JOURNAL OF VIROLOGY, Mar. 1994, p. 1790–1796
0022-538X/94/$04.00+0

Vol. 68, No. 3

Antigenicity of the N8 Influenza A Virus Neuraminidase: Existence of an Epitope at the Subunit Interface of the Neuraminidase

T. SAITO,[1] G. TAYLOR,[2] W. G. LAVER,[3] Y. KAWAOKA,[1,4] AND R. G. WEBSTER[1,4]*

Department of Virology and Molecular Biology, St. Jude Children's Research Hospital, Memphis, Tennessee 38101 [1]; Department of Biochemistry, University of Bath, Claverton Down, Bath BA2 7AY, United Kingdom[2]; John Curtin Medical School, The Australian National University, Canberra, Australian Capital Territory 2601, Australia[3]; and Department of Pathology, University of Tennessee, Memphis, Tennessee 38163[4]

Received 27 September 1993/Accepted 10 December 1993

To locate antigenic epitopes on the N8 neuraminidase (NA), we generated a panel of 97 monoclonal antibodies (MAbs), 66 of which inhibited NA activity (NI antibodies). Three groups of NI MAbs were identified from their different reactivities with escape mutants. Group 1 antibodies recognized the peptide loop containing residues 344 to 346, which appears to be an immunodominant region on the rim of the enzyme center of the N8 NA. Group 2 antibodies recognized a novel epitope containing residues 150, 199, 367, 399, and 400 (N2 numbering). From the location of these residues on the three-dimensional structure of the N8 NA, the epitope appears to be located at the interface of two adjacent monomers in the tetrameric NA, one contributing residues 150 and 199 and the other contributing residues 367 and 399 to 400. The available evidence indicates that the MAbs of this group react with the NA only after it is fully assembled. The third group of antibodies recognized the peptide loops containing residues 367 and 399 to 400. All of the amino acid substitutions in N8 escape mutants which affect the NI activity of antibodies were located in the peptide loops known to form epitopes in the N2 and N9 subtypes, indicating that antigenic regions in the NA head inducing NI antibodies appear to be similar among different subtypes of influenza A viruses. The MAbs used in this study will be valuable in studying the role of each N8 NA epitope in host immune defense systems and in the kinetics analysis of the biosynthesis of the enzyme.

The neuraminidase (NA) surface glycoprotein of influenza A and B viruses is a homotetramer in which each monomer consists of a hydrophobic membrane anchor, a stalk, and a head region that contains the catalytic and antigenic domains (2). Of the influenza virus NAs that have been crystallized, three-dimensional structures have been determined for the N2 (7, 24), the N9 (22), and the influenza B virus NAs (6). The availability of crystallized forms of the N8 NA has permitted X-ray diffraction study of the molecule. Preliminary findings (21) suggested considerable similarity between the folding patterns of the N8 and those of the N2 and N9 NAs. Antigenic mapping of the N2 and N9 subtypes (1, 13, 14, 26–28) has revealed at least two antigenic epitopes that are involved in enzyme inhibitory activity and has suggested another antigenic site(s) on the bottom of the head region.

Only one NA epitope, termed the NC-41 epitope on the N9 NA, has been studied in detail (23). This discontinuous epitope comprises five peptide loops located at the rim of the enzyme active site. All but one of the escape mutants obtained for N9 NA have amino acid substitutions within the NC-41 epitope (23, 26), consistent with results of earlier studies on the antigenicity of the N2 subtype (1). Hence, the NC-41 epitope appears the dominant one for enzyme-inhibiting antibodies on the NA molecule.

Other epitopes for enzyme-inhibiting antibodies involve the peptide loops containing amino acid residue 150, 199, or 220. An escape mutant with a substitution at residue 220 was isolated in studies of the N9 subtype (26), whereas mutants with substitutions at residues 150 and 221, respectively, were isolated in the N2 study (1). Colman et al. (7), from studying the three-dimensional structure of the N2 NA and amino acid sequence changes among human influenza virus N2 isolates, suggested that residues 153 and 197 to 199 were part of the antigenic determinants. Amino acid sequence comparison between equine and avian N8 isolates showed that NA residues 149 and 199 were variable among equine isolates (18). Thus, there are obviously other epitopes on the NA molecules to be characterized.

To characterize the epitopes that have not been defined on the N2 and N9 NAs, we carried out antigenic mapping of the N8 NA. Here we describe a novel epitope on the N8 NA which seems to span two neighboring monomers and which may not bind antibody until the NA is fully assembled.

MATERIALS AND METHODS

Viruses. NWS-duck/Ukraine/1/63 (H1N8) (NWS-N8) is a reassortant virus with hemagglutinin from A/NWS/33 (H1N1) and NA from A/duck/Ukraine/1/63 (H3N8). The virus was cloned by limiting dilution twice in embryonated eggs before selection of escape mutants.

Monoclonal antibodies (MAbs). Hybridoma cells were obtained by the method of Köhler and Milstein (11). Briefly, BALB/c mice were infected intranasally with NWS-N8, and 0.01% amantadine was given in the water supply on the following day to prevent death from this highly lethal virus. The hybridoma fusions were carried out 3 days after intravenous booster immunization with dissolved crystals of N8 NA (21). The hybridoma cells were screened for antibody produc-

* Corresponding author. Mailing address: Department of Virology and Molecular Biology, St. Jude Children's Research Hospital, 332 N. Lauderdale, P.O. Box 318, Memphis, TN 38101. Phone: (901) 522-0400. Fax: (901) 523-2622.

tion by both the NA inhibition (NI) assay and the enzyme-linked immunosorbent assay (ELISA).

