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Antigenic Structure and Variation in an Influenza Virus N9 Neuraminidase

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We previously determined, by X-ray crystallography, the three-dimensional structure of a complex between influenza virus N9 neuraminidase (NA) and the Fab fragments of monoclonal antibody NC-41 [P. M. Colman, W. G. Laver, J. N. Varghese, A. T. Baker, P. A. Tulloch, G. M. Air, and R. G. Webster, Nature (London) 326:358–363, 1987]. This antibody binds to an epitope on the upper surface of the NA which is made up of four polypeptide loops over an area of approximately 600 Å^2 (60 nm^2). We now describe properties of NC-41 and other monoclonal antibodies to N9 NA and the properties of variants selected with these antibodies (escape mutants). All except one of the escape mutants had single amino acid sequence changes which affected the binding of NC-41 and which therefore are located within the NC-41 epitope. The other one had a change outside the epitope which did not affect the binding of any of the other antibodies. All the antibodies which selected variants inhibited enzyme activity with fetuin (molecular weight, 50,000) as the substrate, but only five, including NC-41, also inhibited enzyme activity with the small substrate *N*-acetylneuramin-lactose (molecular weight, 600). These five probably inhibited enzyme activity by distorting the catalytic site of the NA. Isolated, intact N9 NA molecules form rosettes in the absence of detergent, and these possess high levels of hemagglutinin activity (W. G. Laver, P. M. Colman, R. G. Webster, V. S. Hinshaw, and G. M. Air, Virology 137:314–323, 1984). The enzyme activity of N9 NA was inhibited efficiently by 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid, whereas hemagglutinin activity was unaffected. The NAs of several variants with sequence changes in the NC-41 epitope lost hemagglutinin activity without any loss of enzyme activity, suggesting that the two activities are associated with separate sites on the N9 NA head.

The influenza virus N2 neuraminidase (NA) has been crystallized (12), and the three-dimensional structure has been determined at a 2.9-Å (0.29-nm) resolution (7, 21). Topographical mapping of N2 NA revealed four antigenic sites, two on the enzyme active face and two others possibly on the bottom face of the tetramer (23). Sequence analysis of antigenic variants selected with monoclonal antibodies to N9 NA revealed a series of amino acid substitutions ringing the concave substrate-binding site (2, 16); there was a clear correlation between the positions of the sequence changes and the properties of the antibodies (23).

N9 NA from a tern influenza virus has also been crystallized (13), and its three-dimensional structure has been determined (A. T. Baker, I. N. Varghese, W. G. Laver, G. M. Air, and P. M. Colman, Proteins, in press). This NA agglutinates chicken erythrocytes as efficiently as the hemagglutinin (HA) does (13). In the present study, a panel of monoclonal antibodies to N9 NA was prepared and used to select antigenic variants. One of these antibodies, NC-41, yielded Fab fragments which, when complexed with N9 NA heads, formed large crystals of X-ray diffraction quality (15), and the three-dimensional structure of the complex was determined (6).

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The present studies indicate that antibodies which inhibit NA activity bind near the enzyme active site, and some also inhibit the HA activity of the NA. Sequence analysis of antigenic variants shows that the majority of the single amino acid substitutions occur in a rather large antigenic area that in the three-dimensional structure (Baker et al., in press) is on the rim of the substrate-binding cavity and includes the NC-41 epitope (6). The single amino acid substitutions in several of these variants resulted in the complete loss of HA activity with no loss of NA activity.

MATERIALS AND METHODS

Viruses. The N9 NA was from A/tern/Australia/G70C/75 (H11N9) influenza virus isolated from apparently healthy noddy terns (*Anous minutus*) on North West Island of Australia's Great Barrier Reef (8). A recombinant virus with A/NWS/34 (H1) HA and N9 NA was prepared from one of the tern virus isolates (22). This virus, designated NWS-Tern (H1N9), was used in the experiments reported in this paper.

Isolation of NA. Intact N9 NA molecules were isolated from the H1N9 recombinant virus by electrophoresis on cellulose acetate strips after disruption of the virus particles with sodium dodecyl sulfate (14). NA heads were isolated from the H1N9 recombinant virus following pronase digestion of the virus (12).

