Neutralization Map of the Hemagglutinin-Neuraminidase Glycoprotein of Newcastle Disease Virus: Domains Recognized by Monoclonal Antibodies That Prevent Receptor Recognition

RONALD M. IORIO,* RICHARD J. SYDDALL, JOHN P. SHEEHAN, MICHAEL A. BRATT,
RHONA L. GLICKMAN, AND ANNE M. RIEL

*Department of Molecular Genetics and Microbiology, University of Massachusetts
Medical School, 55 Lake Avenue, North, Worcester, Massachusetts 01655*

Received 26 March 1991/Accepted 6 June 1991

Monoclonal antibodies (MAbs) to the hemagglutinin-neuraminidase (HN) glycoprotein of Newcastle disease virus delineate seven overlapping antigenic sites which form a continuum on the surface of the molecule. Antibodies to five of these sites neutralize viral infectivity principally by preventing attachment of the virion to cellular receptors. Through the identification of single amino acid substitutions in variants which escape neutralization by MAbs to these five antigenic sites, a neutralization map of HN was constructed, identifying several residues that contribute to the epitopes recognized by MAbs which block the attachment function of the molecule. These epitopes are defined, at least in part, by three domains on HN: residues 193 to 201; 345 to 353 (which include the only linear epitope we have identified in HN); and a C-terminal domain composed of residues 494, 513 to 521, and 569. To identify HN residues directly involved in receptor recognition, each of the variants was tested for its ability to agglutinate periodate-modified chicken erythrocytes. One variant with a single amino acid substitution at residue 193 was 2.5- to 3-fold more resistant to periodate treatment of erythrocytes than the wild-type virus, suggesting that this residue influences the binding of virus to a sialic acid-containing receptor(s) on the cell surface.

The hemagglutinin-neuraminidase (HN) glycoprotein of Newcastle disease virus (NDV) possesses both the receptor recognition and neuraminidase (NA) activities associated with the virus. In virions, HN is found as spikes protruding from the cellularly derived viral membrane, in which it is anchored near its amino terminus (33).

The amino acid sequences of the HN glycoproteins of several NDV isolates have been deduced from the nucleotide sequences of cloned genes (20, 23, 24, 31, 32). There is extensive amino acid homology between the HN glycoproteins of the different isolates, approximately 90% in any pair-wise comparison (29). Each has a hydrophobic stretch of amino acids near its amino terminus that is presumed to be the membrane-spanning domain. Six potential asparagine-linked glycosylation sites, predominantly in the carboxy-terminal half of the molecule, are also highly conserved in the HN of most of the isolates (29).

There is also extensive conservation of the number and position of cysteine residues among the HN sequences of different NDV isolates. Twelve of the 13 cysteine residues carboxy terminal to the proposed membrane-spanning region are conserved. Only the cysteine at residue 123 is conspicuous by its absence in the majority of the NDV isolates sequenced thus far (34). This is probably causally related to the observation that the HN of NDV apparently can exist in virions in either a disulfide-linked or a non-disulfide-linked form, depending on the isolate, but does not appear to be associated with any obvious difference in HN function or antigenic structure (25, 27, 35). The presence of cysteine at residue 123 correlates with the existence of disulfide-linked dimeric HN in virions, implicating this cys-

teine in intermolecular disulfide bonding between HN monomers.

We initially characterized a panel of monoclonal antibodies (MAbs) to antigenic sites 1, 2, 3, and 4 on the surface of the HN glycoprotein of the AV isolate (13). However, many MAbs produced by hybridomas isolated in subsequent fusions were shown to map to sites which overlap two of the original four sites and were named to reflect this, hence sites 12, 14, and 23 (12, 18). Since any one site overlaps at least one other in competition antibody binding assays, our panel of MAbs recognizes seven overlapping antigenic sites which form a linked continuum (sites 4-14-1-12-2-23-3) on the surface of the molecule. Functional inhibition studies revealed that MAbs to three overlapping sites (12, 2, and 23) inhibit the NA activity of the virus (12, 18). However, analysis of variants, as well as other studies, indicated that site 23 is probably closest to the actual NA site (19). MAbs to these three sites, as well as those to sites 1 and 14, inhibit hemagglutination (HA) (12, 14) and neutralize predominantly by preventing viral attachment to chick cells (18). MAbs to the two remaining sites (3 and 4) inhibit neither HA-attachment nor NA but do inhibit the hemolytic activity of the virus (14) and presumably neutralize at a postattachment step.

We selected and characterized a variety of antigenic variants which escape neutralization by an antibody to each of the five sites on HN involved in viral attachment. Individual amino acid substitutions were identified in the HN proteins of these variants, making it possible to construct a map of the neutralizing domains of HN and providing insight into its three-dimensional structure. These substitutions clustered in three domains of the molecule. One domain (amino acid residues 193 to 201) is part of a more extensive region (171 to 205) previously implicated in the NA activity of HN (19). The remaining two regions include (i) residues

* Corresponding author.

345 to 353 and (ii) residues 513 to 521 plus 494 and 569. The former of these is a linear epitope which coincides with one recognized in another NDV isolate by MABs which also inhibit HA (30).