Serological tests. The hemagglutination, NA, and NI assays were performed by methods recommended by the World Health Organization (4). Fetuin was used as a substrate for the NI assay if not otherwise stated. ELISA was done as described previously except that purified virus diluted in phosphate-buffered saline (PBS) was used as an antigen (10).

Selection of antigenic variants. Antigenic variants (escape mutants) were selected in embryonated eggs. NWS-N8 in allantoic fluid (1 μl; 5×10^6 50% egg infective doses) was inoculated into eggs with 100 μl of undiluted ascitic fluid containing anti-N8 MAb (28). The virus was cultivated with the antibody twice to eliminate residual parental virus. Then allantoic fluids were injected into eggs at a 1/1,000 dilution to amplify the escape mutants. The mutants were cloned twice at limiting dilution in eggs and tested in the NI assay to determine their reactivity with various MAbs.

Sequencing of escape mutants. Viral RNA was prepared from allantoic fluid as described elsewhere (5). Synthetic oligonucleotides 5′-GTAACTCAATGGCACAATAC-3′ (primer 1) and 5′-AACAAGGAGTTTTTTAA-3′ (primer 2) were used for cDNA synthesis and PCR. Primer 1 corresponds to the NA sequence of Duck/Ukraine/1/63 (nucleotides 204 to 223), and primer 2 is complementary to the sequence for nucleotides 1435 to 1454. cDNA was made with avian myeloblastosis virus reverse transcriptase (United States Biochemical Corp., Cleveland, Ohio) and primer 1. PCR was carried out with *Taq* polymerase (Promega, Madison, Wis.) and primers 1 and 2. After purification of the PCR products (DNA Purification System; Promega), sequencing was performed by means of an fmol sequencing system (Promega).

Metabolic labeling of NA and immunoprecipitation. Confluent monolayers of Madin-Darby canine kidney (MDCK) cells in 25-cm^2 culture flasks were infected with 10^9 50% egg infective doses of NWS-N8 in modified Eagle's medium (MEM) with 4% bovine serum albumin (BSA) and incubated for 1 h at 37°C. After addition of 4 ml of MEM-BSA, incubation was continued for another 4 h at 37°C in 5% CO_2. The cells were then washed twice with prewarmed PBS, and 1 ml of methionine-free MEM was added for 15 min. Then 200 μCi of [^{35}S]methionine (Tran ^{35}S; ICN, Irvine, Calif.) was added to the medium for pulse-labeling. After 5 min of labeling, the cells were washed twice with warm PBS, and the medium was replaced with MEM containing 10 times the concentration of unlabeled methionine for chase. At the end of the chase, cells were washed twice with cold PBS and harvested. Cells were lysed in 50 mM Tris (pH 7.5)–100 mM NaCl–1% Triton X-100–20 mM iodoacetamide for 30 min on ice. After removal of cell debris by centrifugation with a microcentrifuge, cell lysates were incubated with MAbs overnight at 4°C. Antigen-antibody complexes were precipitated by rabbit anti-mouse immunoglobulin (heavy plus light chains) bound to solid beads (Immunobead reagent; Bio-Rad, Richmond, Calif.). Immunoprecipitates were resuspended in sample buffer prepared without 2-mercaptoethanol. Samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% polyacrylamide) after being boiled for 4 min.

Gradient centrifugation. Virus-infected cells were pulse-labeled for 5 min with [^{35}S]methionine and chased for 20 min with excess unlabeled methionine. The cells were lysed in the Triton buffer described above, and after microcentrifugation the cell lysates were loaded onto 5 to 25% sucrose gradients containing 0.1% Triton X-100 in the same buffer. Gradients were centrifuged for 16.5 h at 174,500 × *g* at 20°C in an SW41 rotor (Beckman). Thirty 400-μl fractions were collected from each tube, and divided into two lots, and the odd-numbered fractions (starting at the bottom of the tube) were immunoprecipitated with MAbs. Immunoprecipitates were analyzed by SDS-PAGE (10% polyacrylamide) under nonreducing conditions. BSA (4.9S), aldolase (8.6S), and catalase (11.3S) were used as molecular markers.

Peptide-*N*-glycosidase F treatment. Virus-infected cells were pulse-labeled with [^{35}S]methionine and chased for 20 min with unlabeled methionine as described above. The cells were then lysed in 100 mM sodium phosphate buffer (pH 7.1)–20 mM EDTA–0.2% SDS–1% Triton X-100. After removal of cell debris by centrifugation, lysates were divided into two lots. One sample was treated with 6 U of peptide-*N*-glycosidase F (PNGase F; Boehringer Mannheim Biochemicals, Indianapolis, Ind.) overnight at 37°C; the other served as a control. After overnight incubation, each sample was divided into two parts, and then each part was immunoprecipitated with either MAb N8-4 or N8-10. Immunoprecipitates were analyzed by SDS-PAGE (16% polyacrylamide) under reducing conditions.

RESULTS

Characterization of MAbs. The hybridomas were screened for antibody production by using the NI assay and ELISA. Of the 97 MAbs generated, 66 were positive by both assays (NI antibody) and 31 were positive only by ELISA (ELISA antibody). The titers of the NI MAbs, determined with fetuin or *N*-acetylneuramin-lactose (NAL) as the substrate, are reported in Table 1. Some of the MAbs (e.g., N8-1, N8-2, and N8-3) showed similar titers with the two substrates, whereas others (e.g., N8-5, N8-6, and N8-8) inhibited enzyme activity only with fetuin, suggesting that there may be differences in the ways in which these MAbs inhibit enzyme activity.