Serological assays. HA and hemagglutination inhibition

TABLE 1. Biological activities of monoclonal antibodies to N9 NA[a]

Monoclonal antibody	Isotype	NI[b] with:		HI[c]	Variant selection[d]
		Fetuin	NAL		
NC-4	IgG1	<1	<1	<1	–
NC-62	IgG1	<1	<1	<1	–
NC-8	IgG1	<1	<1	1.6	–
NC-30	IgG1	<1	<1	2.5	–
NC-28	IgG2a	3.25	<1	1.3	–
NC-50	IgG2a	3.25	<1	2.2	–
NC-52	IgG1	3.25	3.5	1.3	+
NC-41	IgG2a	3.25	3.7	1.9	+
NC-32	IgG1	4.25	3.75	4.3	+
NC-33	IgG1	>4.5	>4	4.3	+
NC-34	IgG1	4.0	3.25	4.3	+
NC-42	IgG2a	3.75	3.25	4.3	+
NC-44	IgG2a	>4.5	3.25	1.6	+
NC-45	IgG2a	>4.5	3.25	2.2	+
NC-46	IgG2a	>4.5	3.25	3.4	+
NC-47	IgG2a	4.25	3.25	4.3	+
NC-70	IgG1	3.75	3.5	3.1	+
NC-61	ND[e]	3.50	<1	4.3	+
NC-10	ND	4.50	<1	4.3	+
NC-11	IgGl	4.00	<1	2.8	+
NC-15	IgG2a	4.25	<1	4.3	+
NC-17	IgGl	4.5	<1	4.3	+
NC-20	IgGl	>4.5	<1	4.3	+
NC-23	IgGl	>4.5	<1	4.3	+
NC-24	IgG2a	3.75	<1	2.8	+
NC-25	IgGl	>4.5	<1	4.3	+
NC-31	IgGl	4.3	<1	4.3	+
NC-35	IgG2a	>4.7	<1	4.3	+
NC-36	IgG2a	4.25	<1	1.6	+
NC-37	IgG2a	4.2	<1	1.9	+
NC-43	IgGl	>4.5	<1	4.3	+
NC-49	IgG2a	4.5	<1	4.3	+
NC-66	IgGl	4.0	<1	4.3	+
32/3	ND	3.75	<1	3.7	+
NC-38	IgG2a	3.5	<1	3.4	+

[a] Monoclonal antibodies to NWS-Tern (H1N9) N9 NA were prepared as described in Materials and Methods; ascitic fluid from BALB/c mice was used in the assays.

[b] NI assays were done with either fetuin or NAL as the substrate as described in Materials and Methods; antibody titers are expressed in $\log_{10}$ terms.

[c] HI assays were done at 0°C; antibody titers are expressed in $\log_{10}$ terms.

[d] Antigenic variants (escape mutants) were selected in MDCK cells as described in Materials and Methods. –, No variant was selected; the virus was inhibited to the same titer as the parent in NI assays. +, Variant was selected; monoclonal antibody binding was either abolished or markedly reduced (Table 4).

[e] ND, Not done.

(HI) assays were done at 0°C in microtiter plates (17). NA assays were done by the method recommended by the World Health Organization (4). Enzyme-linked immunosorbent assays (ELISA) were done as described previously (9).

Monoclonal antibodies. Hybridoma cell lines producing antibodies to N9 NA were prepared by the method of Kohler and Milstein (11). The spleen cell donor was a BALB/c mouse immunized with one intraperitoneal injection of purified intact NWS-Tern (ca. 2 μg of NA protein), and fusion was done 1 to 2 months later, 4 days after a similar intravenous booster dose of antigen. The SP_2/O Ag 14 cell line (19) was used, and the hybridomas producing antibodies to N9 NA were cloned by limiting dilution and grown as ascites in BALB/c mice. Ascitic fluid was collected from pristane-primed BALB/c mice. To facilitate the purification of antibodies for crystallization studies, we also grew the hybridoma cells in serum-free medium (HB 101; HANA Media, Inc., Berkeley, Calif.).

Selection of antigenic variants. Antigenic variants (escape mutants) were selected in MDCK cell cultures in 24-well tissue culture plates. Cells were inoculated with undiluted N9 monoclonal antibody (100 μl) and NWS-Tern (5 μl containing 10^6 PFU) in 0.4 ml of minimal essential medium containing bovine serum albumin and trypsin (20). After 4 days of incubation in 5% CO_2 at 37°C, samples of the supernatant (0.1 ml) were injected into 11-day-old embryonated eggs. Virus growth was detected by hemagglutination after 48 h of incubation at 34°C. The viruses were cloned at limiting dilutions in embryonated eggs and tested in ELISA and NA inhibition (NI) assays to determine their reactivity with monoclonal antibodies.

