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Mechanism of Antigenic Variation in an Individual Epitope on Influenza Virus N9 Neuraminidase

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Monoclonal antibodies which inhibit influenza virus neuraminidase (NA) and which therefore indirectly neutralize virus infectivity bind to epitopes located on the rim of the active-site crater. The three-dimensional structure of one of these epitopes, recognized by monoclonal antibody NC41, has previously been determined (W. R. Tulip, J. N. Varghese, R. G. Webster, G. M. Air, W. G. Laver, and P. M. Colman, Cold Spring Harbor Symp. Quant. Biol. 54:257–263, 1989). Nineteen escape mutants of influenza virus A/tern/Australia/G70c/75 (N9) NA selected with NC41 were sequenced. A surprising restriction was seen in the sequence changes involved. Ten mutants had a Ser-to-Phe change at amino acid 372, and six others had mutations at position 367. No escape mutants with changes at 369 or 370 were found, although these mutations were selected with other antibodies and rendered the epitope unrecognizable by antibody NC41. Another N9 NA, from A/ruddy turnstone/NJ/85, which differs by 14 amino acids from the tern virus NA, still bound antibody NC41. Epitope mapping by selecting multiple escape mutants with antibody NC41 thus identified only three of the five polypeptide loops on NA that contact the antibody. Escape mutants selected sequentially with three different monoclonal antibodies showed three sequence changes in two loops of the NC41 epitope. The multiple mutants were indistinguishable from wild-type virus by using polyclonal rabbit antiserum in double immunodiffusion tests, but NA inhibition titers were fourfold lower. The results suggest that although the NC41 epitope contains 22 amino acids, only a few of these are so critical to the interaction with antibody that a single sequence change allows selection of an escape mutant. In that case, the variety of amino acid sequence changes which can lead to polyclonal selection of new epidemic viruses during antigenic drift might be very limited.

Influenza virus neuraminidase (NA) is a tetrameric surface glycoprotein with enzyme activity which cleaves off terminal sialic acid and thus destroys receptors for the virus. Although the role of this activity in viral infection has never been defined beyond assisting in viral release and spread (20), antibodies against NA are effective in suppressing virus, and they protect chickens against death (31).

The NA enzyme and antigenic activities are located in a globular head, and this can be released from the fibrous stalk and hydrophobic membrane anchor sequence by proteases. NA heads, which retain tetrameric configuration, can be crystallized after release from several influenza viruses. The three-dimensional structures of NA heads from two subtypes of influenza A virus (N2 and N9) have been determined (7, 25), and crystal structures of two complexes of N9 NA with Fab fragments of monoclonal antibodies, NC41 and NC10 (9, 24), have been reported. Crystals of NA heads have also been obtained from two strains of influenza B virus (8, 17a) and influenza B virus NA complexed with antibody Fab (14), and these structure determinations are in progress.

We have previously reported that neutralizing epitopes on NA heads of two N2 strains (1, 17, 28, 29) and one N9 strain (27) are located on the rim surrounding the catalytic site crater in the NA monomer (10). The NC41 epitope on influenza virus NA of the N9 subtype involves several segments of polypeptide surrounding the enzyme active site on the top surface of the NA monomer. The epitope as seen by X-ray structure analysis of crystals of NA complexed with the Fab fragment of antibody NC41 (24) contains portions of five loops of polypeptide, but the 0.3-nm map does not establish the relative importance of each of the 22 contacting amino acids on the NA. This paper reports further characterization of the particular epitope which binds monoclonal antibody NC41.

MATERIALS AND METHODS

Viruses and serological tests. The reassortant influenza virus A/NWS/34 (HA)-tern/Australia/G70C/75 (NA) (NWS-tern) and other viruses possessing the N9 NA were from the influenza virus repository at St. Jude Children's Research Hospital. NA and NA inhibition (NI) assays were done as described with fetuin as the substrate (6).

Monoclonal antibody and selection of escape mutants. Monoclonal antibodies to the N9 NA were used to select escape mutants in MDCK cell cultures as described previously (27). Multiple selections of escape mutants of NWS-tern were carried out in MDCK cells in 24-well plates (27). Each escape mutant was derived from a separate culture and was cloned as previously described (27).