Epitope mapping with escape mutants. To locate antigenic epitopes on the N8 NA, we selected escape mutants by cultivating NWS-N8 in the presence of antibodies. For each of the 19 escape mutants, we sequenced the head region of the NA, which corresponds to amino acids 90 to 470 (18). Amino acid substitutions were found at residues 150, 199, 284, 344, 346, 367, 399, and 400 (N2 numbering) (Table 1; Fig. 1a). All of the mutants but one had a single-amino-acid substitution in their enzyme head; the exception, V-88, had two mutations, at residues 284 and 367. However, the mutation at residue 284 is located at the bottom of the NA head (Fig. 1a) and is therefore not likely to be responsible for the change in antigenicity. Therefore the mutation at residue 367 is probably responsible for the antigenic phenotype of V-88.

It was possible to divide the MAbs into three groups based on their reactivity patterns with the escape mutants (Tables 1 and 2). The first group either lost or had reduced reactivities to mutants with a substitution at position 344 or 346. Among the 66 clones of NI antibodies, 37 qualified for this group, with the peptide loop at 344 to 346 constituting the immunodominant region (Fig. 1a). MAbs which lost or had decreased NI activities toward the variant with a substitution at 150 made up the second group. One of these antibodies, N8-4, selected a variant that had a substitution at residue 199; it did not show NI activities with variants having substitutions at 150, 199, 367, 399, or 400 (Table 2). Interestingly, all of the MAbs examined in group 2 (N8-1, N8-2, N8-3, N8-4, N8-11, N8-12, N8-13, and N8-14) inhibit enzyme activity at high titers with both fetuin and NAL as substrates (Table 1). The third group of antibodies lost or had reduced reactivity toward variants with a substitution at position 367 or 399.

We could not select escape mutants with MAbs N8-14 and

TABLE 1. MAbs and escape mutants of N8 NA

MAb (group)[a]	NI titer[b] with:		Variant selected	Nucleotide		Amino acid	
	Fetuin	NAL		Position[c]	Change	Position[d]	Change
N8-1 (2)	3.1	2.7	V-01	462	A to G	150 (148)	Lys to Glu
N8-2 (2)	3.8	>4	V-02	462	A to G	150 (148)	Lys to Glu
N8-3 (2)	3.8	>4	V-03	462	A to G	150 (148)	Lys to Glu
N8-4 (2)	>4	>4	V-04	612	T to C	199 (198)	Ser to Pro
N8-5 (1)	3.4	<1	V-05	1043	T to A	344 (341)	Asn to Lys
N8-6 (1)	>4	<1	V-06	1047	G to A	346 (343)	Gly to Arg
N8-7 (1)	>4	2.5	V-07	1047	G to A	346 (343)	Gly to Arg
N8-8 (1)	>4	<1	V-08	1047	G to A	346 (343)	Gly to Arg
N8-86 (1)	2.5	<1	V-86	1047	G to A	346 (343)	Gly to Arg
N8-85 (1)	3.3	1.9	V-85	1047	G to A	346 (343)	Gly to Arg
N8-9 (1)	>4	>4	V-09.2	1047	G to A	346 (343)	Gly to Arg
N8-10 (1)	3.6	<1	V-10	1048	G to A	346 (343)	Gly to Glu
N8-88 (3)	>4	<1	V-88	868	A to G	284 (283)	Asp to Gly
				1111	G to A	367 (364)	Ser to Asn
N8-83 (3)	>4	<1	V-83	1203	G to A	399 (395)	Asp to Asn
N8-87 (3)	>4	<1	V-87	1203	G to A	399 (395)	Asp to Asn
N8-11 (2)	3.9	3.9	V-11	1203	G to A	399 (395)	Asp to Asn
N8-82 (3)	>4	<1	V-82	1206	A to T	400 (396)	Asn to Tyr
N8-12 (2)	>4	>4	V-12	1208	T to A	400 (396)	Asn to Lys
N8-13 (2)	>4	>4	V-13	1208	T to A	400 (396)	Asn to Lys
N8-14 (2)	2.8	2	—[e]				
N8-90[f]	<1	ND[g]	—				
N8-81 (1)	>4	1.7	NS[h]				
N8-15 (3)	3.2	<1	NS				
N8-16 (1)	3.2	<1	NS				
N8-84 (1)	3.2	<1	NS				

[a] Antibody groups based on the NI test with escape mutants (Table 2).
[b] The NI assay was done with either fetuin or NAL as the substrate; antibody titers required for 50% reduction of the NA activity are expressed in $\log_{10}$ units.
[c] Although only one escape mutant was selected, it probably represents the most prevalent mutant (3).
[d] Reported in the N2 numbering system with N8 numbers in parentheses.
[e] —, no escape mutants could be selected despite multiple attempts.
[f] ELISA-positive antibody.
[g] ND, not determined.
[h] NS, selection of escape mutants was not attempted.

N8-90. This failure can be attributed to the low titer of N8-14 (28) and to the lack of NI activity by N8-90, which was used as a representative of ELISA antibodies (26, 27).