Preparation of viral RNA. Virus was pelleted by centrifugation at 40,000 × *g* for 3 h. After the pellet was soaked in 10 mM Tris (pH 7.4)–10 mM KCl–1.5 mM $MgCl_2$ containing 0.2% sodium dodecyl sulfate overnight, the suspension was brought to 5 ml, and 2 mg of proteinase K (Boehringer GmbH, Mannheim, Federal Republic of Germany) was added. This mixture was incubated at 56°C for 20 min. The

TABLE 2. Nucleotide and amino acid sequence changes in escape mutants of NWS-Tern N9 NA[a]

Variant (name)	Nucleotide		Amino acid		HA titer[b]
	Position	Change	N2	Change	
Tern (N9)					30,000
NC-38 V1	683	G→A	220 (222)	Arg→Gln	17,000
32/3 V2 (Ox2)	1009	A→G	329 (331)	Asn→Asp	30,000
NC-35 V1	1009	A→G	329 (331)	Asn→Asp	
NC-10 V1	1009	A→G	329 (331)	Asn→Asp	
NC-47 V1a	1121	G→A	367 (368)	Ser→Asn	0
NC-41 V1	1121	G→A	367 (368)	Ser→Asn	
NC-24 V1	1124	T→G	368 (369)	Ile→Arg	17,000
NC-31 V1	1127	C→A	369 (370)	Ala→Asp	
NC-20 V1	1127	C→A	369 (370)	Ala→Asp	0
NC-45 V1a	1127	C→A	369 (370)	Ala→Asp	100
NC-66 V1a	1127	C→A	369 (370)	Ala→Asp	200
NC-17 V1	1127	C→A	369 (370)	Ala→Asp	
NC-11 V1	1127	C→A	369 (370)	Ala→Asp	
32/3 V1 (Ox1)	1130	C→T	370 (371)	Ser→Leu	0
NC-44 V1	1136	C→A	372 (373)	Ser→Tyr	200
NC-41 V2	1218	C→A	400 (400)	Asn→Lys	
NC-42 V1	1218	C→A	400 (400)	Asn→Lys	100
NC-34 V1a	1320	G→T	432 (434)	Lys→Asn	5,000

[a] The nucleotide sequence of each of the variants was determined as described in Materials and Methods. The deduced amino acid sequences are given in N2 numbering; values in parentheses represent N9 numbering.

[b] HA titers were determined for sodium dodecyl sulfate-isolated N9 NA adjusted to contain the same NA and protein content; tests were done at 0°C as described in Materials and Methods. No entry indicates that tests were not done.

RNA was extracted with phenol-chloroform and then precipitated three times with ethanol (5). The RNA concentration was determined by measuring the A_{260}.

Sequencing of cDNA. The sequence of the cDNA transcribed from the viral RNA was determined by the dideoxy chain termination method (1, 2, 18) with synthetic oligonucleotide primers (3).

RESULTS

Biological activities of N9 monoclonal antibodies. The hybridoma cell lines were initially screened by NI assays and ELISA with NWS-Tern (H1N9). The specificity of the monoclonal antibodies was determined by their ability to react with solubilized crystals of N9 NA in ELISA and by their ability to inhibit hemagglutination at 0°C (Table 1) by isolated intact N9 NA rosettes. The isotypes of the monoclonal antibodies are shown in Table 1; all were either immunoglobulin G1 (IgG1) or IgG2a.

Although antibodies to the NA of influenza virus do not prevent attachment or replication, they do interfere with virus release (10) and permit the selection of antigenic variants in vitro (24). Each of the N9 monoclonal antibodies was tested for its ability to select antigenic variants in MDCK cells.

Two monoclonal antibodies (NC-4 and NC-62) did not inhibit NA or HA activity and failed to select variants. Another two monoclonal antibodies (NC-8 and NC-30) inhibited HA activity but failed to inhibit NA activity or select variants. Two other monoclonal antibodies (NC-28 and NC-50) inhibited NA and HA activities but failed to select variants. The majority of the monoclonal antibodies (Table 1) inhibited NA and HA activities and selected variants.

The monoclonal antibodies were also tested for their ability to inhibit enzyme activity with fetuin (molecular weight, 50,000) or *N*-acetylneuramin-lactose (NAL) (molecular weight, 600) as the substrate. Only a subset of the monoclonal antibodies which inhibited enzyme activity on fetuin also inhibited enzyme activity on NAL.

