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Review Article

The Neuraminidase of Influenza Virus

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ABSTRACT **It is the enzyme neuraminidase, projecting from the surface of influenza virus particles, which allows the virus to leave infected cells and spread in the body. Antibodies which inhibit the enzyme limit the infection, but antigenic variation of the neuraminidase renders it ineffective in a vaccine. This article describes the crystal structure of influenza virus neuraminidase, information about the active site which may lead to development of specific and effective inhibitors of the enzyme, and the structure of epitopes (antigenic determinants) on the neuraminidase.The 3-dimensional structure of the epitopes was obtained by X-ray diffraction methods using crystals of neuraminidase complexed with monoclonal antibody Fab fragments. Escape mutants, selected by growing virus in the presence of monoclonal antibodies to the neuraminidase, possess single amino acid sequence changes. The crystal structure of two mutants showed that the change in structure was restricted to that particular sidechain, but the change in the epitope was sufficient to abolish antibody binding even though it is known in one case that 21 other amino acids on the neuraminidase are in contact with the antibody.**

Key words: neuraminidase (sialidase) of influenza virus, structure, enzyme active site, antigenic sites, escape mutants, structures of epitopes, structures of neuraminidase, Fab complexes

PROLOGUE

Early in the 1940s, at the Rockefeller Foundation in New York City, George Hirst noticed that allantoic fluid from embryonated chicken eggs inoculated with influenza virus caused red blood cells to heavily agglutinate.[1] When the red cells, agglutinated by virus particles, were warmed to 37°C they dispersed as the virus eluted and were not reagglutinated by fresh infectious allantoic fluid. On the other hand, virus which had eluted from the red cells at 37°C could agglutinate fresh red blood cells. Hirst interpreted these phenomena as resulting from some enzymic activity of the virus, where the substrate (virus receptor) was located on the surface of the red cell. In the 1950s, Alfred Gottschalk, working at the Walter and Eliza Hall Institute in Melbourne, realized that if the receptor-destroying activity was an enzyme, this might remove something from the surface of the red cells. He therefore set about looking for the "split product." This was eventually identified as *N*-acetylneuraminic acid (sialic acid) and the enzyme was called sialidase or neuraminidase,[2] now designated acylneuraminyl hydrolase (EC 3.2.1.18).

INTRODUCTION

Influenza viruses are classified into three types, A, B, and C. Flu C is rather different to the other two types and has a different receptor-destroying enzyme, and only types A and B will be discussed here.

The genome of the influenza A and B viruses consists of single-stranded RNA of negative sense existing in eight pieces, packaged in orderly fashion within the virion by some as yet unknown mechanism. Each piece codes for one of the major viral proteins, and in some cases minor proteins are also coded, using overlapping reading frames (for review see ref. 3). The RNA is associated with a nucleoprotein (NP) and three proteins involved in RNA replication and transcription (PA, PB1, and PB2). This replication complex is enclosed within a membrane composed of matrix protein associated with a lipid bilayer. Embedded in the lipid bilayer are two surface glycoprotein spikes, the hemagglutinin (HA) and the enzyme neuraminidase (NA) (Fig. 1).

Each of the viral genes has been sequenced completely, some from many isolates of different virus subtypes, and the three-dimensional structures

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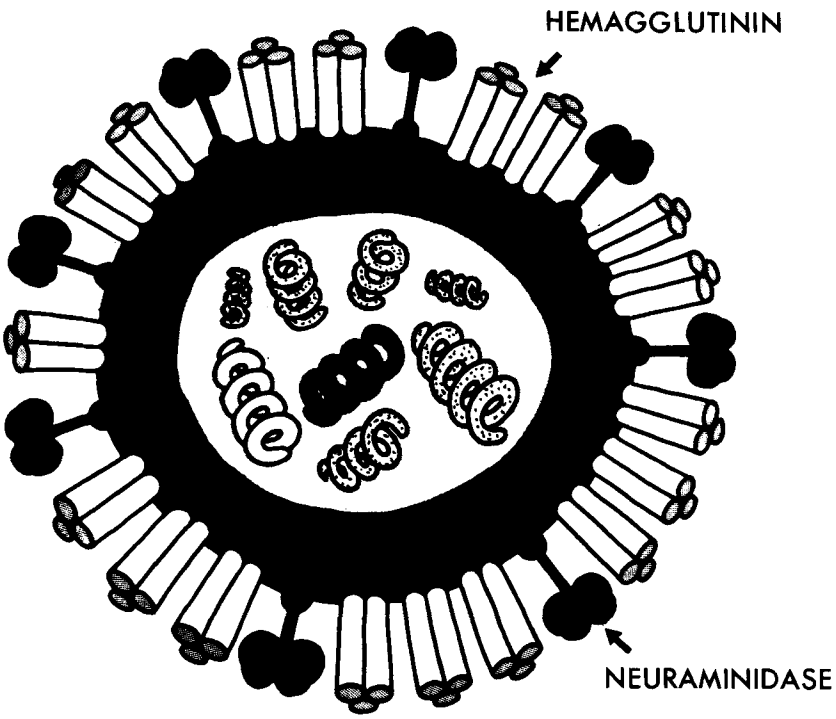


Fig. 1. Schematic of an influenza virion. Shown are the two glycoprotein surface antigens, the hemagglutinin (a trimer) and the neuraminidase (tetramer). The eight RNA segments are coated with nucleocapsid protein and associated with the polymerase proteins.

of the two surface glycoproteins have been determined.[4,5]

Despite this wealth of information, influenza remains a potentially devastating disease of man, lower mammals, and birds. No effective vaccine exists, and no cure is available once infection has set in.