Location of substitution on the three-dimensional structure. We next sought to find the locations of epitopes on the N8 NA. The epitope recognized by MAb N8-4 involved peptide loops containing residues 150, 199, 367, and 399 to 400 (Fig. 1b). We thought that MAb N8-4 might recognize a previously uncharacterized epitope, since the changes in the escape mutants (at residues 150 and 199) were not in equivalent peptide loops to those recognized by the NC-41 antibody on N9 (23). As shown in Fig. 1b, residues 150 and 199 are situated on the rim of the enzyme active center opposite residues 367 and 399 to 400. The distance between these two groups of residues within a monomer is much greater than their distance between neighboring monomers (Table 3). For example, the intramolecular distance between amino acids 199 and 399 is 42.5 Å (4.25 nm), but the distance between them on two neighboring monomers is 14.4 Å (1.44 nm). The greatest distance between residues involved in the NC-41 epitope on N9 is 34 Å (3.4 nm). In several Fab fragments, the immunoglobulin hypervariable loops that interact with antigen have a diameter of approximately 30 Å (3 nm) (15, 17, 19). Therefore, the intramolecular distance of 42.5 Å appears too large to be recognized by a Fab fragment. Thus, it is possible that the epitope recognized by MAb N8-4 involves residues 150 and 199 in one monomer and residues 367 and 399 to 400 of a neighboring monomer.

Characterization of a novel epitope on N8 NA. Our working hypothesis was that MAb N8-4 would recognize an epitope spanning more than one NA monomer. To test this prediction, we attempted to immunoprecipitate pulse-labeled NA with MAb N8-4, representing the MAbs recognizing the putative novel epitope, and MAb N8-10, representing group 1 MAbs which seemed to bind to an epitope on a monomer. As shown in Fig. 2a, MAb N8-10 immunoprecipitated a small amount of monomer and appreciable amounts of dimer forms of NA from the beginning of the chase period. The low level of monomer detection in these experiments probably reflects the incomplete folding of newly synthesized monomers (9), which in turn would prevent precipitation by MAbs recognizing conformational epitopes. A similar situation has been seen with other viral antigens (8). Tetrameric NA could not be detected by gel electrophoresis, probably because the tetramer was dissociated into the dimer during electrophoresis (9).

On the other hand, MAb N8-4 did not precipitate any NA at 0 min of chase and precipitated a very small amount of dimer at 5 min. Both MAbs precipitated larger amounts of NA at 20 min of chase. The failure of MAb N8-4 to precipitate either monomeric or dimeric NA at 0 min of chase suggests that it recognizes the epitope existing only on the oligomeric form of the NA and that dimerization is not sufficient to form this epitope. Thus the NA dimer precipitated by MAb N8-4 might be from the tetrameric form of the NA, which was dissociated into dimers by electrophoresis. The reactivity patterns of MAbs N8-1 and N8-11 were similar to that of N8-4 (data not

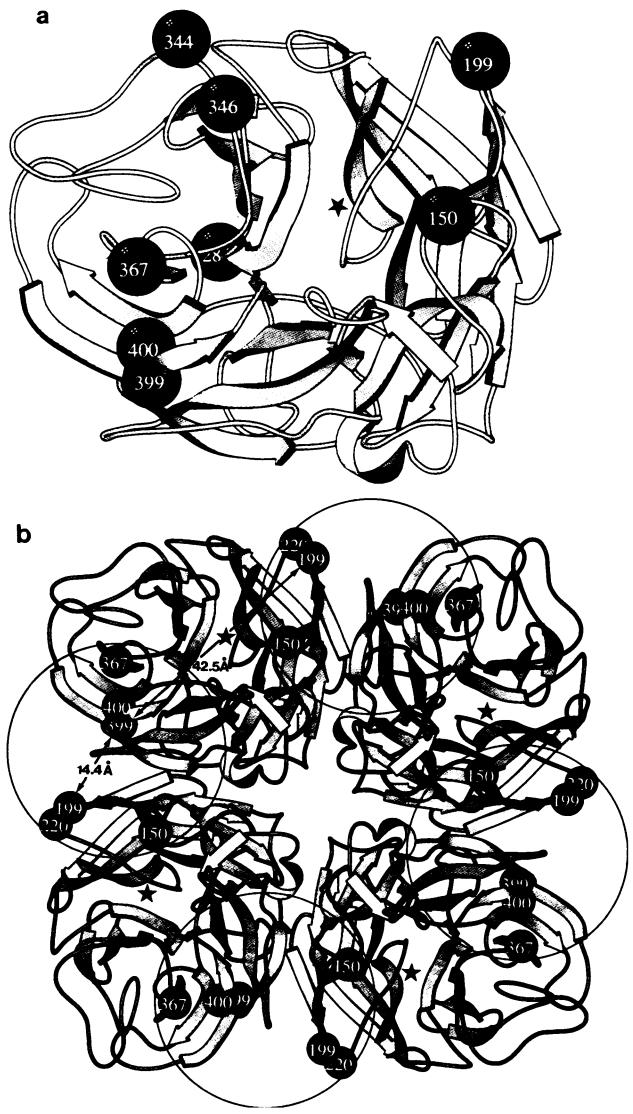

FIG. 1. Schematic diagrams of chain folding in the N8 NA viewed from the top of the fourfold symmetry axis. Figures were produced with the program MOLSCRIPT (12). (a) Amino acid substitutions found in N8 escape mutants are plotted on the monomer. (b) Amino acids recognized by MAb N8-4 are plotted on the N8 NA tetramer together with residue 220, which is antigenic in N9 (29) and is 5.4 Å (0.54 nm) away from residue 199. The predicted locations of the epitope recognized by MAb N8-4 are shown as circles of approximately 30 Å (3 nm) in diameter. The enzyme active sites are shown as stars.

shown), suggesting that antibodies of group 2 may recognize a similar epitope to that recognized by MAb N8-4.