Sequential variants. Escape mutants of NWS-tern were selected in MDCK cells in 96-well plates. The escape mutant selected with monoclonal antibody NC41 was cloned at limiting dilution in embryonated eggs and tested against a panel of antibodies with epitopes overlapping the NC41 epitope (27). Antibodies that inhibited the initial variant in NI tests were used to select the second round of variants; a third round of variants was also selected in this fashion. Each of the variants was cloned twice at limiting dilution in embryonated eggs and was then grown in chicken embryos

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TABLE 1. Escape mutants of N9 NA selected with monoclonal antibody NC41

Variant	Nucleotide		Amino acid	
	Position	Change	Position (N2)	Change
NC41v1	1121	G→A	367	Ser→Asn
NC41v4	1121	G→A	367	Ser→Asn
NC41v9	1121	G→A	367	Ser→Asn
NC41v12	1121	G→A	367	Ser→Asn
NC41v2A	1120	A→G	367	Ser→Gly
NC41v13	1122	C→A	367	Ser→Arg
NC41v5	1136	C→T	372	Ser→Phe
NC41v6	1136	C→T	372	Ser→Phe
NC41v7	1136	C→T	372	Ser→Phe
NC41v8	1136	C→T	372	Ser→Phe
NC41v10	1136	C→T	372	Ser→Phe
NC41v11	1136	C→T	372	Ser→Phe
NC41v15	1136	C→T	372	Ser→Phe
NC41v18	1136	C→T	372	Ser→Phe
NC41v20	1136	C→T	372	Ser→Phe
NC41v21	1136	C→T	372	Ser→Phe
NC41v17	1218	C→A	400	Asn→Lys
NC41v2	1218	C→A	400	Asn→Lys
NC41v3	1318	A→G	432	Lys→Glu
and	1327	AA→GG	435	Lys→Gly

and purified by differential sedimentation through a 25 to 70% sucrose gradient in a Beckman SW 28 rotor.

Preparation of viral RNA and sequence analysis. Viral RNA preparation and sequence analysis were performed as previously described (1).

Nucleotide sequence accession number. The sequence of A/ruddy turnstone/NJ/85 cDNA coding for the viral NA is deposited in the GenBank data base under accession number M37511.

RESULTS

The repertoire of sequence changes in 19 escape mutants selected with monoclonal antibody NC41. Previous experiments showed that if a monoclonal antibody was used to select two escape mutants in independent experiments, it sometimes selected the same amino acid change twice, sometimes selected two different amino acid substitutions at the same place, and sometimes selected different amino acid changes altogether. We were therefore interested in the escape mutants that were selected by NC41, and in particular, whether all of the contacting amino acids would be represented if enough escape mutants were selected. Table 1 shows the sequence changes in the NA gene and corresponding amino acid changes when antibody NC41 was used 19 times in completely separate selection experiments on A/tern/Australia/G70c/75 (tern virus) NA. One variant (NC41v3) had three nucleotide changes and two amino acid changes compared with the parental (wt) tern virus NA. All the others had a single nucleotide change giving a single amino acid change. Seven different amino acid substitutions were found at five sites. Ten of the 19 independently selected variants had the same change (Ser to Phe at position 372). Mutant viruses with a change in the NA at Ser-367 were selected six times: four variants with a Ser-to-Phe change, one with a Ser-to-Gly change, and one with a Ser-to-Arg change.

Interestingly, some variants which were expected were not selected. Six other monoclonal antibodies each selected a change of Ala-369 to Asp (27). In NI and enzyme-linked immunosorbent assays this change was found to abolish binding to NC41, yet NC41 did not select variants with this substitution. One explanation is that there is sufficient binding of NC41 to this mutant to prevent escape of the mutant in the more rigorous conditions of the selection experiment, even though the binding is not detectable in the assays used. Similarly, variants selected by other antibodies, Ser-370 to Leu, Ser-372 to Tyr, and Lys-432 to Asn (27), were not selected by NC41, although no binding of NC41 to these variants could be detected. These results are summarized in Fig. 1.