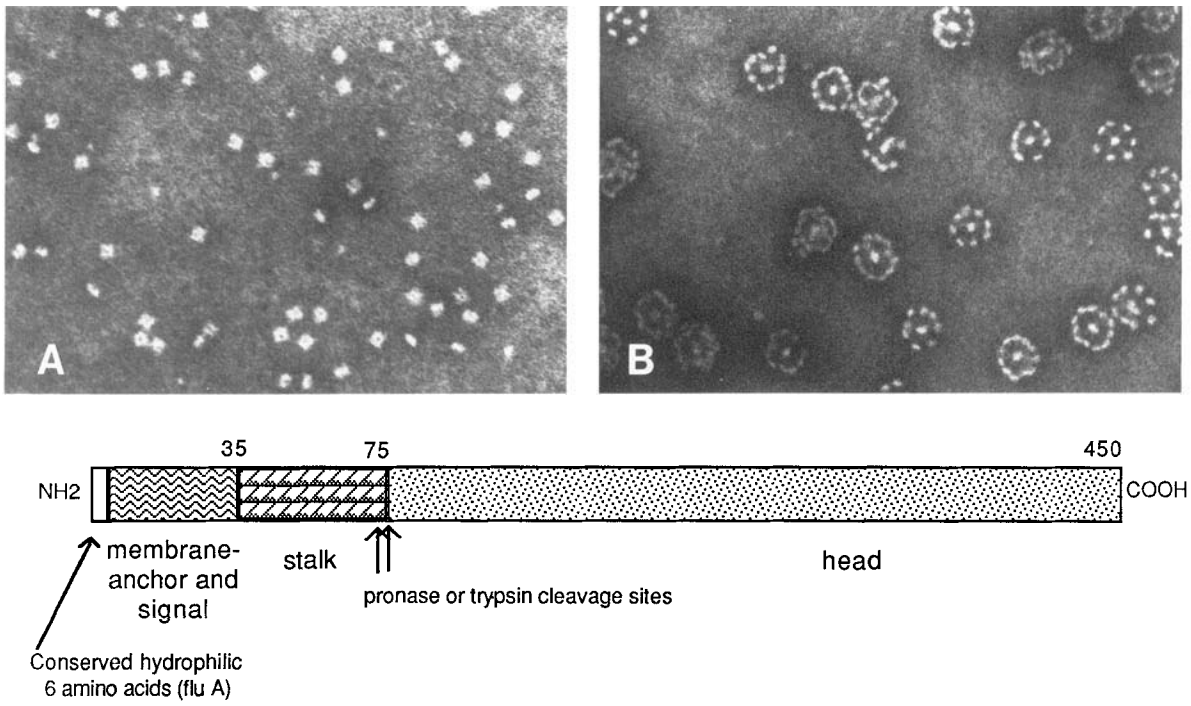
Influenza viruses continually undergo antigenic variation in the two surface antigens (HA and NA) toward which neutralizing antibodies are directed. Because of this rapid evolution, vaccines have not been very effective, and attention is now being directed to potential targets within the virus against which antiviral agents can rationally be designed.

At the moment, three such targets can be identified: (1) the replication complex, which reproduces the viral genomic RNA and which produces the mRNA molecules from which viral proteins are translated; (2) the HA, whose function is to attach the virus to cell receptors and mediate entry of the virus into the cell by a membrane fusion process; and (3) the NA, whose function seems to be release of newly budded virus from infected cells and the spread of virus in the body.

This review will discuss the three-dimensional structure of the NA, the structure of the active site against which inhibitors can be designed, the structure of epitopes on the enzyme, the way in which antibodies recognize these epitopes, and the way in which epitopes change during antigenic drift.

THE NEURAMINIDASE (NA)

The NA accounts for about 5–10% of the virus protein and exists as a mushroom-shaped spike on the surface of the virion. It is a tetramer with a box-shaped head, 100 × 100 × 60 Å, made of four coplanar, roughly spherical subunits and a centrally attached stalk containing a hydrophobic region by which it is embedded in the viral membrane (Fig. 2). The neuraminidase molecule is composed of a single polypeptide chain, coded by RNA segment 6, anchored in the virus membrane by a series of hydrophobic amino acids near the N-terminal end of the polypeptide (Fig. 2). This contrasts to the influenza hemagglutinin, which is anchored by a hydrophobic sequence near the C-terminus. No posttranslational cleavage of the NA polypeptide occurs, no signal peptide is split off, and even the initating methionine is retained.[6] Nor is there processing at the C-terminus; the C-terminal sequence, -Met-Pro-Ile predicted from the gene sequence for N2 NA is found in intact NA molecules isolated from the virus. A sequence of six polar amino acids at the N-terminus of the NA polypeptide, which are totally conserved in each of the nine different influenza A NA subtypes,[7,8] but not in influenza B,[9] is followed by a sequence of hydrophobic amino acids that must represent the transmembrane regions of the NA.[10] This sequence is not conserved at all between subtypes (apart from conservation of hydrophobicity). For bio-