Most of the antibodies inhibited the HA activity of NA, but there was a wide range of HI activity; some antibodies inhibited to titers of 20, and others inhibited to titers of 20,000. There was no correlation between NI activity and HI activity; for example, with fetuin as the substrate monoclonal antibody NC-31 had an NI titer of 20,000 and an HI titer of 20,000, while NC-36 had an NI titer of 18,000 and an HI titer of 40.

Sequence changes in escape mutants. The NA genes of a number of the escape mutants were completely sequenced. Single nucleotide changes were found in each of the variants. The nucleotide and deduced amino acid sequence changes in 18 variants are shown in Table 2. The N2 NA numbering of the amino acids will be used throughout this publication to maintain consistency with the published structure (21). In the three-dimensional structure of the NA, all but one of the changes in the variants were on one side of the top surface of the molecule (Fig. 1). Six of the variants had identical amino acid sequence changes at residue 369; other changes were at residues 367, 368, and 370. On adjacent loops of the structure there were changes at residues 329, 400, and 432. Two variants selected with the same monoclonal antibody (32/3) had sequence changes at different amino acid residues (329

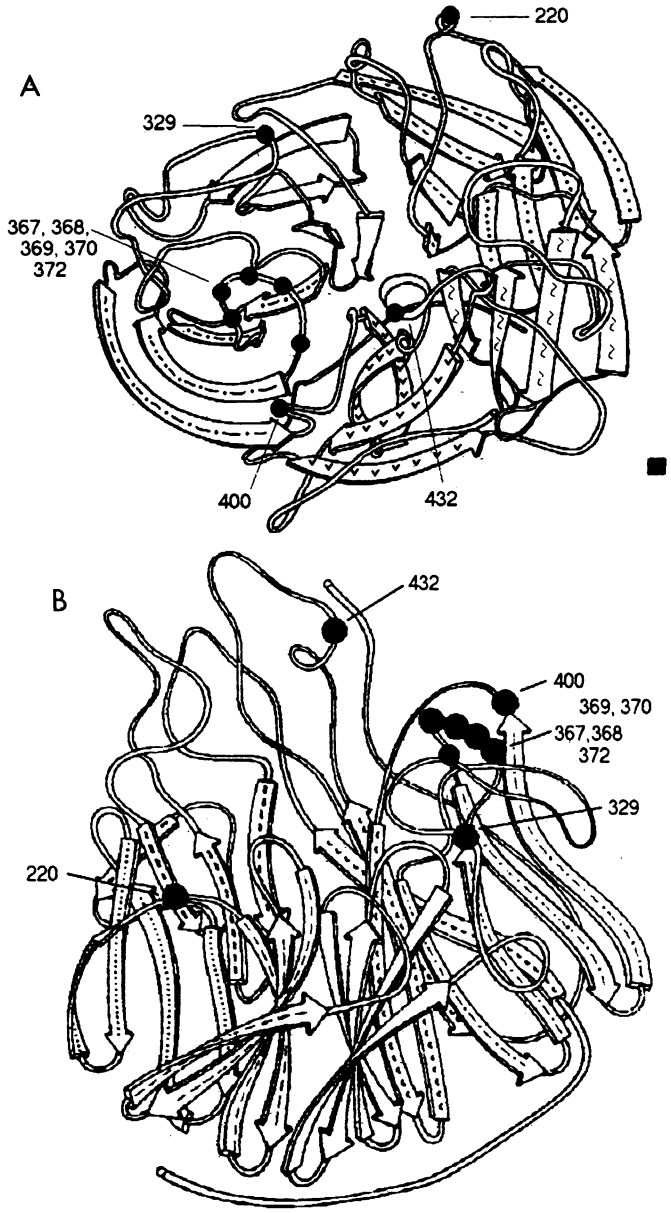

FIG. 1. Schematic diagrams of chain folding in N9 NA viewed down the fourfold axis (A) and perpendicular to this axis (B). The symmetry axis is at the lower right in panel A and standing vertically at the left rear in panel B. Mutations at the numbered positions abolish or reduce the binding of NC-41 antibody to NA (Table 4). A mutation at residue 220, which falls outside the NC-41 epitope, has no effect on NC-41 binding to NA (Table 4).

and 370); similarly, two variants selected with NC-41 had changes at residues 367 and 400. The only variant with a change outside this region was selected by NC-38 (Arg→Gln [residue 220]) (Table 2). The results are summarized in Table 3.