Higher-molecular-weight species of the NA appeared at the later periods (20 min) along with the species whose molecular weight was similar to that found at the early time (Fig. 2a), as a result of the completion of oligosaccharide processing in the Golgi (9). Since both MAbs recognized the immature and mature species of NA, this suggests that they recognize the epitope that does not require oligosaccharide processing. This was further confirmed by treatment of cell lysates with PNGase F, which cleaves all types of N-linked oligosaccharides from protein (20), before immunoprecipitation (Fig. 2b). PNGase F treatment did not alter the precipitation abilities of either MAb. The molecular weight of NA digested by PNGase F was similar to that predicted for unglycosylated NA from amino acid sequences. Therefore, neither MAb appears to recognize oligosaccharide as a part of the epitope.

To examine whether the NAs immunoprecipitated by MAb N8-4 are dimers or tetramers, we performed gradient centrifugation of pulse-labeled NA in the cell lysates followed by immunoprecipitation (Fig. 3). At 0 min of chase, the group 1 antibody N8-10 precipitated NAs in fractions 11 through 17, with a peak at fraction 17. At 20 min, MAb N8-10 precipitated NAs in fractions 7 through 17, with two peaks at fractions 11 and 17. The first peak corresponded to tetrameric NA, and the second corresponded to dimeric NA. Monomeric NA was not detected in the experiment because of its small amount. At 0 min, antibody N8-4 did not precipitate NAs, consistent with results in Fig. 2a. It did, however, precipitate NAs at 20 min in fractions that corresponded to tetrameric NAs (fractions 9 to 13). No precipitation was detected at fraction 15 or 17 by MAb N8-4 even after longer exposure. Thus, these results suggest that MAb N8-4 recognizes a tetramer-specific epitope.

Amino acids 367, 399, and 400 are involved in two distinct epitopes. Group 3 MAbs recognized peptide containing residues 367 and 399 to 400, which have been shown to be components of the NC-41 epitope on N9 (23). These peptide loops were also involved in the epitope recognized by MAb N8-4. This suggested that these two loops were involved in multiple epitopes. To investigate this possibility, we performed radioimmunoprecipitation experiments with group 3 MAbs N8-15, N8-82, N8-83, and N8-88. Although the antibodies showed similar reactivity patterns in NI tests (Table 2), they produced different patterns in radioimmunoprecipitation experiments (Fig. 2c). MAb N8-82 precipitated NA at both 0 and 20 min of chase, showing similar reactivity to MAb N8-10, a group 1 antibody. However, MAbs N8-15, N8-83, and N8-88 did not precipitate any NA at 0 min of chase and then precipitated NA at 20 min of chase. This reaction pattern is similar to that of MAb N8-4. These results indicate that loops containing residues 367 and 399 to 400 are involved in at least two epitopes and that group 3 MAbs should be subdivided further.

DISCUSSION

Only one epitope on influenza virus NA has been characterized by X-ray crystallography. This is the epitope on the N9 NA, termed the NC-41 epitope, which binds NC-41 antibody; the characterization was done with crystals of the N9 NA complexed with NC-41 Fab fragments (23). This epitope has an area of about 900 $Å^2$ (9 nm^2) and is discontinuous, comprising five peptide loops with a total of 19 amino acids making contact with 17 residues on five of the six complementarity-determining regions of the antibody.

To characterize the epitopes on the N8 NA, we have generated a panel of MAbs to the N8 NA; Fab fragments from some of these have been complexed with the N8 NA heads, and the complexes have been crystallized. So far, none of the complex crystals are of X-ray diffraction quality, but some may be suitable for high-resolution electron microscopy (15a).

In the meantime, we have determined the general location of some epitopes on the N8 NA by determining the sequence changes in escape mutants of the N8 NA selected with MAbs with NI activity. It has been shown previously that the residues which change in escape mutants are actually located in the epitope recognized by that particular MAb used in the selection and that no overall change in conformation of the protein occurred by the selection of escape mutants (22).

TABLE 2. Reactivity patterns and amino acid changes in escape mutants of N8 NA

Group[a] and MAbs	NI titer[b] for amino acid substitutions in variants:								
	150 (V-01)	199 (V-04)	344 (V-05)	346 (V-06)	346 (V-10)	367 (V-88)	399 (V-87)	400 (V-82)	NWS-N8 (parent)
I									
N8-5			<1						3.4
N8-6			2	<1	<1				>4
N8-10			2.3	<1	<1				3.6
N8-16			<1	<1	1.1				3.2
II									
N8-1	<1								3.1
N8-4	<1	<1				<1	<1	<1	>4
N8-11	<1					<1	<1	<1	3.9
N8-14	1.5								2.8
III									
N8-15						2.0	<1		3.2
N8-82						<1	1.7	<1	>4
N8-83						<1	<1	<1	>4
N8-88						<1	2.9		>4

[a] A total of 66 NI antibodies are divided into three groups based on their reactivity patterns with escape mutants. Group 1, group 2, and group 3 contained 37, 16, and 13 antibodies, respectively.

[b] Only representative NI data are shown; the titers are expressed in $\log_{10}$ units. No entry indicates that there was no significant difference from NWS-N8.