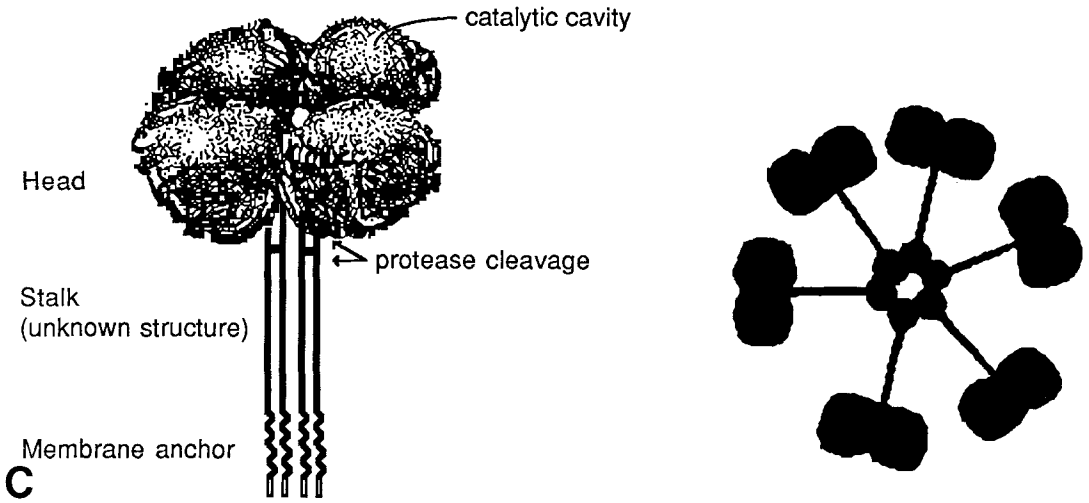


Fig. 2. Electron micrographs of neuraminidase molecules. **A:** Pronase-released NA heads. **B:** Whole NA molecules isolated from detergent-disrupted virus. The tetrameric heads can be seen in (**A**) (both en face and side views appear); in (**B**) the molecules (from which detergent has been removed) associate by the hydrophobic N-terminal sequences as diagrammed in (**C**). Protease digestion releases NA heads, which retain all the enzymatic and antigenic activity. Deletion in the stalk region can result in "stubby" NA molecules.[11] Micrographs by Nick Wrigley.

chemical studies, a soluble form of the NA can be released from the virus particles by treatment with proteases, which cleave the polypeptide in the positions shown (Fig. 2), removing the stalk and releasing the enzymatically and antigenically active head of the NA that, in some cases, can be crystallized. Viruses have been obtained with "stubby" neuraminidase molecules in which the stalk is shortened by deletions of up to 18 amino acids.[11]

Several roles have been suggested for the neuraminidase. The enzyme catalyzes cleavage of the α-ketosidic linkage between terminal sialic acid and an adjacent sugar residue. In mucin, removal of sialic acid lowers the viscosity and permits access of the virus to the epithelial cells. Neuraminidase also destroys the hemagglutinin receptor on the host cell, thus allowing elution of progeny virus particles from infected cells.[12] The removal of sialic acid from the

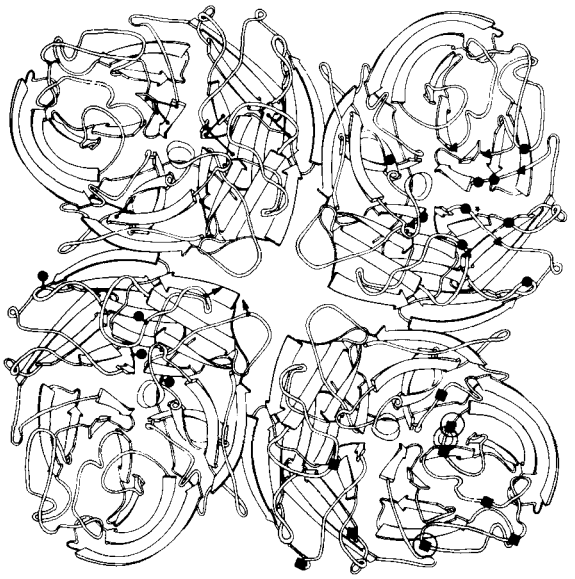


Fig. 3. Schematic diagram of the NA tetramer viewed from above down the symmetry axis. The four subunits highlight different features of the structure [17]. Top left: disulfide bonds (joined points). Bottom left: carbohydrate attachment sites at positions 86, 146, 200, and 234 (circle) and metal ligands Asp 113 and Asp 114 (arrows). Bottom right: residues which change in variants selected with monoclonal antibodies (squares) N2 variants; (circles) N9 variants. Top right: conserved acidic (circles) and basic (triangle) residues in infleunza A and B neuraminidase and the sialic acid binding site (star).

carbohydrate moiety of newly synthesized hemagglutinin and neuraminidase is also necessary to prevent self-aggregation of the virus. In general, then, the role of neuraminidase may be to facilitate mobility of the virus both to and from the site of infection, and although this role is not well defined, the NA induces antibodies that protect against lethal influenza viruses.[13] In type A influenza, nine subtypes of NA that lack any antigenic cross-reactivity have been classified. N1 and N2 have been found in viruses infecting man and N1–N9 in viruses infecting other animals, mainly wild birds.

STRUCTURE OF N2 NEURAMINIDASE

The three-dimensional structure of N2 neuraminidase heads determined from an electron-density map at 2.9 Å resolution shows that each monomer is composed of six topologically identical β-sheets arranged in a propeller formation.[5] The tetrameric enzyme has circular fourfold symmetry stabilized in part by metal ions bound on the symmetry axis. The catalytic sites are located in deep pockets that occur on the upper corners of the box-shaped tetramer. Sugar residues are attached to four of the five potential glycosylation sequences, and in one case the carbohydrate contributes to the interaction between subunits in the tetramer (Fig. 3).