Cross-reactions between antibodies and variants. Antigenic sites can be operationally defined by the ability of the antigenic changes in a certain group of variants to prevent the binding of a corresponding group of monoclonal antibodies but, at the same time, to have no effect on the binding of other monoclonal antibodies which recognize different antigenic sites on the same molecule.

The cross-reactions in NI assays of the monoclonal antibodies and the variants selected by them are shown in Table 4. All except one of the antibodies clearly fell into one group, and each recognized an epitope overlapping the NC-41 epitope. The one antibody outside this group was NC-38, which recognized a residue (220) outside the NC-41 epitope.

HA activity. (i) Antigenic variants. The HA activity of the isolated intact NA from the parent and antigenic variants was determined at 0°C (Table 2). Amino acid substitutions at residues 220, 329, and 432 had a minimal effect or no effect on hemagglutination by N9 NA, whereas mutations at residues 367, 369, 370, and 372 markedly reduced or abolished this property. Interestingly, a mutant N9 NA with a substitution at residue 368 (Ile→Arg) hemagglutinated as efficiently as did the parental N9 NA.

Figure 2 shows the three-dimensional structure of the putative HA site in N9 NA in comparison with the same site in nonhemagglutinating N2 NA.

(ii) Morphology. Electron microscopic examination of intact NA molecules isolated from the parent virus NWS-Tern and the variants showed that HA-negative NA variants formed NA rosettes indistinguishable from those of HA-positive NA variants (results not shown).

(iii) Inhibition by DANA. Previous studies showed that 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid (DANA) partially inhibited the NA activity of N9 NA on the fetuin substrate but failed to inhibit the HA activity of N9 NA (13). These studies were repeated with NAL as the substrate and N9 NA isolated from virions by electrophoresis on cellulose acetate strips after disruption of the virions by sodium dodecyl sulfate. At 10^{-2} M DANA, the NA activity was 100% inhibited, but there was no inhibition of the HA activity (Fig. 3).

(iv) Sialic acid binding. To determine if the HA-binding site on N9 NA recognizes sialic acid residues, we did the following experiments. Chicken erythrocytes were treated with receptor-destroying enzyme and tested for HA activity with isolated N9 NA rosettes at 0°C. The receptor-destroying-enzyme-treated cells were not agglutinated by N9 NA, while the untreated cells were agglutinated (results not shown).

Both fetuin (molecular weight, 50,000) and NAL (molecular weight, 600) were tested for their ability to inhibit the HA activity of isolated N9 NA at 0°C. Fetuin inhibited HA activity to a concentration of 10^{-4} M, whereas NAL had no inhibiting activity. Neither fetuin nor NAL inhibited the HA activity of H3 NA from A/Aichi/68 (H3N2) influenza virus.

These studies indicate that the HA activity of N9 NA is analogous to that of the HA molecules on the virion in binding to sialic acid residues; however, the reactivities could be distinguished by inhibition with fetuin.

DISCUSSION

Monoclonal antibodies specific for N9 NA were characterized. Two monoclonal antibodies (NC-4 and NC-62) failed to inhibit either NA or HA activity yet bound at high titers to N9 NA in ELISA. These antibodies presumably recognize sites that are distant from the biologically active center(s), maybe on the bottom or side of the globular head of the NA.

Four other monoclonal antibodies (NC-8, NC-30, NC-28, and NC-50) failed to select antigenic variants even though NC-28 and NC-50 inhibited NA activity. Inhibition curves suggested that NC-28 and NC-50 bound with low affinity.

The majority of monoclonal antibodies inhibited NA activity on fetuin and selected variants in vitro. Sequence

TABLE 3. Summary of antibody binding grouped according to area of N9 recognized

Monoclonal antibody	Binding of monoclonal antibodies to indicated variant (site of amino acid change)[a]:								NI with:	
	NC-38 V1 (220)	NC-35 V1 (329)	NC-41 V1 (367)	NC-24 V1 (368)	NC-20 V1 (369)	32/3 V1 (370)	NC-42 V1 (400)	NC-34 V1 (432)	Fetuin	NAL
NC-41		?	0	?	0	0	0	0	Yes	Yes
NC-66		0		0	0	0		0	Yes	No
NC-35		0	0		0			0	Yes	No
NC-10		0			0	0		0	Yes	No
NC-24		0		0				0	Yes	No
NC-34		0			0			0	Yes	Yes
NC-17		0		0	0	0			Yes	No
NC-45			0		0		0	0	Yes	Yes
NC-47			0		0		0		Yes	Yes
NC-42			0		0		0		Yes	Yes
NC-31				0	0				Yes	No
NC-20				0	0				Yes	No
NC-11				0	0				Yes	No
NC-38	0								Yes	No