We have now located a novel epitope on the N8 NA which seems to involve surface loops on two adjacent monomers. The antibody directed to this epitope does not seem to bind until the NA is assembled into its final tetrameric form. This epitope on the N8 NA head contains peptide loops that involve residues 150, 199, 367, and 399 to 400. The residues involved in this epitope and the physical distances between them suggest that the epitope is present at the interface of adjacent monomers of the tetrameric NA. Colman et al. predicted the existence of an antigenic determinant across the subunit interface which involved residues 153, 197 to 199, and 400 to 403; this was based on the three-dimensional structure of the N2 NA and sequence variation among human influenza virus N2 isolates (7). Although we would expect that the epitope at the subunit interface should exist in both dimeric and tetrameric forms of NA, we found that MAb N8-4 did not bind NA dimers and that it bound only to tetramers (Fig. 3). The N8 NA monomer has one cysteine residue at position 45 in the stalk (18), and this is the only cysteine participating in intermolecule disulfide linkage to form dimeric forms of the NA, whereas 16 cysteines in the head region form intramolecule disulfide linkages. A pair of disulfide-bound dimers form the tetramer by noncovalent binding. The subunit interface in a tetramer is characterized by hydrophobic interactions and hydrogen bonding (24). The inability of MAb N8-4 to precipitate the dimer form of NA suggests that completion of these interface interactions requires tetramerization and that interface conformations are therefore different between dimeric and tetrameric forms of NA. Alternatively, conformational change on dimers may occur upon tetramerization, which does not alter the epitope recognized by MAb N8-10.

TABLE 3. Distance between amino acids in the N8-4 epitope

Amino acid	Intramolecular distance (Å) for amino acid:			Intermolecular distance[a] (Å) for amino acid:		
	367	399	400	367	399	400
150	27.8	31.1	30.5	26.3	16.4	21.0
199	36.5	42.5	41.0	24.1	14.4	18.3

[a] Distances between the amino acids in two neighboring monomers were measured.

All of the MAbs examined in group 2 show high NI titers with both fetuin and NAL as substrates. By contrast, only a limited number of antibodies in group 1 inhibit enzyme activity with both substrates. How do group 2 MAbs inhibit NA activity with a small substrate such as NAL? Since group 2 antibodies were shown to recognize residue 150, binding of antibody to residue 150 may have a special characteristic. Binding of antibody to residue 150 might inhibit enzyme activity because of its proximity to Asp-151, which has been implicated in catalysis by structural analysis on N2 NA complexed with sialic acids (25). Direct interaction of Asp-151 with sialic acid has also been demonstrated for the influenza B virus NA, whose structure is similar to that of the influenza A virus NA (6). Therefore, antibody binding to residue 150 may sterically interfere with the interaction between Asp-151 and substrate. The other explanation for the NI activity of group 2 MAbs with both fetuin and NAL is that these antibodies completely cover the enzyme active site by recognizing residues 150, 199, 367, 399, and 400 on the same NA monomer. This possibility is, however, much less likely since the evidence obtained in this study suggests that these MAbs recognize the epitope at the interface of the monomers.

The epitope at the interface appears to contain four peptide loops; we cannot exclude the involvement of additional loops. For example, the NC-41 epitope on N9 comprises five loops; however, escape mutants with a substitution in one of the five loops (loop 343 to 347) have never been isolated by MAbs against N9 (3, 26). Amino acids 220 and 221 have been shown to be part of the antigenic sites on both N2 (27) and N9 (26). The distance between amino acid 199, which is involved in the epitope recognized by MAb N8-4, and amino acid 220 within the same molecule is 5.3 Å (0.53 nm). One could postulate that the loop containing residue 220 is also involved in the epitope because of the proximity to residue 199 (Fig. 1b).

The peptide loop containing residues 344 to 346 appears to be immunodominant at the rim of the enzyme center, because

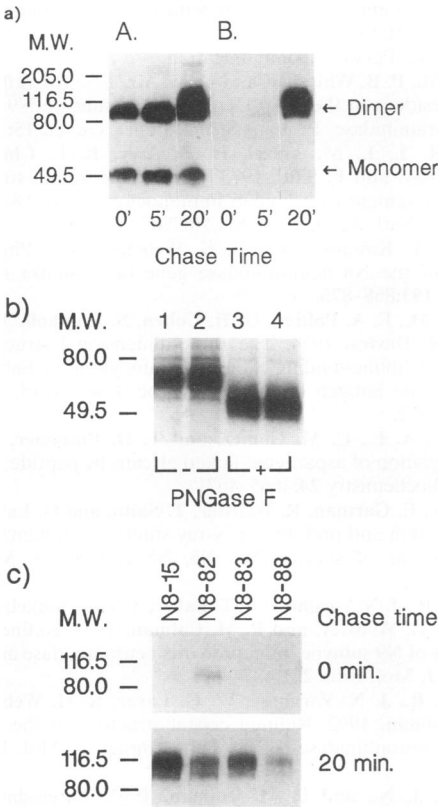

FIG. 2. Immunoprecipitations of NA by MAbs. (a) The infected cells were chased with unlabeled methionine for 0, 5, and 20 min. Immunoprecipitations were performed with MAb N8-10 (lanes A) or MAb N8-4 (lanes B). (b) The cell lysates were treated with PNGase F prior to immunoprecipitation. Lanes: 1 and 3, MAb N8-10; 2 and 4, MAb N8-4. (c) The infected cells were chased for 0 min (top) and 20 min (bottom). Immunoprecipitations were performed with MAbs N8-15 (lane 1), N8-82 (lane 2), N8-83 (lane 3), and N8-87 (lane 4). Immunoprecipitates were run under nonreducing conditions (panels a and c) or reducing conditions (panel b).