STRUCTURE OF N9 NEURAMINIDASE

The three-dimensional structure of the neuraminidase antigen of subtype N9 from an avian influenza virus (A/tern/Australia/G70c/75) that shares 50% sequence identity with the human N2 influenza virus neuraminidase has been determined by X-ray crystallography[14] and shown to be folded similarly to N2 neuraminidase. This result demonstrates that lack of immunological cross-reactivity is not related to differences in polypeptide chain folding. Small differences in the way in which the subunits are organized around the molecular fourfold axis are observed. Insertions and deletions with respect to subtype N2 neuraminidase occur in four regions, only one of which is located within the major antigenic determinants around the enzyme active site.

INFLUENZA TYPE B NEURAMINIDASE

Type B influenza virus RNA segment 6 codes for two distinct glycoproteins, NA and NB, using overlapping reading frames. The amino acid sequence of polypeptide NB deduced from the nucleotide sequence of the B/Lee/40 strain consists of 100 amino acids.[9] The sequence contains four potential glycosylation sites, and two of these have been found to be glycosylated in infected cells. NB has not been found in virions but is present on the surface of infected cells.[15] A protein analogous to NB has not been found in influenza A virus and this represents a major difference between the two virus types. No subtypes of influenza B virus exist and, in marked contrast to influenza A, influenza B has so far been found only in the human population.

Influenza B/Lee/40 neuraminidase has less than 25% sequence identity when compared with either N2 or N9 neuraminidase in the head region. Conservation of 16 cysteine residues that form disulfide bonds in N2 and N9 neuraminidases and of several amino acid side chains that line the active site pocket suggests that the polypeptides may be similarly folded, but this can be confirmed only by a complete structure determination.

Crystals of neuraminidase heads from two different influenza B virus strains, B/Lee/40 and B/Hong Kong/8/73, have been grown and unit cell parameters determined.[16] On further analysis these crystals showed disorder, making the solution of the structure difficult if not impossible. However, when the B/Lee/40 NA is complexed with Fab fragments of a monoclonal antibody (see below), crystals are obtained that are well ordered, and the route to the structure determination of influenza B NA may be through its complex with antibody.

ACTIVE CENTER OF NEURAMINIDASE

The influenza virus NA provides a unique opportunity to characterize an enzyme active center that is conserved amid enormous variation in amino acid

TABLE I. Site-Directed Mutations in the NA Active Site

Mutant	Expression	Transport to surface	Correct folding	Enzyme activity (%)	Other
wt	+	+	+	100	
N 146 S	+	(+)	0	0	
R 152 K	+	+	+	0	
R 152 I	+	+	+	0	
W 178 L	+	+	+	0	
D 198 N	+	+	+	0	
I 222 V	+	+	+	90–100	
H 274 Y	+	+	+	70–80	Low pH activity
H 274 N	+	+	+	70–80	Low pH activity
E 276 D	+	+	+	0	
E 276 Q	+	+	+	0	
E 277 D	+	+	+	0	
R 371 K	+	+	+	5–10	
Y 406 F	+	(+)	0	0	

sequence. There is up to 75% sequence variation between NAs of influenza A and B, and hence those residues that are conserved in all known sequences are likely to be essential for enzyme function. In the structure determined to 2.9 Å resolution[17] several such conserved residues line the substrate binding pocket, and these were obvious targets for site-specific mutagenesis to delineate the mechanism of enzyme action.

Oligonucleotide-directed site-specific mutagenesis was used to study the enzyme active site of the influenza virus neuraminidase.[18] Previous studies described the catalytic properties of wild-type NA including pH optimum, substrate specificity, and kinetics,[19,20] but little was known about the mechanism of enzyme action. Conserved amino acids lining the active site pocket were therefore altered and the properties of these mutants used to locate a possible center of catalysis.

Fourteen mutations were made, as shown in Table I. To minimize the likelihood of altering the folding of the protein, most mutations were designed to be conservative. Correct folding was monitored by binding of a mixture of monoclonal antibodies, which were shown to be specific for native NA.

The mutant NA genes were inserted into an SV40 vector in which the NA gene replaced the SV40 late region, and the recombinant SV40 DNA was transfected into African Green monkey kidney cells in the presence of a "helper" SV40 DNA. Synthesis of the NA was followed by immunofluorescence. The NA polypeptides were seen to be transported to the cell surface and could be assayed for NA activity on two substrates, fetuin, a glycoprotein, and *N*-acetylneuraminyl lactose (NANL), a trisaccharide.

Two mutations (N146S, abolishing a carbohydrate addition site, and Y406F) altered the three-dimensional structure of the protein as the mutant NA proteins were no longer recognized by a mixture of monoclonal antibodies specific for the properly folded protein. The enzyme activity was lost in most of the mutant NA polypeptides.[18] At two sites, 152 and 371, the seemingly conservative change of Arg to Lys almost completely abolished enzyme activity, possibly because the Arg and Lys side chains are unequal in length. The most interesting change in enzyme activity occurred when His-274 was changed to either Tyr or Asn. Both of the mutants behave identically, having 70–80% wild-type activity with NANL, and only 40–60% of wild-type activity toward fetuin under the standard assay conditions, partly due to a shift in pH optimum from 5.5–6.5 in the wild-type to pH 5.0 in both mutants.