Amino acid substitution in the attachment site on HN could influence the receptor recognition properties of the virus. Periodate treatment oxidizes the sialic acid moieties on the surface of erythrocytes, rendering them resistant to agglutination by viruses that recognize a sialic acid-containing receptor (37). Thus, substitution at amino acid residues important to the recognition of cellular receptors might be expected to alter the sensitivity of the virus-erythrocyte interaction to periodate treatment. This was shown to be the case for MAB-selected variants of influenza virus (38). We show here that a substitution at position 193 of the HN of the AV isolate renders the HA activity of the virus almost threefold more resistant to periodate treatment of chicken erythrocytes, indicating that this residue influences receptor recognition.

MATERIALS AND METHODS

Virus. The Australia-Victoria (1932) (AV) isolate of NDV, as well as variants and revertants derived from it, and the Illinois-Great Lakes (GL) (1953) and Mexico-Textcoco (MT) (1950) isolates were grown in the allantoic sacs of 10-day-old embryonated hen eggs at 37°C from a stock of virus one egg passage from cloning (2). After the death of the majority of the embryos, allantoic fluid was harvested and virus was purified as described previously (4, 41).

Cell culture. Primary and secondary chicken embryo cells were prepared and maintained as described previously (2, 41). Confluent secondary cultures in 60-mm tissue culture dishes were used for plaque assays.

Hybridomas and MABs. The preparation of hybridomas and the initial characterization of MABs have been described previously (12, 13, 18).

Selection of variants and isolation of revertants. Variants were selected from cloned, passaged stocks of AV by escape from neutralizing MAB, using rabbit anti-mouse immunoglobulin (16). Each variant originated from a different passaged stock, ensuring that they are independent isolates.

Variant 2_a-1 is temperature sensitive, unable to form plaques at the nonpermissive temperature of 41.8°C. Revertants, no longer temperature sensitive for plaque formation, were selected by plating the variant at the permissive temperature (37°C), passaging virus from individual plaques in eggs, and replating at the nonpermissive temperature. Only one revertant from each stock was characterized.

ELISA and neutralization assay. MAB recognition of virus was determined by assaying binding of hybridoma supernatants to intact virions in an enzyme-linked immunosorbent assay (ELISA) (18). The ability of MABs to block viral infectivity was determined in a plaque neutralization assay (15). Rabbit anti-mouse immunoglobulin treatment (16) was used to verify binding by those MABs which did not result in neutralization.

Primer extension and dideoxy sequencing. The nucleotide sequence of the entire HN gene of each variant and revertant was determined by using 17-mer oligonucleotides complementary to that of the AV isolate (23) to prime dideoxynucleotide chain termination-sequencing reactions. The primers, their purification, the primer extension reactions, and the sequencing gel protocols have all been described previously (34), as has the preparation of virion RNA template (19).

Western blots (immunoblots). Virion proteins (15 to 20 µg per lane) (22) were resolved by electrophoresis in sodium dodecyl sulfate (SDS)-10% polyacrylamide gels (21) in the presence of 0.5 M 2-mercaptoethanol. After presoaking for 2 h in 25 mM Tris (pH 8.3)-192 mM glycine-50 mM NaCl-7 M urea, proteins were transferred to Hybond-C Extra hybridization transfer membrane (0.45-µm pore size; Amersham Corp., Arlington Heights, Ill.) for 16 to 18 h at 100 mA in 25 mM Tris (pH 8.3)-192 mM glycine-150 mM NaCl. Antibody binding was detected by using the ECL Western Blotting Detection system (Amersham) according to protocols provided by the company. Briefly, the filters were blocked for 1 h with 5% nonfat milk in phosphate-buffered saline (PBS) containing 0.5% Tween 20 (PBS-T) before being cut into strips and incubated for 1 h with ascites fluid preparations of MAB at a concentration (14) of 5 µg/ml in PBS-T containing 0.5% milk. The strips were reacted for 1 h with a 1/10,000 dilution of horseradish peroxidase-conjugated goat anti-mouse antibody (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.). All incubations were done on a rotary shaker at room temperature, and the strips were washed repeatedly with PBS-T between steps. After reaction for 1 min with the detection reagents provided by the company, the strips were aligned in plastic wrap and exposed to Kodak X-Omat film. After evaluation of a preliminary 30-s exposure, longer exposures were made for strips when necessary.

Peptides. Peptides corresponding to amino acid residues 336 to 355 (NH₂-IYKRYNDTCPDEQDYQIRMA-COOH), 337 to 351, and 341 to 355 were purchased from Multiple Peptide Systems (San Diego, Calif.). Each was synthesized with an N-terminal cysteine residue to facilitate cross-linking to a carrier molecule for related experiments.

To determine MAB recognition of peptides, the ELISA protocol (18) was modified such that 1 µg of peptide in PBS was adsorbed overnight to each well of a microtiter plate, blocked for 4 h with 0.25% nonfat milk in PBS, and incubated overnight with serial twofold dilutions (starting concentration of 25 µg/ml) of MAB-containing ascites fluid. All incubations were done in a moist chamber at room temperature.

Periodate sensitivity test. To determine the effect of periodate treatment of erythrocytes on HA activity, we used a modification of the method of Underwood (37). Erythrocytes washed three times in PBS (10 mM Na₂PO₄, 3 mM KCl, 1.8 mM KH₂PO₄, 8.5% NaCl) containing 1% CaCl₂ and MgCl₂ were incubated for 30 min at room temperature with 0.1 volume of various concentrations (0.5 to 10 mM) of freshly prepared sodium metaperiodate. A 0.1 volume of 40% glucose was added to stop periodate oxidation before use of the erythrocytes in an HA assay (19). The concentration of periodate which decreased the HA activity of each virus by 4 HA units was determined.

