strains, therefore, does not correlate with the presence or absence of any of these three sites on the M protein. The M protein may not be an important virulence factor. Alternatively, epitopes other than the three described by these antibodies or other nonantigenic areas of the M protein may be involved in virulence. A similar conclusion was drawn from M protein antigenic variations among nine strains of measles virus (19). A larger panel of monoclonal antibodies, representing additional sites on M, may reveal an epitope which is important to virulence.

In this report, the NP, P, M, HN, and F proteins of NDV have all been localized to the cytoplasm or membranes of infected cells with specific monoclonal antibodies. However, a portion of the M protein was found associated with the nucleus, as briefly noted previously (6, 16). All five monoclonal antibodies against the M protein described in the present report, representing three antigenic sites, stained the strain AV-infected cell nucleus. It is highly unlikely that a host cell protein induced by NDV infection would contain all three M protein antigenic sites. In fact, another strain of NDV induced nuclear staining by one antibody which reacted with virions of that strain but failed to induce nuclear staining by another antibody which did not react with virions of that strain. This correlation has been found consistently in cells infected with other NDV strains. These results indicate that the nuclear staining is caused by the NDV M protein and not by a host cell protein induced by infection.

Since NDV is considered to be a cytoplasmic virus, the simplest explanation for its nuclear location would seem to be that the M protein is associated with the cytoplasmic surface of the nucleus. However, we have recently designed a method to distinguish intranuclear from nuclear surface antigens and used it to demonstrate that the M protein is actually within the nucleus (Peeples, in press).

Earlier studies, using polyvalent antisera against NDV, located the viral antigens to the cytoplasm of infected cells (20, 24). It is possible that the antisera used in those studies had a low reactivity to the M protein. Our experience in generating M protein-specific hybridomas (Table 1) or antisera (data not shown) indicates that it may not be an efficient immunogen. In fact, enriched M protein preparations were more efficient in inducing B cells for hybridomas against contaminating viral proteins than against the M protein. Presently, we are systematically comparing the immunogenicity of the M protein with that of the other NDV proteins.

ACKNOWLEDGMENTS

The authors thank L. Edward Cannon for help in the generation of the hybridoma cell lines; Valdis I. Kirsis for excellent technical assistance; and Ronald M. Iorio, Rhona L. Glickman, and Michael A. Bratt for the M2b and NP3 monoclonal antibodies, purified virions of NDV strains other than AV, and technical advice. We also thank Trudy G. Morrison, Ronald M. Iorio, Michael A. Bratt, and Rhona L. Glickman for critical reading of this manuscript and Alice F. Robinson and Kaarin Soma for assistance in the manuscript preparation.

This work was supported by a Basic Research Support Grant administered by Rush-Presbyterian-St. Luke's Medical Center and by Public Health Service grant AI 21924 from the National Institute of Allergy and Infectious Diseases.

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