Sequential variants. Since we have shown (above) that selection of escape mutants may be a more rigorous test of abolition of binding than any other assays we have used, we tested the independence of regions of the NC41 epitope (27) in sequential selection experiments by using three monoclonal antibodies which all have epitopes overlapping that of NC41. The first antibody used was NC41 itself, to give variant NC41v1, with a change of Ser-367 to Asn. This was tested for reactivity with a large panel of N9 monoclonal

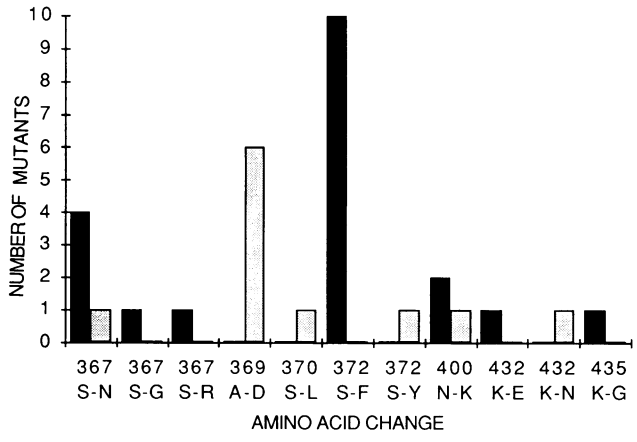

FIG. 1. Amino acid sequence changes in escape mutants selected in 19 independent experiments by antibody NC41 (■) compared with escape mutants selected by other antibodies which did not bind NC41 (□).

TABLE 2. Reactivity of sequential variants with N9 monoclonal antibodies

Monoclonal antibody[a]	NI with following escape mutants[b]:		
	NC41[c] (V1)	NC10[d] (V5 a/b)	NC33[e] (V1/V2/V6)
NC35 (329)	0	0	0
NC36	0	0	0
NC41 (367)	0	0	0
NC42 (400)	0	0	0
NC44 (372)	0	0	0
NC45 (369)	0	0	0
NC46	0	0	0
NC47 (367)	0	0	0
NC49	0	0	0
NC61	0	0	0
NC70	0	0	0
NC11 (369)	±	0	0
NC15	±	0	0
NC17 (369)	±	0	0
NC20 (369)	±	0	0
NC23	±	0	0
NC24 (368)	±	0	0
NC25	±	0	0
NC31 (369)	±	0	0
NC52	±	+	0
NC66 (369)	±	0	0
NC10 (329)	+	0	0
NC32	+	±	0
NC33	+	+	0
NC34 (432)	+	±	0
NC37	+	0	0
NC43	+	0	0
NC50	+	+	±

[a] Number in parentheses indicates the residue changed when single escape mutants were selected with these antibodies (27).

[b] Symbols: 0, escape mutant was not inhibited (fetuin substrate); ±, escape mutant was partially inhibited; +, escape mutant was still inhibited by that monoclonal antibody.

[c] An escape mutant of NWS-tern was selected with antibody NC41v1 (Fig. 4).

[d] The escape mutant from footnote *c* with a sequence change at 367 was selected with antibody NC10 (v5A and v5B).

[e] The escape mutant from footnote *d* with changes at 367 and 329 was selected with antibody NC33 (v1, v2, and v6).

antibodies (Table 2). These antibodies gave three reactivity patterns: those that inhibited NA activity to levels similar to that with the parent (wt) NWS-tern virus, those that gave partial inhibition, and those that failed to neutralize. The antibodies that inhibited partially or completely were used to select sequential variants. The antibodies that showed partial inhibition failed to select escape mutants, whereas those that showed complete inhibition did select mutants.

The antibodies that showed partial inhibition with mutant NC41v1 had selected escape mutants from wt virus at residues 369 (NC11, NC17, NC20, NC31, and NC66) or 368 (NC24), suggesting that their main center of interaction was at the tip of the 366 to 373 loop and the change at 367 in escape mutant NC41v1 caused some reduction of binding. The antibodies that bound variant as wt (complete inhibition of NA) and selected escape mutants of NC41v1 had selected mutants from wt NA changed at residues 329 (NC10) and 432 (NC34).

Antibody NC10 was used to select two variants of NC41v1 (NC10v5A [Pea] and NC10v5B [Bean]), and then variants of NC10v5B were selected with NC33. The NC10 epitope encompasses the same region of N9 NA as NC41 does, but with gaps when compared with NC41 (see Table 3 of reference 27); some amino acid changes which are recognized by NC41 are not recognized by NC10. All amino acid substitutions recognized by NC10 are also seen by NC41, so the reciprocal experiment could presumably not have been done. Antigenic properties and variant selection by NC33 have not been reported previously. The reactivity patterns of the sequential variants with monoclonal antibodies are included in Table 2.