The location and orientation of conserved amino acids in the substrate binding pocket are diagrammed in Figure 4. So far, no one has reported obtaining quantities of purified NA from any expression system sufficient to undertake detailed kinetic or structural analyses of the mutants, but the shift in pH optimum to the acidic side when His-274 was altered suggested a possible mechanism for the catalytic activity of the influenza neuraminidase by analogy with catalysis by lysozyme.[21,22] The proposed NA mechanism is shown schematically in Figure 5, and involves the proposal that His-274 elevates the pK_a of nearby Glu-276, which would function as the proton donor for the reaction. In the absence of His at residue 274, the pK_a of Glu-276 would assume a more typical value accounting for the lowered pH required for reaction in the two mutants.

An obvious question is whether substrate (or product) does indeed bind in this region. Although crystals containing bound sialic acid were reported,[17] no further information has been released. Thus, at present, there is no way of comparing the sialic acid binding site of NA with that demonstrated in the HA by X-ray crystallography.[23]

The enzyme activity of the neuraminidase protein is conserved among all the different strains and sub-

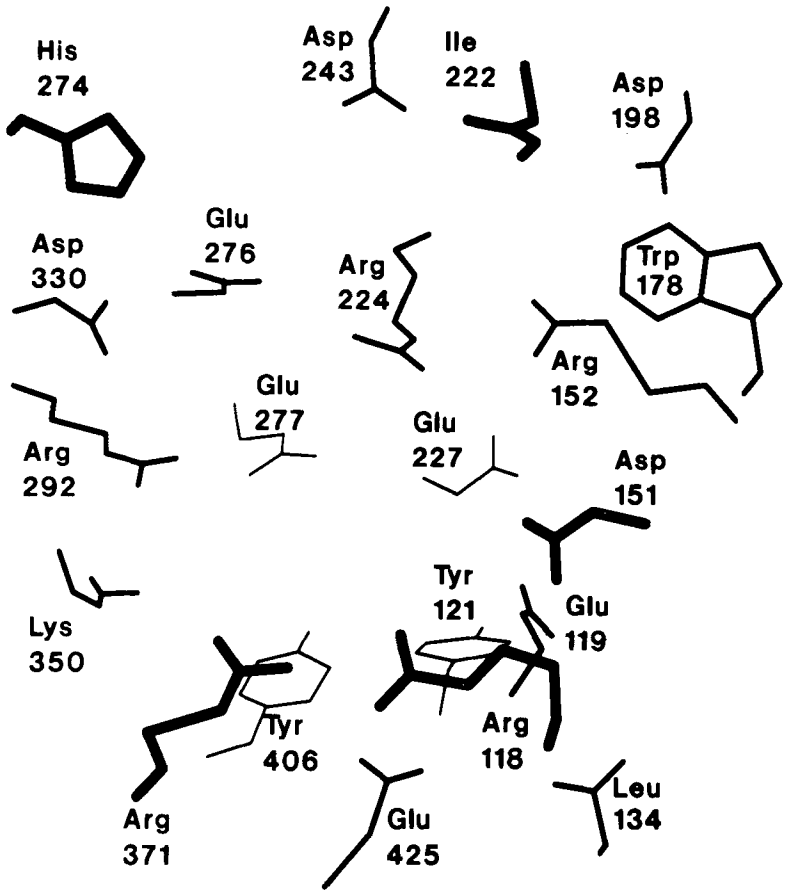


Fig. 4. Structure of the neuraminidase active site.[18] The conserved residues located in the substrate binding pocket are shown. Mutations were made in residues 152, 178, 198, 222, 274, 276, 277, 371, and 406. Side chains implicated in the catalytic mechanism are His-274 and Glu-276.

types of influenza virus and depends on conservation of the amino acid side chains lining the active site pocket. Thoughts of using this conserved region to develop a (universal) vaccine against all flu strains are dashed by the knowledge from the three-dimensional structure that they are inaccessible to antibodies. A similar situation of conserved amino acids inaccessible to antibody surrounded by variable amino acids was noted in rhinoviruses and has become known as the "canyon hypothesis."[24] Detailed information about the activity and structure of the active site of influenza neuraminidase may enable design of new inhibitors to effectively control the virus regardless of its antigenic structure.

ANTIGENIC PROPERTIES OF THE NEURAMINIDASE

Two distinct kinds of antigenic variation have been demonstrated in influenza viruses: antigenic drift and major antigenic shifts. The first consists of relatively minor changes in the surface glycoprotein antigens, HA and NA, that occur gradually within a family of strains, all of which are clearly related to each other with respect to both internal and surface antigens. Among influenza A strains infecting man, each successive variant replaces preexisting ones. This may be due to a selective advantage possessed by antigenic variants in overcoming immunological host resistance. Antigenic variation also occurs in type B influenza viruses, but it does not follow a sequential pattern as in influenza A.[25,26]

The second kind of antigenic variation that has been described only for influenza type A viruses involves much more dramatic antigenic changes. These are the major antigenic shifts that occur when a new influenza virus suddenly appears in the human population with HA, and sometimes also NA molecules, of a different subtype to those of the virus that was circulating before the new virus appeared. Although the origin of these new viruses, which