[a] 0 indicates that an amino acid substitution at this residue markedly reduced the binding of that particular monoclonal antibody. ? indicates a limited reduction in binding (Table 4). No entry indicates no reduction in binding. The HA titers of NC-38 V1, NC-35 V1, NC-24 V1, and NC-34 V1 were less than fourfold different from that of the parent.

analysis of these variants indicated that all antibodies except one (NC-38) recognized epitopes on one side of the top surface of the NA. Since all of these antibodies were prepared from two mice, the antigenic regions which were defined may simply reflect the areas that were dominant in these animals; however, the antigenic region recognized was the same as for N2 NA (2, 23), suggesting that this is indeed the dominant antigenic region on the NA molecule. In the three-dimensional structure (7, 21), the amino acid substitutions in the variants were all located in a series of four polypeptide loops on or near the rim of the concave substrate-binding pocket. The three-dimensional structure of the Fab fragments complexed with N9 NA (6) showed that the NC-41 epitope involves surface loops containing residues 432, 400, 367 to 370, and probably 329 (Fig. 1).

Each of the antigenic variants of N9 NA had a single amino acid substitution which markedly reduced or abolished antibody binding. A summary of the different residues substituted and their effect on the binding of antibody to antigen is shown in Table 3. Monoclonal antibody NC-41 had markedly reduced binding to antigenic variants that had amino acid substitution at residues 367, 369, 370, 400, and 432 and marginally reduced binding to a variant with a change at residue 329. Recent studies on the three-dimensional structure of a crystal complex of NC-41 Fab fragments with N9 NA at a 3-Å (0.3-nm) resolution have established that this antibody makes contact with residues 368 to 370, 400 to 403, 430 to 434, and part of 325 to 350 (6). The contact residues on the loop containing residues 325 to 350 are still not completely defined. Amino acid substitutions were detected in each of the four loops on N9 NA where antibody NC-41 makes contact.

The binding of antibodies to the antigenic variants showed different patterns of reactivity. Antibodies which reacted with residue 367 did not react with residue 368 and vice versa. This separation of residues 367 and 368 correlated with the behavior of the HA activity of mutants at those sites. The three-dimensional structure in this region (Fig. 2)

TABLE 4. Cross-reactions between antibodies and variants[a]

Monoclonal antibody	Reactivity of NWS-Tern	Reactivity of the following escape mutant (site of sequence change):																	
		NC-38 V1 (220)	NC-10 V1 (329)	32/3 V2 (329)	NC-35 V1 (329)	NC-47 V1 (367)	NC-41 V1 (367)	NC-24 V1 (368)	NC-11 V1 (369)	NC-17 V1 (369)	NC-20 V1 (369)	NC-31 V1 (369)	NC-45 V1 (369)	NC-66 V1 (369)	NC-70 V1 (ND)	32/3 V1 (370)	NC-44 V1 (372)	NC-42 V1 (400)	NC-34 V1 (432)
NC-11	4.0							2.0	<1	<1	<1	<1	<1	<1	<1				
NC-20	>4.5							3.0	<1	<1	<1	<1	<1	<1	<1				
NC-31	4.3							<1	<1	<1	<1	<1	<1	2.5	<1				
NC-45	>4.5					<1	<1		<1	<1	1.5	<1	<1	2.0	1.25		<1	<1	<1
NC-17	4.5		1.5	1.75	2.0			<1	2.25	3.25	3.25	2.5	2.5	3.0		<1			
NC-66	4.0		<1	<1	<1			<1	2.5	2.5	2.25	2.0	2.0	2.25	3.25	<1	3.5		1.75
NC-41	3.75		3.0	2.75	3.5	<1	<1	2.75	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
NC-47	4.25					<1	<1		<1	<1	<1	<1	<1	<1	<1		2.0	<1	
NC-10	4.5		<1	<1	<1				2.5	2.0	2.25	3.5	2.0	2.5	2.5	<1	3.75		2.25
NC-35	>4.5		1.5	1.5	1.75	2.0	1.75		1.25	2.5	1.5	3.5	1.0	2.0	1.75		2.25		1.8
NC-42	3.75					<1	<1		<1	<1	<1	<1	<1	<1	<1		1.75	<1	
NC-34	4.0		1.75	2.25	2.75				2.5	2.5	2.5	2.5	2.0	1.8	3.0		3.0		2.5
NC-24	3.75		<1	<1	<1			<1								<1	<1		<1
NC-44	4.5		<1	<1	<1	<1	<1	3.25	<1	<1	<1	<1	<1	<1	1.5	3.5	<1	<1	2.3
NC-38	3.5	<1																	