Amino acid sequence homology and hydrophilicity profile. The HN amino acid sequences of different paramyxoviruses were aligned by using the DNASTAR Align program. The published nucleotide sequence of the HN gene of the AV isolate of NDV (23) predicted a protein of 570 amino acids lacking the tyrosine at residue 184 of other isolates (20, 24). We have since found that this tyrosine residue is, indeed, present in the HN of the AV isolate (data not shown). This eliminates the need to insert a space in the AV sequence at this position to achieve maximum alignment (19). Previously published variant amino acid substitutions, all of which are carboxy terminal to residue 184 (19), have been renumbered (plus 1) in this report.

Secondary structure was predicted by the method of Chou

FIG. 1. Reactivity of MAbs with variants and revertants. MAb binding to virus was determined in an ELISA, and its ability to neutralize viral infectivity was determined in a standard plaque assay. Those for which antibody recognition of virus resulted in detectable neutralization (persistent fraction of <80%) were then tested for anti-immunoglobulin neutralization of antibody-sensitized virus. The results were as follows: □, MAb does not recognize virus (<20% of the binding to the wild-type virus) and does not neutralize viral infectivity (persistent fraction of >80%); ■, MAb neutralizes virus to an extent similar to its neutralization of wild type (18); ▨, MAb recognizes virus in the ELISA (>50% relative to the wild type) but neutralizes its infectivity ineffectively compared with the wild type (>50% persistent fraction). Addition of anti-immunoglobulin, however, results in complete neutralization.

and Fasman (3). The hydrophilicity profile of the HN protein was generated from the published sequence (23) by using the normalized consensus hydrophobicity scale of Eisenberg et al. (6, 7), which is then used to predict the location of antigenic sites.

RESULTS

Reactivity of anti-HN MAbs with antigenic variants. Competitive antibody binding and additive neutralization assays indicate that a panel of neutralizing MAbs recognize epitopes within seven overlapping antigenic sites on HN (12, 13, 18). Since none of the anti-HN MAbs is sufficiently neutralizing to allow the selection of variants in a simple plaque neutralization assay, variant isolation required the use of anti-immunoglobulin to neutralize MAb-sensitized infectious virus (16).

Antigenic variants selected by escape from neutralization with antibodies to each site were screened for binding and neutralization by all MAbs to that site to map epitopes within sites. Representative MAbs to heterologous sites were also assayed to explore the possibility of an amino acid substitution in one site causing either a direct or a conformational change in other sites (Fig. 1). There were three different outcomes of the interaction of the neutralizing MAbs with the variants: (i) failure to neutralize because the variant is

TABLE 1. Nucleotide and deduced amino acid substitutions in variants and revertants

Variant	Nucleotide substitution	Amino acid substitution
23 _a -3	668, T→C	193, Phe→Leu
23 _a -1	671, T→C	194, Ser→Pro
23 _f -1	672, C→T	194, Ser→Phe
23 _a -2	693, A→C	201, His→Pro
1 _a -3	1124, C→T	345, Pro→Ser
1 _a -1; 1 _b -1; 1 _b -2; 1 _c -1	1125, C→A	345, Pro→Gln
1 _a -2	1125, C→T	345, Pro→Leu
14 _a -7	1131, A→C	347, Glu→Ala
	1569, A→G	493, Asp→Gly
14 _b -1; 14 _d -1; 14 _f -1	1131, A→G	347, Glu→Gly
14 _c -1; 14 _e -1	1139, T→C	350, Tyr→His
14 _a -8; 14 _b -2	1149, G→A	353, Arg→Gln
12 _a -2	1571, G→A	494, Asp→Asn
2 _a -3	1628, C→A	513, Arg→Ser
2 _a -2; 2 _b -1	1629, G→A	513, Arg→His
2 _a -1 ^a	1632, T→C	514, Ile→Thr
2 _a -1R11 ^a	1632, T→C	514, Ile→Thr
	1652, A→G	521, Arg→Gly
2 _a -1R6 ^a	1632, T→C	514, Ile→Thr
	1654, A→T	521, Arg→Ser
12 _c -1	1637, C→T	516, Arg→Trp
12 _c -2	1638, G→A	516, Arg→Gln
2 _b -2	1795, T→A	569, Asp→Glu

^a Each of these viruses also has a conservative nucleotide substitution at position 1705 (T→C).

not recognized by the MAb; (ii) neutralization similar to the wild-type virus; and (iii) recognition, but ineffective neutralization, of the variant by the MAb. In the last case, binding was verified by ELISA or by enhanced neutralization mediated by the addition of anti-immunoglobulin. Examples of antibody binding to virions without neutralization include several MAbs to site 14 and variants 14_a-8 and 14_c-1 (Fig. 1); some of the site 23 MAbs and variants 23_a-3 and 23_f-1 (19); and the site 2 MAbs and variant 2_a-1 (16).

Variant 2_a-1 is temperature sensitive for plaque formation (17). Two revertants of this variant, 2_a-1R6 and 2_a-1R11, formed plaques at the nonpermissive temperature of 41.8°C. These viruses also reverted for neutralization by the selecting antibody, 2_a.