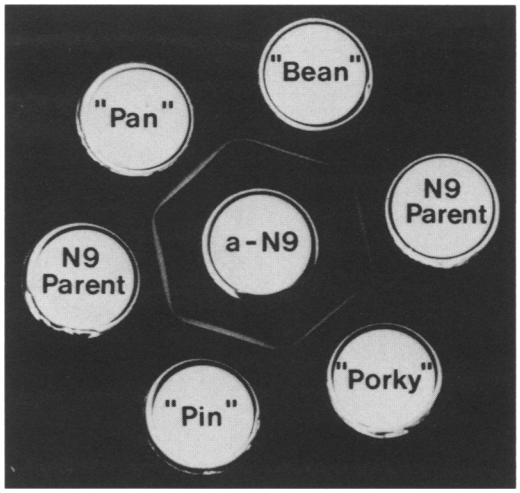

FIG. 2. Double immunodiffusion test of the triple variants. The center well contained antiserum against purified NA of A/tern/Australia/G70c/75 (parent). Two wells contained NA of wt virus; three wells contained NA from the triple variants (NC41v1/NC10v5B/NC33v1 [Pin], NC41v1/NC10v5B/NC33v2 [Pan], and NC41v1/NC10v5B/NC33v6 [Porky]); and one well contained the double variant NC41v1/NC10v5B (Bean).

In several previous studies of single-change escape mutants selected with monoclonal antibodies, there was no detectable difference between mutant and wt virus when they were tested with polyclonal antisera (30). Therefore we tested the triple escape mutants with polyclonal rabbit antiserum. In immunodiffusion tests there was no difference between any of the mutants and wt virus (Fig. 2). However, there were fourfold reductions in the NI titers of two of the triple escape mutants and twofold reductions in the titers of the other (Fig. 3).

The sequence of the NA gene of each mutant was determined completely, and the amino acid changes derived from changes in the nucleotide sequences are shown in Fig. 4. The change Asn-329 to Asp was the same as when NC10 was used to select an escape mutant from wt virus (27). The other change selected by NC10, of Ala-369 to Thr, has not been seen before, although the change form Ala-369 to Asp abolished binding by NC10. The change from Ala-369 to Thr was also selected in the third selection three times (by antibody NC33); in one case the Asn-329 to Asp change was found to have reverted (Fig. 4).

The results confirm that all these antibodies recognize the same general area of the NA, but in very different ways; the epitopes are overlapping, but the contacts are very different.

Sequence of another N9 NA. Viruses with subtype N9 NA are frequently found in influenza viruses from gulls and shorebirds. We have previously determined the nucleotide sequence of an N9 NA from an influenza virus isolated from a whale (A/whale/Maine/1/84) (3). We have now completed the sequence analysis and determined antigenic properties of another N9 NA isolated from a bird virus (A/ruddy turn-