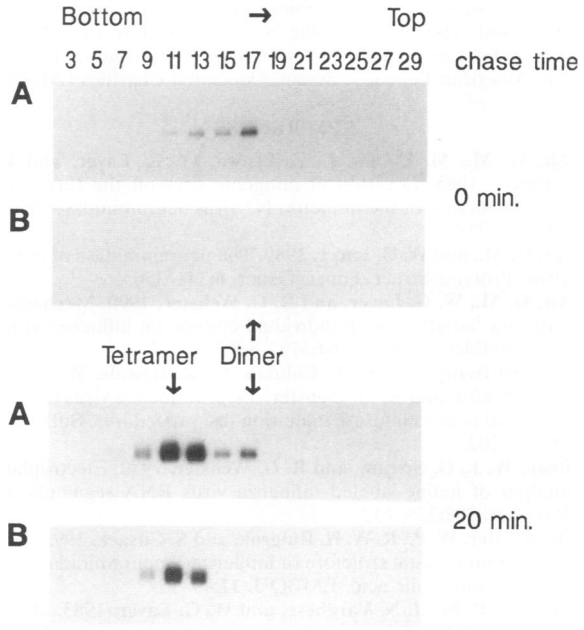

FIG. 3. Tetramer specificity of MAb N8-4 demonstrated by gradient centrifugation of cell lysates followed by immunoprecipitation. Virus-infected cells were pulse-labeled for 5 min and then chased for 0 min (top two panels) or 20 min (bottom two panels) with unlabeled methionine. MAbs N8-10 (A) and N8-4 (B) were used for immunoprecipitation after fractionation of virus-infected cell lysates through 5 to 25% sucrose gradients. Under identical conditions, the marker proteins sedimented as follows: catalase (11.3S), fraction 5; aldolase (8.6S), fraction 17; BSA (4.3S), fraction 21.

more than 50% of the MAbs with enzyme-inhibitory activity recognized substitutions in this loop. Among the antibodies recognizing the loop containing residues 344 to 346, seven MAbs selected variants with a substitution at 346 and one selected a variant with a substitution at 344. This preference may indicate that amino acid 346 is one of only a few residues in the epitope that make critical contact with antibody to allow selection of escape mutants or reduce antibody binding, as has been observed for the NC-41 epitope on N9 (3, 16).

The immunoprecipitation data suggest that peptide loops comprising amino acids 367 and 399 to 400 were involved in two distinct epitopes. Since MAbs N8-82 and N8-83 recognized these loops differently, the same antigenic peptide loops could be recognized as part of different epitopes. Hence even if one MAb loses reactivity against an escape mutant to another MAb, it does not necessarily mean that they recognize the same epitope.

The use of escape mutants allowed us to show that antigenic regions in the N8 subtype are similar to those of N2 and N9 NAs. The amino acid substitutions at 150, 199, 284, 344, 346, 367, 399, and 400 were found in the escape mutants of N8 NA. The mutants with a substitution at residue 150 have been isolated in antigenic studies of N2 (1), and residues 153, 197, and 199 are variable among human influenza virus N2 isolates (7). The substitution at residue 284 is not likely to affect the NI activity of antibodies because it is located at the bottom of the NA head (Fig. 1a). The substitutions found in N8 escape mutants other than residues 150, 199, and 284 were all within the equivalent loops to those of the NC-41 epitope on N9 NA (23). Thus, all of the substitutions in N8 escape mutants which affect the enzyme-inhibitory activity of antibodies were found within the equivalent peptide loops considered to be antigenic on the N2 and N9 subtypes. This indicates that regardless of the subtypes, antigenic regions in the NA head of influenza A viruses appear to be similar to each other. Indeed, the protein-folding patterns of N8 NA are similar to those of N2 and N9 NA (21).

In this study, we show that one of the epitopes of N8 NA appears to exist at the interface of the two adjacent monomers. The MAb recognizing this epitope seems to bind specifically to tetrameric forms of NA. This MAb will be useful in dissection of the biosynthetic process of the NA in greater detail, especially with regard to its oligomerization. Moreover, since antibodies against different epitopes on NA function differently in defense against virus infection (29), it will be important to learn whether this antibody has a unique protective role in host immunity.

ACKNOWLEDGMENTS

We thank Rachel Aston and Mark Powell, who were summer students visiting St. Jude Children's Research Hospital from the University of Bath, Bob Sealy, Hybridoma Laboratory, St. Jude Children's Research Hospital, and Avlikki Koskinen, John Curtin Medical School, for technical assistance; John Gilbert for scientific editing; and Dayna Anderson for typing the manuscript.

This work was supported by Public Health Service research grants AI-08831 and AI-29599 from the National Institute of Allergy and Infectious Diseases, Cancer Center Support (CORE) grant CA-21765, and the American Lebanese Syrian Associated Charities (ALSAC).