Two of the site 14 variants, 14_c-1 (Fig. 1) and 14_e-1 (data not shown), were not recognized by antibodies to site 1. Variant 2_a-1 and its revertants were not recognized by MAbs to site 12. These are the only examples among the panel of variants of a substitution in one site resulting in escape from neutralization by an MAb to a different site. However, it is important to note that, for each of these variants, the heterologous site topologically overlaps the homologous site, suggesting that either the two sites share amino acid residues or the substitutions in the variants induce a local conformational change in HN. Therefore, it can be concluded that the amino acid substitutions responsible for escape from neutralization by the selecting MAbs do not result in gross changes in the antigenic structure of HN.

MAbs which prevent virus attachment recognize epitopes in three domains of HN. The complete nucleotide sequences of the HN genes of variants selected with MAbs to sites 14, 1, 12, 2, and 23 were determined. The nucleotide and deduced amino acid substitutions for each are listed in Table 1. With one exception, each of the variants had a single nonconser-

FIG. 2. Antigenic sites on the hydrophilicity profile of HN. Hydrophilic regions of HN (peaks) are predicted by using the normalized consensus (NCS) hydrophobicity scale of Eisenberg et al. (6, 7), which predicts the localization of antigenic sites.

vative amino acid substitution in HN. The epitopes recognized by antibodies to these sites could be localized to three widely separated domains in the linear amino acid sequence of the molecule.

(i) **Site 23: residues 193 to 201.** The amino acid substitutions at residues 193, 194, 200, and 201 in variants selected with MABs to antigenic site 23 have been described previously in the course of identification of this domain as important to the NA activity of HN (19).

(ii) **Sites 1 and 14: residues 345 to 353.** All six of the variants selected with antibodies to site 1 had a substitution (glutamine, leucine, or serine) for the proline residue at position 345 in the amino acid sequence of wild-type HN (Table 1). Site 14 MABs selected variants with substitutions at residue 347, 350, or 353. Variant 14_a-7, selected with an MAB isolated in a separate fusion, was the only one found to have two amino acid substitutions, at residues 347 and 493.

(iii) **Sites 12 and 2: residues 513 to 521 and 494 and 569.** Antibodies to site 2 selected variants with substitutions at residue 513, 514, or 569. Revertants 2_a-1R6 and 2_a-1R11 are pseudorevertants of 2_a-1. Reversion of the temperature-sensitive phenotype and neutralization by antibody are the result of second-site substitutions, both at residue 521. Two variants selected with site 12 antibodies had a substitution at residue 516, and a third had a substitution at residue 494.

The three domains are all in hydrophilic regions of the molecule. The localization of the three domains recognized by MABs that prevent attachment relative to the hydrophilicity profile of HN showed that the region of the molecule recognized by site 14 and 1 MABs (residues 345, 347, 350, and 353) aligns with the broadest peak of hydrophilicity in the linear amino acid sequence of HN (Fig. 2). Similarly, the remaining two domains also coincide with peaks of hydrophilicity, but both are much sharper peaks centered around residues 199 (site 23) and 516 (sites 12 and 2), respectively. The hydrophilic regions around residues 258 and 279 coincide with determinants recognized by MABs which do not block attachment (data not shown). None of the MABs to the HN glycoproteins of any of the paramyxoviruses selected a variant with a substitution coinciding with the remaining hydrophilic peak. This peak represents the 234-NRKSCS-239 sequence that is completely conserved among the *Paramyxoviridae* (26). Thus, we now have MABs to most of

FIG. 3. Binding of MABs to HN in Western blots. Virion proteins were electrophoresed in SDS-10% polyacrylamide gels after boiling and reduction. Separated proteins were then electrophoretically transferred to nitrocellulose and cut into strips which were reacted with MABs as follows: lane 1, no MAB; lane 2, 12_a; lane 3, 12_e; lane 4, 12_r; lane 5, 23_a; lane 6, 23_r; lane 7, 14_a; lane 8, 14_b; lane 9, 14_r; lane 10, MAB to nucleocapsid (NP) were detected with the enhanced chemiluminescence system, protein. Bound MABs were detected with the system, using an exposure time of 10 min for lanes 1 to 7 and 30 s for lanes 8 to 10.

the major regions of HN predicted to be exposed at the surface of the molecule.

MABs to site 14 recognize a continuous epitope. To determine whether any of the antibody-binding epitopes recognized by these antibodies is a continuous determinant, we assayed MABs to each of the five sites for their ability to recognize denatured HN. Figure 3 shows that site 14 MABs (except 14_a) very efficiently recognized the non-disulfide-linked monomeric form produced by boiling and reducing HN, suggesting that they bind to a continuous epitope in the sequence of HN. In contrast, MABs to sites 12 and 23 could not be shown to recognize boiled, reduced HN even after a 20-fold longer exposure (Fig. 3, lanes 2 to 6).

MAB 14_a had a distinctly different pattern of reactivity with the site 14 variants compared with the other site 14 MABs (Fig. 1) and bound comparatively weakly in the Western blots (Fig. 3, lane 7), also requiring a significantly longer exposure for even weak detection. This suggests that 14_a binds to an epitope that is slightly more dependent on conformation than those recognized by the other site 14 MABs. This may be related to the fact that one of its variants, 14_a-7, has a second substitution outside the domain defining site 14.