[a] Escape mutants were selected in MDCK cells as described in Materials and Methods. Escape mutants and the parent were tested in NI assays; the titers are expressed in $\log_{10}$ terms. No entry indicates that the titers were not different from those of the parent. The reactivity patterns in NI assays were confirmed in ELISA.

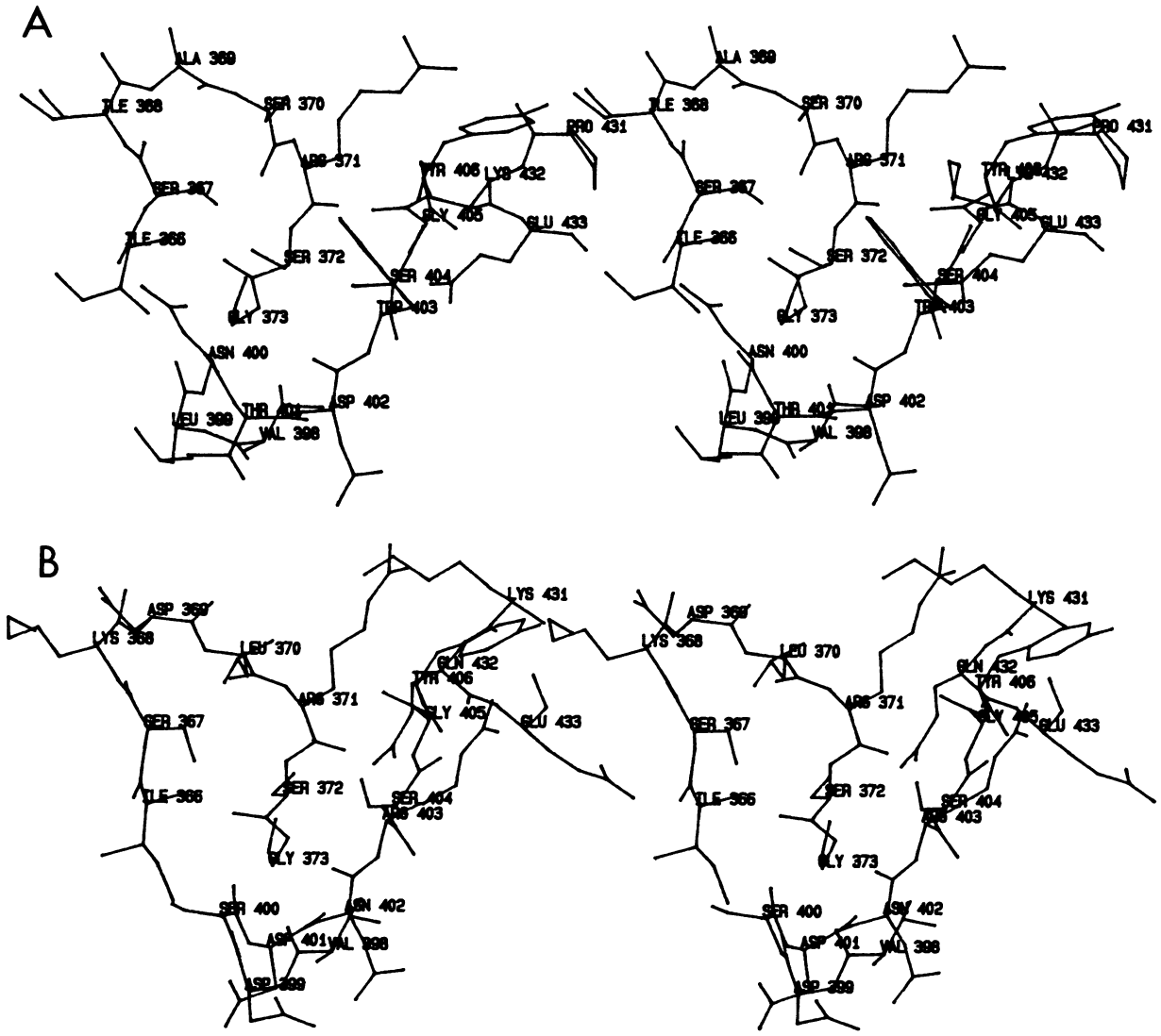

FIG. 2. Stereo diagrams of the putative HA site in N9 NA (A) and the corresponding site in N2 NA (B). The two structures are not yet completely refined (J. N. Varghese, A. T. Baker, and P. M. Colman, unpublished data). N2 numbering is used in both diagrams. Note in particular the spatial clustering of residues 369, 370, 372, and 400 (positions at which mutants have no HA activity) and the proximity of residues 368 and 432 (positions at which mutants have reduced HA activity). Arg-371 points into the enzyme active site, which is separated from the site shown here by the loop at residues 367 to 372. The view here is as in the schematic in Fig. 1A.

revealed that the side chains of residues 367 and 368 pointed in opposite directions. Similarly, reactivity with residue 368 precluded reactivity with residue 400 and vice versa (Fig. 2). The binding of monoclonal antibody NC-11 was decreased only by substitutions in a single loop (residues 369 and 370).

The receptor-binding site on H3 HA recognizes sialic acid side chains and is a shallow pocket at the top of the globular head (25). The HA-binding site on N9 NA is similar in recognizing sialic acid side chains and being a shallow pocket; however, any atomic similarity between the two remains to be determined. The functional significance of the hemagglutinating activity on N9 has not been determined; antibodies to N9 NA do not inhibit virus attachment (13).