REFERENCES

1. **Air, G. M., M. C. Els, L. E. Brown, W. G. Laver, and R. G. Webster.** 1985. Location of antigenic sites on the three-dimensional structure of the influenza N2 virus neuraminidase. Virology **145:**237–248.
2. **Air, G. M., and W. G. Laver.** 1989. The neuraminidase of influenza virus. Proteins Struct. Funct. Genet. **6:**341–356.
3. **Air, G. M., W. G. Laver, and R. G. Webster.** 1990. Mechanism of antigenic variation in an individual epitope on influenza virus N9 neuraminidase. J. Virol. **64:**5797–5803.
4. **Aymard-Henry, M., M. T. Colman, W. R. Dowdle, W. G. Laver, G. C. Schild, and R. G. Webster.** 1973. Influenza virus neuraminidase and neuraminidsase inhibition test procedures. Bull. W.H.O. **48:**199–202.
5. **Bean, W. J., G. Sriram, and R. G. Webster.** 1980. Electrophoretic analysis of iodine-labeled influenza virus RNA segments. Anal. Biochem. **102:**228–232.
6. **Burmeister, W. P., R. W. H. Ruigrok, and S. Cusack.** 1992. The 2.2 Å resolution crystal structure of influenza B neuraminidase and its complex with sialic acid. EMBO J. **11:**49–56.
7. **Colman, P. M., J. N. Varghese, and W. G. Laver.** 1983. Structure of the catalytic and antigenic sites in influenza virus neuraminidase. Nature (London) **303:**41–44.
8. **Earl, P. L., B. Moss, and R. W. Doms.** 1991. Folding, interaction with GRP78-Bip, assembly, and transport of the human immunodeficiency virus type 1 envelope protein. J. Virol. **65:**2047–2055.
9. **Hogue, B. G., and D. P. Nayak.** 1992. Synthesis and processing of the influenza virus neuraminidase, a type II transmembrane glycoprotein. Virology **188:**510–517.
10. **Kida, H., L. E. Brown, and R. G. Webster.** 1982. Biological activity of monoclonal antibodies to operationally defined antigenic regions on the hemagglutinin molecule of A/Seal/Massachusetts/1/80 (H7N7) influenza virus. Virology **122:**38–47.
11. **Köhler, G., and C. Milstein.** 1975. Continuous culture of fused cells secreting antibody of pre-defined specificity. Nature (London) **256:**495–497.
12. **Kraulis, P. J.** 1991. MOLSCRIPT: a program to produce both detail and schematic plots of protein structures. J. Appl. Crystallogr. **24:**946–950.
13. **Laver, W. G., G. M. Air, R. G. Webster, and L. J. Markoff.** 1982. Amino acid sequence changes in antigenic variants of type A influenza virus N2 neuraminidase. Virology **122:**450–460.
14. **Lentz, M. R., G. M. Air, W. G. Laver, and R. G. Webster.** 1984. Sequence of the neuraminidase gene of influenza virus A/Tokyo/3/67 and previously uncharacterized monoclonal variants. Virology **135:**257–265.
15. **Matsunami, M., M. Marquart, T. A. Jones, P. M. Colman, K. Bartels, and R. Huber.** 1978. Crystal structure of the human Fab fragment Kol and its comparison with the intact Kol molecule. J. Mol. Biol. **121:**441–459.

15a. **Murti, K. G.** Personal communication.

16. **Nuss, J. M., P. B. Whitaker, and G. M. Air.** 1993. Identification of critical residues in the NC-41 epitope of a subtype N9 influenza virus neuraminidase. Proteins Struct. Funct. Genet. **15:**121–132.
17. **Poljak, R. J., L. M. Amzel, H. P. Avey, B. L. Chen, R. P. Phizackerley, and F. Saul.** 1973. Three-dimensional structure of the Fab′ fragment of a human immunoglobulin at 2.8-Å resolution. Proc. Natl. Acad. Sci. USA **70:**3305–3310.
18. **Saito, T., Y. Kawaoka, and R. G. Webster.** 1993. Phylogenetic analysis of the N8 neuraminidase gene of influenza A viruses. Virology **193:**868–876.
19. **Segal, D. M., E. A. Paldan, G. H. Cohen, S. Rudikoff, M. Potter, and D. R. Davies.** 1974. The three-dimensional structure of a phosphorylcholine-binding mouse immunoglobulin Fab and the nature of the antigen binding site. Proc. Natl. Acad. Sci. USA **71:**4298–4302.
20. **Tarentino, A. L., C. M. Gomez, and T. H. Plummer, Jr.** 1985. Deglycosylation of asparagine-linked glycans by peptide:n-glycosidase F. Biochemistry **24:**4665–4671.
21. **Taylor, G., E. Garman, R. Webster, T. Saito, and G. Laver.** 1993. Crystallization and preliminary X-ray studies of influenza A virus neuraminidase of subtype N5, N6, N8 and N9. J. Mol. Biol. **230:**345–348.
22. **Tulip, W. R., J. N. Varghese, A. T. Baker, A. Van Donkelaar, W. G. Laver, R. G. Webster, and P. M. Colman.** 1991. Refined atomic structures of N9 subtype influenza virus neuraminidase and escape mutants. J. Mol. Biol. **221:**487–497.
23. **Tulip, W. R., J. N. Varghese, W. G. Laver, R. G. Webster, and P. M. Colman.** 1992. Refined crystal structure of the influenza virus N9 neuraminidase-NC 41 Fab complex. J. Mol. Biol. **227:**122–148.
24. **Varghese, J. N., and P. M. Colman.** 1991. Three-dimensional structure of the neuraminidase of influenza virus A/Tokyo/3/67 at 2.2 Å resolution. J. Mol. Biol. **221:**473–486.
25. **Varghese, J. N., J. L. McKimm-Breschkin, J. B. Caldwell, A. A. Kortt, and P. M. Colman.** 1992. The structure of the complex between influenza virus neuraminidase and sialic acid, the viral receptor. Proteins Struct. Funct. Genet. **14:**327–332.
26. **Webster, R. G., G. M. Air, D. W. Metzger, P. M. Colman, J. N. Varghese, A. T. Baker, and W. G. Laver.** 1987. Antigenic structure and variation in an influenza virus N9 neuraminidase. J. Virol. **61:**2910–2916.
27. **Webster, R. G., L. E. Brown, and W. G. Laver.** 1984. Antigenic and biological characterization of influenza virus neuraminidase (N2) with monoclonal antibodies. Virology **135:**30–42.
28. **Webster, R. G., V. S. Hinshaw, and W. G. Laver.** 1982. Selection and analysis of antigenic variants of the neuraminidase of N2 influenza virus with monoclonal antibody. Virology **117:**93–104.
29. **Webster, R. G., P. A. Reay, and W. G. Laver.** 1988. Protection against lethal influenza with neuraminidase. Virology **164:**230–237.