We tried to further define site 14 through the use of synthetic peptides corresponding to overlapping sequences in this region of the molecule. Peptides corresponding to HN residues 336 to 355, 337 to 351, and 341 to 355 were adsorbed to microtiter ELISA plates and screened with antibodies to site 14. Representative data for one site 14 MAB, 14_r, are shown in Fig. 4. All the site 14 MABs, even 14_a to a lesser extent, recognized peptides corresponding to residues 336 to 355 and 341 to 355. The peptide corresponding to residues 337 to 351 was not recognized by the site 14 MABs. In addition, none of the peptides are recognized by MABs specific for any other HN antigenic site (data not shown). These studies prove that site 14 is defined by the linear amino acid sequence of HN residues 341 to 355 and that residues 352 to 355 are required for antibody recognition. The binding of site 14 MABs to the peptides was of sufficient

FIG. 4. Binding of MABs to peptides. Twofold serial dilutions of ascites fluid containing MAB 14_r (starting concentration of 25 µg/ml) were reacted in an ELISA with 1 µg of peptide dried onto a microtiter plate. The peptides correspond to HN amino acid residues 336 to 355 (●), 337 to 351 (□), and 341 to 355 (○).

affinity that pretreatment of the MABs with the peptides they recognized could protect the virus from neutralization by the MAB (Fig. 5). Consistent with the ELISA data (Fig. 4), only peptides corresponding to HN residues 336 to 355 and 341 to 355 were protective. Furthermore, neither protected the virus from MABs to other domains, e.g., site 1 (Fig. 5), which topologically overlaps site 14.

Effect of periodate treatment of erythrocytes on HA by variants. Each of the variants was assayed for its ability to agglutinate chicken erythrocytes pretreated with increasing concentrations of periodate to try to identify an amino acid substitution(s) in HN that influences receptor recognition. The results of the periodate sensitivity test are shown in Fig. 6. The concentration of periodate which decreases by 4 units the HA activity of each variant is related to that which similarly decreases the HA activity of the wild type, which is set at unity. Variant 55D7-5, selected with an MAB to the F glycoprotein, and a variant (3_a-1), selected with an MAB to HN that does not prevent viral attachment to the host cell (18), served as controls and did not differ significantly from the wild type in their periodate sensitivity.

The variant with the most significant alteration in periodate sensitivity was 23_a-3, which had a single amino acid

FIG. 5. Peptide inhibition of neutralization of viral infectivity by antibody 14_r. MAB 14_r or 1_a (100 µg/ml) was incubated with a 200-µg/ml solution of peptides corresponding to HN amino acid residues 336 to 355 (A), 337 to 351 (B), or 341 to 355 (C). The bar graph shows the percent persistent fraction surviving treatment by the MAB without peptide and with each peptide.

FIG. 6. Effect of periodate treatment of erythrocytes on HA. Erythrocytes to be used in HA assays were treated with periodate (0.5 to 10 mM). "Resistance to periodate" shows the concentration of periodate which decreased the HA activity of each virus by 4 HA units relative to that which decreased the HA activity of the AV isolate by 4 HA units.

substitution of leucine for the phenylalanine present at residue 193 of the wild type. This substitution rendered receptor recognition nearly threefold more resistant to periodate treatment of erythrocytes than the wild-type HN, indicating that the residue at this position strongly influences receptor recognition.

Of five NDV isolates for which the sequence of the HN gene is known, only AV has a phenylalanine residue at position 193 (19). The majority of the others have leucine at this position. Application of the periodate sensitivity test to two other NDV isolates (MT and GL), which also have leucine at position 193 (data not shown), indicated that they have a level of resistance to periodate similar to that of variant 23_a-3 (Fig. 6). These findings suggest that the amino acid residue at position 193 of HN influences receptor recognition by NDV.

Several other variants, most notably 23_a-1, 1_b-2, 1_c-1, 2_a-3, and 12_c-2, showed decreased resistance to periodate relative to the wild type (Fig. 6). However, unlike 23_a-3, no counterpart in naturally occurring isolates was found, since each of the amino acid residues substituted for in the above variants is, as far as is known, conserved throughout the NDV serotype (29).

DISCUSSION

Variants were selected with MABs to five overlapping antigenic sites on HN, each of which neutralizes viral infectivity by preventing virion attachment to the host cell (18). Escape from neutralizing antibody is attributable to single nonconservative amino acid substitutions, making possible the construction of a detailed neutralization map of the attachment glycoprotein of the virus as summarized in Fig. 7. The epitopes recognized by these MABs are defined, at least in part, by three widely separated domains in the linear amino acid sequence of HN: residues 193 to 201 (site 23), 345 to 353 (sites 14 and 1), and 513 to 521 plus 494 and 569 (sites 12 and 2). Results obtained previously with com-

FIG. 7. Summary of location of amino acid substitutions in variants selected with antibodies that prevent virion attachment to cellular receptors. Positions of the amino acid substitutions in variants (and revertants of 2_a-1) as well as the proposed membrane-spanning region (MSR) and potential glycosylation sites (shaded boxes) are indicated.

petition antibody binding and additive neutralization assays indicated that sites 14 and 1 partially overlap site 12 and that sites 12 and 2 partially overlap site 23 (18). Thus, these three regions are brought together at the surface of the molecule, presumably near its receptor recognition site. This suggests that the receptor-binding site on HN is probably also defined by noncontiguous regions of the molecule brought together in the native structure. These findings are consistent with those from similar studies of the antigenic structure of the attachment proteins of other paramyxoviruses such as Sendai virus (36) and parainfluenza virus type 3 (5).