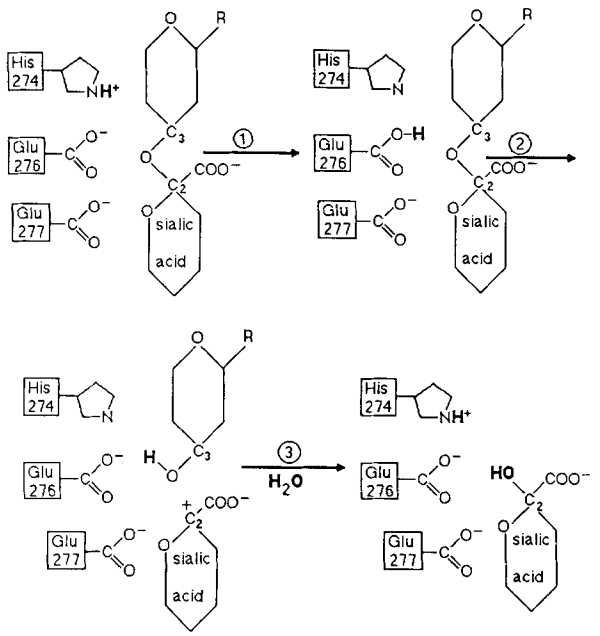


Fig. 5. A proposed catalytic mechanism of neuraminidase. The substrate contains sialic acid linked α(2,3) to any polysaccharide chain (R). After binding to the active site, His-274 donates a proton to the ionized side chain of Glu-276 as shown in step 1. In step 2, Glu-276 uses this proton to break the glycosidic bond, resulting in the release of the polysaccharide minus its terminal sialic acid. A possible ionized intermediate forms, which may be stabilized by the ionized carboxyl of Glu-277 or another conserved acidic residue in the active site. In the final step, a water molecule reprotonates His-274 and contributes a hydroxyl to the transition state intermediate, resulting in free sialic acid that can diffuse from the active site. The enzyme is then ready for another round of catalysis.

cause the major pandemics of influenza, is not known, they may arise by reassortment from influenza viruses infecting animals or birds.

Antigenic variation renders vaccine programs ineffective, and is thus a key factor in the continued epidemiology of influenza in the human population. Drift is caused by mutations in the genes coding for HA and NA, which result in amino acid substitutions in the proteins leading to altered antigenicity and escape from circulating antibodies. Although many data are available on the sequence variation in epidemic strains of flu, it is not possible to identify which of the several changes are important in escape from antibodies. Monoclonal antibodies have proved to be powerful tools in the study of epitopes on the NA. In 1979 Gerhard and Webster[27] showed that if viruses were grown in the presence of monoclonal antibodies to the HA, most of the virus infectivity was neutralized, but mutants with changes in the particular epitope recognized by the antibody were not neutralized, and could be isolated at a frequency of 1 in 10^5. These variants, subsequently called "escape mutants,"[28] were found to have single amino acid sequence changes in the HA.[29]

Antibodies to the NA do not neutralize virus infectivity directly, but if the cells in which virus is growing are bathed in antibody, the enzyme is inhibited, newly formed virus cannot leave the infected cell, and the infection is effectively terminated. Escape mutants of N2 and N9 NA were obtained by growing the viruses in the presence of high concentrations of monoclonal antibodies to the NA.[30,31] The presence of antibodies in the fluid surrounding the cells prevented these from yielding virus and these monoclonal antibodies could be used to select antigenic variants (escape mutants) of the NA. The NA genes of a number of the escape mutants were completely sequenced. Single nucleotide changes were found in each of the variants, which resulted in single amino acid sequence changes in the NA polypeptide.[32,33] Most of these sequence changes occurred on the top of the tetrameric NA head, on the rim around the active site crater, suggesting that neutralizing antigenic determinants (epitopes) were located in these regions. Other epitopes almost certainly exist at the base of the tetramer, but escape mutants have not been isolated, presumably because antibodies binding in this region do not neutralize NA activity.

STRUCTURE OF EPITOPES ON THE NA

A great deal of information has been gathered concerning the structure of combining sites on antibodies and the interactions involved in the binding of small molecular weight haptens, but there is a considerable debate about the way in which antibodies recognize and bind to the surfaces of foreign proteins. In the past, many methods have been used in attempts to define antigenic determinants (epitopes) on protein molecules (reviewed in ref. 34). The methods have included the use of protein fragments to absorb antisera, the production of antipeptide antisera and their reactions with intact proteins, proteolytic digestion of antigens in complexes with antibodies as a probe of protected peptide bonds within the epitope, and the protection of amino acids in the epitope from specific chemical derivatization. Other methods include the characterization of "escape mutants" (variants that do not bind neutralizing monoclonal antibodies) and competitive binding studies of monoclonal antibodies for an antigen.[35] Although data from some experiments have implicated particular amino acid residues as participants in particular epitopes, none has allowed a complete structural description of an epitope.

The first determination of the structure of an epitope on a protein molecule was the determination of the three-dimensional structure of a crystallized complex between lysozyme and the Fab fragment of a monoclonal antibody at 2.8 Å resolution.[36] In this complex, 16 amino acids on the lysozyme and 17 on the antibody form a tightly packed interface from which all water molecules except one are excluded. The interaction has been described as conforming to