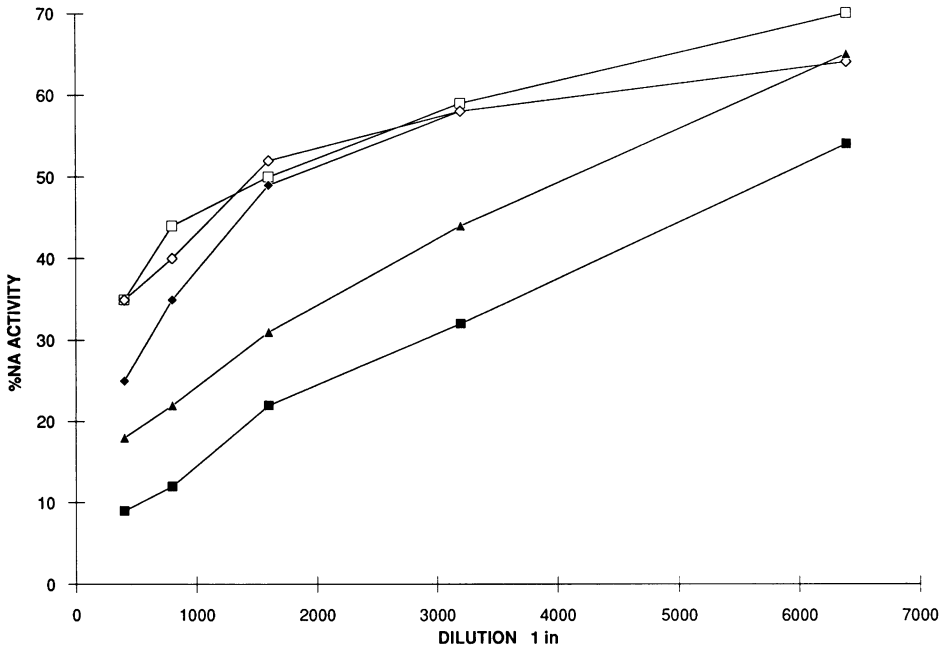

FIG. 3. Inhibition of NA of wt N9 NA compared with that of sequentially selected triple variants. Rabbit antiserum used was raised against the wt NA. The NA activity remaining was determined at each dilution of antibody. The substrate used was fetuin. Symbols: ■, wt N9; □, Pin; ◆, Pan; ◇, Porky; ▲, Bean.

stone/NJ/85). The sequence is more similar to the whale virus N9 than to tern virus N9; there are only 5 amino acid sequence differences between the ruddy turnstone and whale virus NAs versus 14 between tern and turnstone virus NAs (Table 3). A remarkable feature of the sequence comparison between the tern and whale virus NAs was the lack of any changes in the known antigenic regions, and this is almost true for ruddy turnstone virus NA. The one difference in a known epitope is Ile-368 (whale and tern virus NA) to Thr in ruddy turnstone virus NA. A change of Ile-368 to Arg was selected from the tern virus NA by antibody NC24. In NI assays NC24 still inhibits ruddy turnstone virus NA, as do all other antibodies which inhibit the whale virus NA (results not shown); therfore, the less drastic change in side chain of Ile-368 to Thr is not sufficient to abolish the binding of NC24. The nucleotide sequence showed some other changes which did not affect the amino acid sequence.

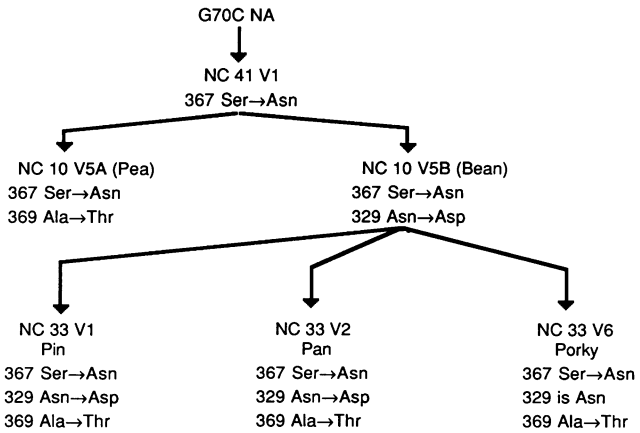

FIG. 4. Derivation and sequence changes in sequentially selected triple variants of N9 NA.

DISCUSSION

The three-dimensional structure of the N9 NA-NC41 Fab complex has been solved by X-ray crystallography and refined to 0.29-nm resolution with an R factor of 0.187 (9, 24). Although virtually all of the main chain and most of the side chains were reported to be observed as continuous electron density, the structural information has not been released beyond a list of contacting amino acids. A major problem in understanding antigenic drift in influenza A virus is in understanding how single amino acid sequence changes in the NA of escape mutants abolish the binding of antibody NC41 when there are 21 other amino acids contacting the antibody. Evidence obtained so far indicates that the

TABLE 3. Amino acid changes in N9 NA

Residue[a]		Amino acid in:		
N9	N2	Tern	Whale	Turnstone
82		I	S	S
84		D	E	E
158	156	A	D	A[b]
189	187	K	R	R
190	188	T	A	A
235	233	H	Q	H[b]
271	269	A	T	T
286	284	R	Q	Q
288	286	E	G	R[b]
289	287	I	V	V
306	304	R	Q	Q
359	358	V	G	G
369	368	I	I	T[b]
388	387	K	R	R
437	435	K	K	R[b]
459	457	D	N	N