Regions of a virion attachment protein directly involved in receptor recognition might be expected to show significant sequence conservation among similar viruses. This would certainly not be the case for antibody-binding epitopes, which are inherently variable. The domain recognized by MAbs to sites 14 and 1 and that recognized by MAbs to sites 12 and 2 are not well conserved in the members of the family *Paramyxoviridae* (Fig. 8). It is possible that the relationship between these antigenic sites and the receptor recognition site in the HN of NDV is similar to that in the hemagglutinin of influenza virus. X-ray crystallographic studies of the structure of the latter protein complexed with cell receptor analogs show sialic acid bound in a pocket in the molecule surrounded by antibody-binding sites (40). This gives the virus a means to escape from neutralizing antibody, yet retain its receptor recognition site.

With our findings concerning the site 1 and 14 MAbs and those obtained with the Beaudette (30, 42) and D26 (10, 28) isolates of NDV, three groups have now characterized MAbs to epitopes localized to the most hydrophilic region of

VIRUS	SITE 23	SITES 14 AND 1	SITES 12 AND 2
NDV	193-FSGCRDHS-201	345-PDEQDYQIR-353	513-RITRVSSSR-521
SV5	182-LN Q VS-190	334-SQN AT VQ-342	512-SSQQFG G-520
Mumps	199-NAN K TS-207	351-SGP QDLQ-359	527-VLAPYGTQG-535
Sendai	213-TQ A IGK-221	358-QQVSQDTCN-366	521-IKDVQLEVA-529
hPF-3	211-TR Q IGK-219	356-GKTQRCN-364	521-IRNKTAL AG-529

FIG. 8. Comparison of the deduced amino acid sequences of residues 193 to 201, 345 to 353, and 513 to 521 for different paramyxoviruses. Only amino acid (single-letter code) differences are shown. Positions of variant substitutions are indicated by arrowheads. The sequences are as follows: NDV AV (23), simian virus 5 (SV5) (11), mumps virus (39), Sendai virus (1), and human parainfluenza virus type 3 (hPF-3) (8).

the molecule in three different isolates of the virus. This region is probably the immunodominant domain on HN. Also, it is very likely that the epitope recognized by the site 14 MAbs corresponds to the A1 epitope previously described for the HN of the Beaudette isolate. MAbs to the latter epitope also inhibit HA, bind in Western blots, and select variants with substitutions at residues 347 and 349 (30, 42).

Unlike the other HN antigenic sites, that defined by HN residues 193 to 201 (our site 23) showed a significant degree of conservation among the paramyxoviruses (Fig. 8). Residues 195, 196, 198, and 199 were almost completely conserved throughout the group. When one broadens the comparison (residues 171 to 206) to include residues on either side of those known to contribute to the site 23 epitope, an even more remarkable degree of conservation is revealed (19).

Previous studies have revealed that this domain is also directly involved in the NA activity of HN. Substitution at residue 194 or 201 results in markedly altered NA (19) but, as shown here, had no significant effect on sensitivity to periodate treatment of erythrocytes (Fig. 6). On the other hand, although substitution at HN residue 193 (variant 23_a-3) has no effect on NA activity (19), the relative periodate resistance resulting from a substitution of leucine for the phenylalanine at residue 193 of the HN of the AV isolate indicates that this residue is probably directly involved in receptor recognition. Thus, the site 23 domain includes residues involved in both functions of the molecule, and substitution within the domain differentially affects the attachment and NA activities of the protein. This speaks to the long-debated question of whether or not the attachment and NA sites on HN are separate. Certainly, a great deal of evidence now exists to support the notion that the two sites are separable (reviewed in reference 19). However, the stretch of amino acids from residues 193 to 201 is clearly involved in both functions, so the two active sites probably overlap to a certain extent. Confirmation of this awaits the solution of the three-dimensional structure of the protein.

The isolation of variants which are not neutralized by the selecting MAb but retain the ability to bind that antibody adequately enough to ensure that the addition of anti-immunoglobulin can effect neutralization has also been described for antibodies to the hemagglutinin of influenza A viruses (9). In fact, in both that system and NDV (17), the antibodies which bind but do not neutralize nonetheless inhibit the ability of the virus to agglutinate erythrocytes. This phenomenon may be explained by considering the prevention of attachment to cellular receptors by antibody as a competition between HN for its receptor on cells and that of the antibody for its epitope on HN. Inhibition of HA but not attachment to chick cells may be explained by a differ-

ence in affinity of the virus for receptors on chicken embryo fibroblasts and erythrocytes. In wild-type HN, antibody binds with sufficient avidity to compete with the binding of HN to its cellular receptor on both erythrocytes and chick cells. Variants which retain the ability to bind the selecting antibody but escape neutralization by it may bind that antibody with reduced avidity such that binding to the receptor on chick cells, but not erythrocytes, is favored. These variants are selected only by chance as they should be indistinguishable from wild type in the anti-immunoglobulin treatment required for variant selection.

ACKNOWLEDGMENTS

We gratefully acknowledge the excellent technical assistance of Jeff Barbon, Denis Bernard, and Theresa Sergel as well as Louissik Shabo for help in preparation of the manuscript. We thank D. J. King of the Southeast Poultry Research Laboratory for providing the GL and MT isolates of NDV.

This investigation was supported by FIRST (AI-24770) and Public Health Service (AI-12467) grants from the National Institute of Allergy and Infectious Diseases and a National Foundation for Infectious Diseases Burroughs-Wellcome Matching Grant for Young Investigators (awarded to R.M.I.).