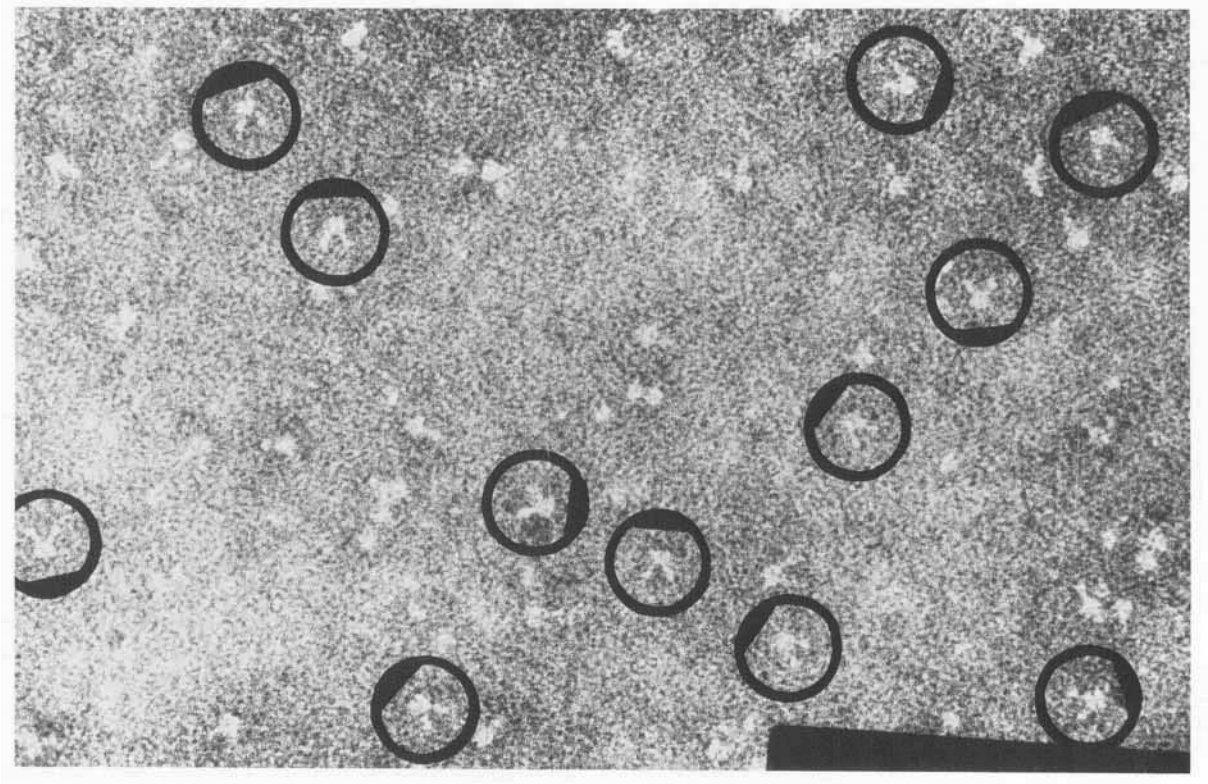


Fig. 6. Electron micrograph of isolated neuraminidase–antibody complexes. Edge projections of individual N9 NA–32/3 Fab complexes (examples circled) are interpreted here as single tetrameric NA heads viewed edge-on, each with four Fab molecules attached and subtending an angle of ~75° to the plane of the NAase head. The upper two Fab arms eclipse the lower two in this projection. Specimens were negatively stained with neutral 2% potassium phosphotungstate.

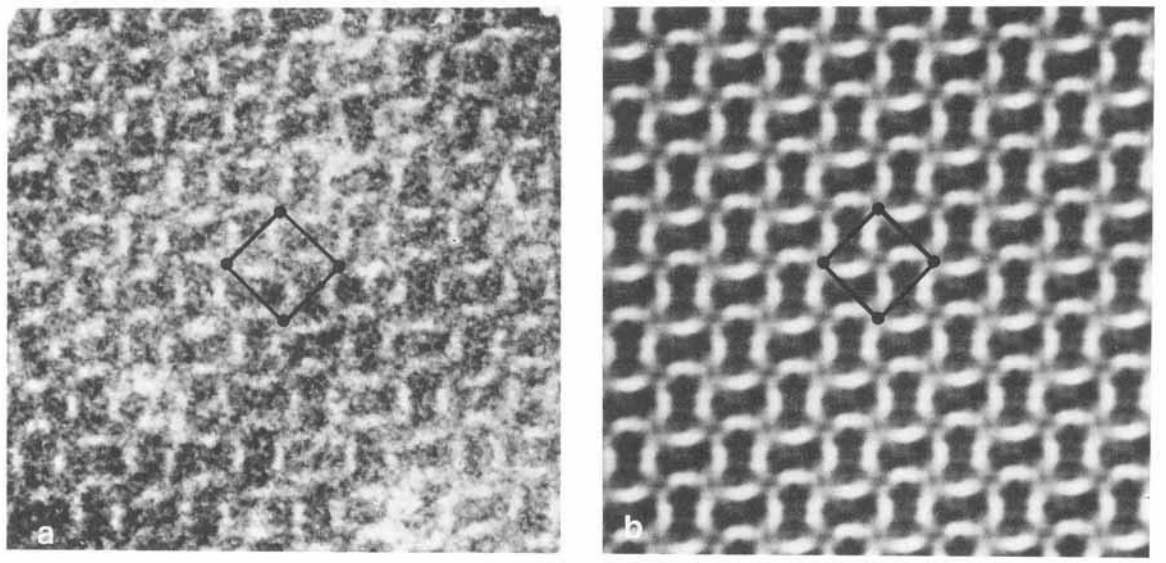


Fig. 7. Electron micrograph of thin crystals of N9 NA complexed with Fab of antibody 32/3. Lattice image of a two dimensional microcrystal of N9 NA–32/3 Fab complex negatively stained with neutral 2% potassium phosphotungstate (**a**) before and (**b**) after computer image processing. The unit cell, with α ~170 Å, is indicated.