[a] Since the N9 structure was described in N2 numbers (7), the N2 equivalents are used in describing sequence changes.
[b] Amino acids that are different from those in the whale virus NA.

changes in escape mutants are restricted to the side chain of the amino acid involved and that there is no overall change to the conformation of the epitope (13, 24, 26, 32). A number of escape mutants selected by a variety of monoclonal antibodies and involving contact amino acids in the NC41 epitope either abolished or reduced binding of the NC41 antibody. Many of these, however, were not isolated during the selection process. One aim of the work reported here was to see, if we set up a large number of identical selection experiments with antibody NC41, whether we would eventually find escape mutations at all of the contacting amino acids or whether the changes would be restricted to certain amino acids (and perhaps certain types of substitutions).

To our bewilderment, 19 escape mutants of N9 NA selected with antibody NC41 show a marked restriction in the sequence changes involved. Six mutants had a change at position 367 (four of Ser to Asn and one each of Ser to Gly and Arg). At position 400 two mutants had the same sequence change (Asn to Lys), and, remarkably, at position 372 10 mutants has the same sequence change (Ser to Phe) while none had the change to Tyr which was selected by another antibody and which did not seem to bind NC41. Equally surprising was the finding that no escape mutants with changes at 369 or 370 were found, even though mutants with changes at these residues (selected with other antibodies) rendered the epitope unrecognizable by antibody NC41.

Escape mutants isolated in the manner described above do not bind the antibody used for their selection, and although these may in fact occur infrequently in the population, they are the only ones we can study. Experiments in which site-directed mutagenesis is used to create changes at other sites are in progress. Escape mutants at positions 329 and 368 (selected by antibodies NC10 and NC24, respectively) were not selected by NC41, clearly because this antibody still bound partially, and changes at many of the other positions may also have resulted in reduced binding by NC41 but remained undetected because of the selection procedure.

The NC41 epitope as currently understood is shown in Fig. 5. The amino acids which contact antibody as determined from the crystal structure of the NA-Fab complex (24) are shown together with escape mutations which partially or completely abolish the binding of NC41 to the N9 NA. The reason why changes in only 4 of the 22 amino acids in the NC41 epitope were selected as escape mutants is not clear. It is unlikely that in N9 NA this region cannot tolerate changes. The amino acid sequences in N9 NAs are highly conserved between ruddy turnstone virus and whale virus in the Atlantic and tern virus in Australia, but this is in keeping with a general lack of antigenic selection in avian viruses (2, 11, 12). The most likely explanation is that these are the key contacts and the other interactions are less important. A prime candidate for an escape mutation would seem to be amino acid 369, which contacts both the light and heavy chains of the antibody. A change of Ala-369 to Asp was selected by six other antibodies; therefore, we know that this mutant exists in the virus population which was used for selection of escape mutants by antibody NC41. Although the mutant NA with Asp-369 did not bind antibody NC41 when tested by enzyme-linked immunosorbent assay or NI, it was not selected by NC41 in 19 experiments.

It is clear that we need to look at a wider variety of changes to fully characterize the NC41 epitope, and future experiments will use site-directed mutagenesis with a eucaryotic expression system to further study the NC41 epitope. Epitope mapping by selection of multiple escape mutants with a single monoclonal antibody (NC41) identified