REFERENCES

- Blumberg, B., C. Giorgi, L. Roux, R. Raju, P. Dowling, A. Chollet, and D. Kolakofsky. 1985. Sequence determination of the Sendai virus HN gene and its comparison to the influenza virus glycoproteins. *Cell* 41:269-278.
- Bratt, M. A., and W. R. Gallaher. 1969. Preliminary analysis of the requirements for fusion from within and fusion from without by Newcastle disease virus (NDV). *Proc. Natl. Acad. Sci. USA* 64:536-543.
- Chou, P. Y., and G. D. Fasman. 1974. Prediction of protein conformation. *Biochemistry* 13:222-244.
- Clavell, L. A., and M. A. Bratt. 1972. Hemolytic interaction of Newcastle disease virus and chicken erythrocytes. II. Determining factors. *Appl. Microbiol.* 23:461-470.
- Coelingh, K. L., C. C. Winter, E. D. Jorgensen, and B. R. Murphy. 1987. Antigenic and structural properties of the hemagglutinin-neuraminidase glycoprotein of human parainfluenza type 3 virus: sequence analysis of variants selected with monoclonal antibodies which inhibit infectivity, hemagglutination, and neuraminidase activities. *J. Virol.* 61:1473-1477.
- Eisenberg, D., R. Weiss, T. Terwilliger, and W. Wilcox. 1982. Hydrophobic moments and protein structure. *Faraday Symp. Chem. Soc.* 17:109-120.
- Eisenberg, D., W. Wilcox, and A. McLahlan. 1986. Hydrophobicity and amphiphilicity in protein structure. *J. Cell. Biochem.* 31:11-17.
- Elango, N., J. E. Coligan, R. C. Jambou, and S. Venkatesan. 1986. Human parainfluenza type 3 virus hemagglutinin-neuraminidase glycoprotein: nucleotide sequence of mRNA and limited amino acid sequence of CNBr peptides of the purified protein. *J. Virol.* 57:481-489.
- Gitelman, A. K., N. V. Kaverin, I. G. Kharitonov, I. A. Rudneva, E. L. Sklyanskaya, and V. M. Zhdanov. 1986. Dissociation of the haemagglutination inhibition and the infectivity neutralization in the reactions of influenza A/USSR/90/77 (H1N1) virus variants with monoclonal antibodies. *J. Gen. Virol.* 67:2247-2251.
- Gotoh, B., T. Sakaguchi, K. Nishikawa, N. M. Inocencio, M. Hamaguchi, T. Toyoda, and Y. Nagai. 1988. Structural features unique to each of the three antigenic sites on the hemagglutinin-neuraminidase protein of Newcastle disease virus. *Virology* 163:174-182.
- Hiebert, S. W., R. G. Paterson, and R. A. Lamb. 1985. Hemagglutinin-neuraminidase protein of simian virus 5: nucleotide sequence of the mRNA predicts an N-terminal anchor. *J. Virol.* 54:1-6.
- Iorio, R. M., J. B. Borgman, R. L. Glickman, and M. A. Bratt. 1986. Genetic variation within a neutralizing domain on the haemagglutinin-neuraminidase glycoprotein of Newcastle disease virus. *J. Gen. Virol.* 67:1393-1403.
- Iorio, R. M., and M. A. Bratt. 1983. Monoclonal antibodies to Newcastle disease virus: delineation of four epitopes on the HN glycoprotein. *J. Virol.* 48:440-450.
- Iorio, R. M., and M. A. Bratt. 1984. Monoclonal antibodies as functional probes of the HN glycoprotein of Newcastle disease virus: antigenic separation of the hemagglutinating and neuraminidase sites. *J. Immunol.* 133:2215-2219.
- Iorio, R. M., and M. A. Bratt. 1984. Neutralization of Newcastle disease virus by monoclonal antibodies to the HN glycoprotein: requirement for antibodies to four sites for complete neutralization. *J. Virol.* 51:445-451.
- Iorio, R. M., and M. A. Bratt. 1985. Selection of unique antigenic variants of Newcastle disease virus with neutralizing monoclonal antibodies and anti-immunoglobulin. *Proc. Natl. Acad. Sci. USA* 82:7106-7110.
- Iorio, R. M., R. L. Glickman, and M. A. Bratt. 1987. Characterization of unique antigenic variants in the hemagglutinin-neuraminidase of Newcastle disease virus and the unique isolation of a neutralization-sensitive revertant of one of these variants, p. 369-374. In B. W. Mahy and D. Kolakofsky (ed.), *The biology of the negative strand viruses*. Elsevier Biomedical Press, New York.
- Iorio, R. M., R. L. Glickman, A. M. Riel, J. P. Sheehan, and M. A. Bratt. 1989. Functional and neutralization profile of seven overlapping antigenic sites on the HN glycoprotein of Newcastle disease virus: monoclonal antibodies to some sites prevent viral attachment. *Virus Res.* 13:245-262.
- Iorio, R. M., R. J. Syddall, R. L. Glickman, A. M. Riel, J. P. Sheehan, and M. A. Bratt. 1989. Identification of amino acid residues important to the neuraminidase activity of the HN glycoprotein of Newcastle disease virus. *Virology* 173:196-204.
- Jorgensen, E. D., P. L. Collins, and P. T. Lomedico. 1987. Cloning and nucleotide sequence of Newcastle disease virus hemagglutinin-neuraminidase mRNA: identification of a putative sialic acid binding site. *Virology* 156:12-24.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* 227:680-685.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
- McGinnes, L. W., A. Wilde, and T. G. Morrison. 1987. Nucleotide sequence of the gene encoding the Newcastle disease virus hemagglutinin-neuraminidase protein and comparisons of paramyxovirus hemagglutinin-neuraminidase protein sequences. *Virus Res.* 7:187-202.
- Millar, N. S., P. Chambers, and P. T. Emmerson. 1986. Nucleotide sequence analysis of the haemagglutinin-neuraminidase gene of Newcastle disease virus. *J. Gen. Virol.* 67:1917-1927.
- Moore, N. F., and D. C. Burke. 1974. Characterization of the structural proteins of different proteins of different strains of Newcastle disease virus. *J. Gen. Virol.* 25:275-289.
- Morrison, T. G. 1988. Structure, function, and intracellular processing of paramyxovirus membrane proteins. *Virus Res.* 10:113-136.
- Nagai, Y., M. Hamaguchi, K. Maeno, M. Iinuma, and T. Matsumoto. 1980. Proteins of Newcastle disease virus. A comparison by partial protease digestion among the strains of different pathogenicity. *Virology* 102:463-467.
- Nishikawa, K., T. Morishima, T. Toyoda, T. Miyadai, T. Yokochi, T. Yoshida, and Y. Nagai. 1986. Topological and operational delineation of antigenic sites of the HN glycoprotein of Newcastle disease virus and their structural requirements. *J. Virol.* 60:987-993.
- Sakaguchi, T., T. Toyoda, B. Gotoh, N. M. Inocencio, K. Kuma, T. Miyata, and Y. Nagai. 1989. Newcastle disease virus evolution. I. Multiple lineages defined by sequence variability of the hemagglutinin-neuraminidase gene. *Virology* 169:260-272.
- Samson, A. C. R., M. Nesbit, A. M. Lyon, and G. Meulemans. 1988. Identification of haemagglutinin-neuraminidase antibody