Approximately half of the monoclonal antibodies examined (Table 3) (NC-41, NC-66, NC-35, NC-10, NC-24, and NC-34) failed to combine with N9 NA with mutations in any of the four loops recognized by NC-41; these antibodies had different reactivity patterns, suggesting that each antibody recognizes a different set of contact amino acids. The remainder of the monoclonal antibodies recognized only some of the variants. Does this indicate differences in the size or shape of the antibody-combining site from antibody to antibody? Information on these questions can be obtained with the influenza virus NA monoclonal antibody system.

Five of the monoclonal antibodies (Table 3) inhibited NA activity on NAL, whereas all the antibodies which selected variants inhibited NA activity on fetuin. These five antibodies failed to combine with variants that had changes at residue 369; four of them recognized changes at residues 367 and 400. The crystal structure of NC-41 Fab fragments bound to N9 NA shows that there is no block to the access of NAL into the active site, and one possibility is that the inhibition may be a result of a lowering of catalytic activity caused by the displacement of residue 371 (R. M. Lentz, R. G. Webster, and G. M. Air, Biochemistry, in press), which points directly into the catalytic site (6). Alternatively,

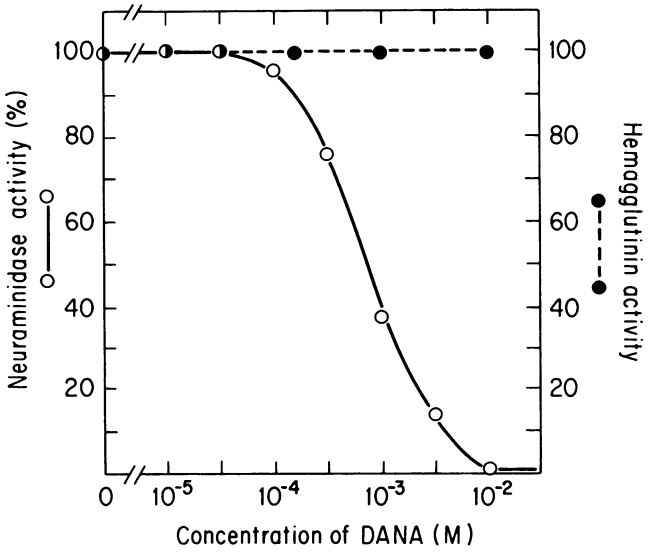

FIG. 3. Selective inhibition of the biological activity of N9 NA by DANA.

the antibody may influence the correct orientation of the substrate for catalysis.

The variants of N9 NA with changes at residues 367, 369, 370, and 372 lost most or all of their HA activity without a concomitant loss of their NA activity. The findings that NA enzyme activity is inhibited by DANA without inhibition of HA activity and that some antibodies inhibit NA activity but not HA activity also suggest that there are separate sites for HA and NA activities. The two sites are separate surface features of the molecule; the enzyme site is a deep pocket separated from the shallow HA site by the loop at residue 370.

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