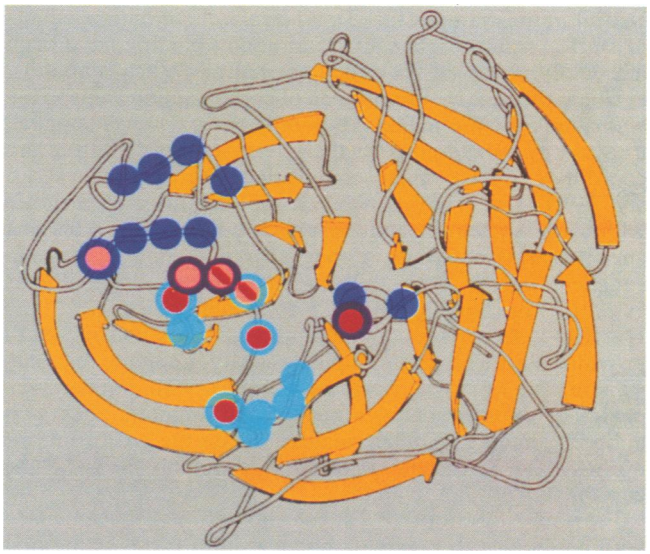

FIG. 5. NC41 epitope on N9 NA. Amino acid side chains identified as contacts with antibody in the X-ray crystal structure (24) are indicated, together with escape mutations selected by NC41 and other escape mutations which abolish or reduce binding. Another escape mutation, at amino acid 220, is located on the opposite side of the monomer and has no effect on NC41 binding. Colors: mid-blue, contacts antibody light chain; light blue, contacts heavy chain; dark blue, contacts both light and heavy chains; red, escape mutants selected with NC41; red stripe, escape mutants selected with other antibodies which do not bind NC41; pink, escape mutants selected by other antibodies which have little effect on binding NC41.

only three of the five polypeptide loops involved in antibody binding (Fig. 5). Sequential selection of escape mutants with three monoclonal antibodies which recognize overlapping epitopes permitted selection of mutants with three changes. Although these changes were in only two polypeptide loops, the resulting variants did not bind any of the antibodies with epitopes overlapping that of NC41.

The only method currently available to study the complete structure of a protein epitope is X-ray diffraction analysis of crystals of antibody-antigen complex, and such experiments have so far been successful for only two protein antigens; two epitopes on influenza virus NA (24) and three epitopes on hen egg lysozyme (5, 19, 22) have been structurally defined. It has been demonstrated by using closely related lysozymes that in the lysozyme system, single amino acid sequence changes can cause a dramatic reduction in antibody binding (23), and more recently site-directed mutagenesis has been used to probe the structural requirements of the epitope (15, 16). Although escape mutant selection experiments cannot be done with lysozyme, the binding studies show that, as in the case of NA, some mutations in contact amino acids cause much more dramatic effect than others on antibody binding. However, to date there are no examples of a mutation within the structural epitope which does not affect binding at all.

The crucial involvement of certain amino acids in antibody interaction was noted for the D1.3 epitope of lysozyme; even at 0.6-nm resolution (4) Gln-121 was seen to occupy a central position in the contact area, explaining why substitution of this amino acid abolishes lysozyme inhibition by antibody D1.3. However, in the case of the NC41 epitope on NA, the amino acids selected as escape mutants are not particularly

central in the epitope (Fig. 5). Although the dramatic effects of single sequence changes on antibody binding have been frequently observed (21), they are not easily explained. To evaluate the relative importance of certain amino acids to the lysozyme-Fab interactions, Novotny et al. (18) have applied free-energy calculations to the energy-minimized structure coordinates of lysozyme complexed with antibodies D1.3 and HyHEL-5. They concluded that a subset of residues within the structural epitope provide most of the binding energy, and the residues of the "energetic epitope" were in excellent accord with those identified experimentally as essential for binding anti-lysozyme antibodies D1.3 and HyHEL-5. The other contact amino acids are considered to provide passive surface complementarity, implying that only substitution of these residues to ones with bulkier side chains or different charges could have dramatic effects on antibody binding. Similar calculations on the NC41 epitope of NA have not been reported, presumably because the coordinates are not available.

Previous selections of escape mutants have been made with antibodies to nonoverlapping epitopes; this is the first study of selection of sequential escape mutants in which the epitopes show extensive overlap. The results indicate that different antibodies, although contacting many amino acids in the domain, may have relatively few key interactions. The requirements for selecting an escape mutant are apparently very strict. None of the large number of antibodies which showed partial binding to NC41v1 selected variants, and escape mutants selected by other antibodies which appeared not to bind NC41 were not selected by NC41. These studies indicate that selection of multiple escape mutants (to determine the key contacts) combined with sequential selection of escape mutants (to identify other contacts) will permit a more extensive analysis of the combining site where an X-ray crystal structure has not been determined or the pertinent information is not released.

The results suggest that although the NC41 epitope contains 22 amino acids, only a few of these are so critical to the interaction that a single sequence change allows selection of an escape mutant. Multiple changes of less important contacting amino acids also allow escape, but in the laboratory these were obtained only from sequential selections. The variety of amino acid sequence changes which can lead to the polyclonal selection of new epidemic viruses during antigenic drift would seem to be very limited.

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