- binding sites by Western blot analysis of antibody-resistant mutants and partial digest fragments of Newcastle disease virus. *J. Gen. Virol.* **69**:473–480.
31. Sato, H., S. Hattori, N. Ishida, Y. Imamura, and M. Kawakita. 1987. Nucleotide sequence of the hemagglutinin-neuraminidase gene of Newcastle disease virus avirulent strain D26: evidence for a longer coding region with a carboxyl terminal extension as compared to virulent strains. *Virus Res.* **8**:217–232.
 32. Schaper, U. M., F. J. Fuller, M. D. W. Ward, Y. Metrotra, H. O. Stone, B. R. Stripp, and E. V. DeBuysscher. 1988. Nucleotide sequence of the envelope protein genes of a highly virulent, neurotropic strain of Newcastle disease virus. *Virology* **165**: 291–295.
 33. Schuy, W., W. Garten, D. Linder, and H.-D. Klenk. 1984. The carboxyterminus of the hemagglutinin-neuraminidase of Newcastle disease virus is exposed at the surface of the viral envelope. *Virus Res.* **1**:415–426.
 34. Sheehan, J. P., R. M. Iorio, R. J. Syddall, R. L. Glickman, and M. A. Bratt. 1987. Reducing agent-sensitive dimerization of the hemagglutinin-neuraminidase glycoprotein of Newcastle disease virus correlates with the presence of cysteine at residue 123. *Virology* **161**:603–606.
 35. Smith, G. W., and L. E. Hightower. 1981. Identification of the P proteins and other disulfide-linked and phosphorylated proteins of Newcastle disease virus. *J. Virol.* **37**:256–267.
 36. Thompson, S. D., and A. Portner. 1987. Localization of functional sites on the hemagglutinin-neuraminidase glycoprotein of Sendai virus by sequence analysis of antigenic and temperature-sensitive mutants. *Virology* **160**:1–8.
 37. Underwood, P. A. 1985. Receptor binding characteristics of strains of the influenza Hong Kong subtype, using a periodate sensitivity test. *Arch. Virol.* **84**:53–61.
 38. Underwood, P. A., J. J. Skehel, and D. C. Wiley. 1987. Receptor-binding characteristics of monoclonal antibody-selected antigenic variants of influenza virus. *J. Virol.* **61**:206–208.
 39. Waxham, M. N., J. Aronowski, A. C. Server, J. A. Smith, J. S. Wolinsky, and H. M. Goodman. 1988. Sequence determination of the mumps virus HN gene. *Virology* **164**:318–325.
 40. Weis, W., J. H. Brown, S. Cusack, J. C. Paulson, J. J. Skehel, and D. C. Wiley. 1988. Structure of the influenza virus haemagglutinin complexed with its receptor, sialic acid. *Nature (London)* **333**:426–431.
 41. Weiss, S. R., and M. A. Bratt. 1974. Polyadenylate sequences on Newcastle disease virus mRNA synthesized in vivo and in vitro. *J. Virol.* **13**:1220–1230.
 42. Yusoff, K., M. Nesbit, H. McCartney, P. T. Emmerson, and A. C. R. Samson. 1988. Mapping of three antigenic sites on the haemagglutinin-neuraminidase protein of Newcastle disease virus. *Virus Res.* **11**:319–333.