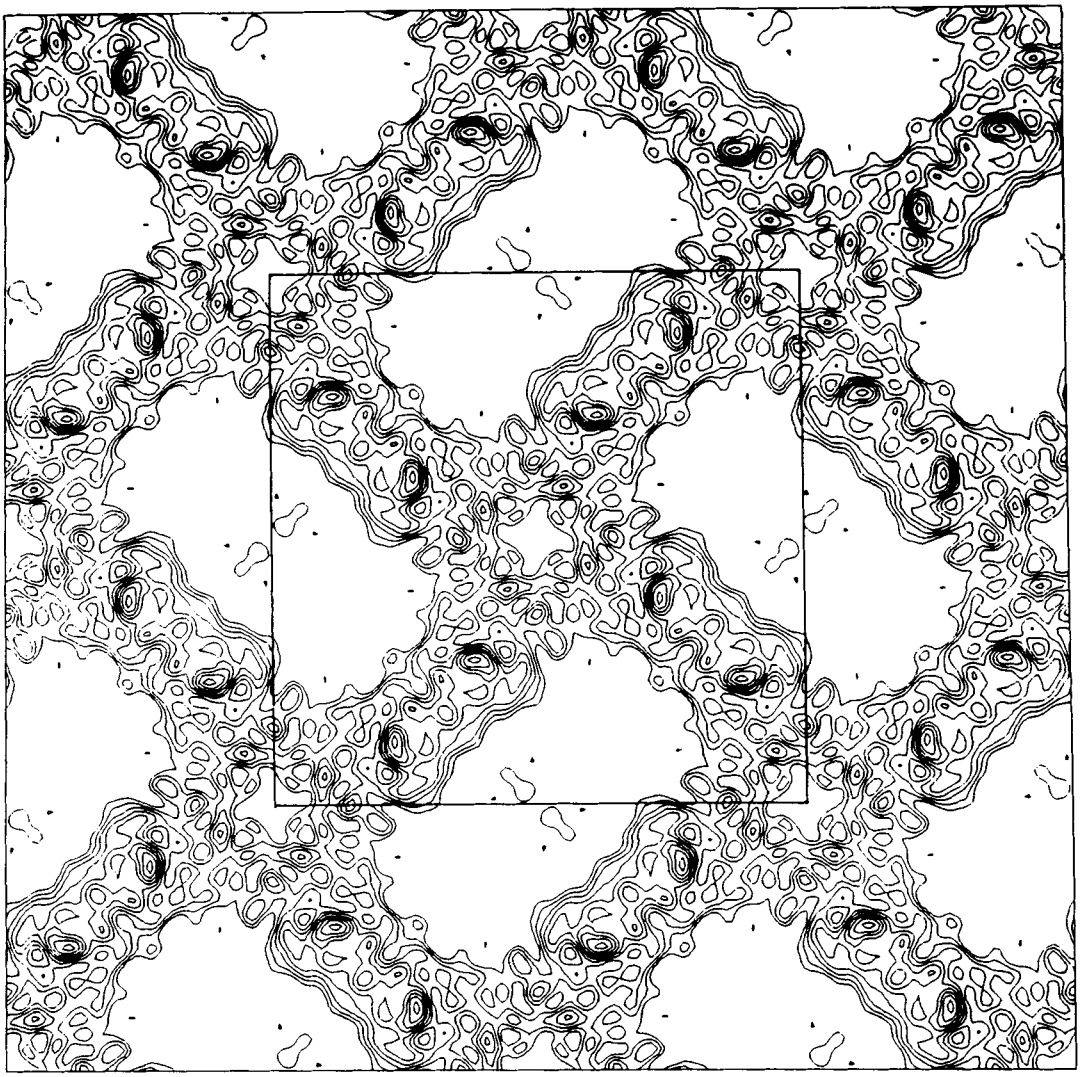


Fig. 8. A 7 Å resolution projection structure of the N9 NA–32/3 Fab complex solved by electron diffraction. One unit cell (plane group p^4gm, $a = b = 159.5$ Å) is outlined in the center. The central complex protomer consists of an approximately square NA head of dimensions 90 Å × 90 Å. It is thought that an Fab arm is attached at each corner of the NA and is pointing up toward the viewer. All nearest neighbor complex protomers are thought to be facing the other way, with the Fab arms pointing into the plane of the paper. Figure after Bullough.[42]

a "lock-and-key" picture of antibody–antigen interaction, in which, apart from some amino acid side chain movements, no structural changes occur in either the antibody or the antigen. Two more lysozyme–Fab complex structures (with antibodies HyHEL-5 and HyHEL-10) have since been determined at high resolution.[37,38] A synthetic peptide containing residues 40–60 of lysozyme (encompassing most of the HyHEL-5 epitope) neither bound to the HyHEL-5 antibody nor inhibited binding of antibody to the lysozyme (S. Smith-Gill, personal communication).

This result is not surprising; since this peptide represented only part of the epitope and is missing 4 amino acids of the 14 in the contacting surface. It is established in several protein antigens that single amino acid changes in the contact surface of the antigen are sufficient to reduce binding of antibody to undetectable levels.

We have grown crystals of monoclonal antibody Fab fragments complexed with influenza virus neuraminidase of the N9 subtype. A number of crystalline complexes were obtained. The first complex yielded small crystals only, which were unsuitable for X-ray diffraction analysis. This complex, between N9 NA and antibody 32/3 Fab (Fig. 6), could be grown as extremely thin, extensive two-dimensional platelet microcrystals (Fig. 7), but all attempts to grow three-dimensional crystals suitable for X-ray diffraction analysis have failed.[39] However, recent advances in high-resolution electron microscopy suggest that considerable structural information may be gleaned from such crystals. Using new methods for

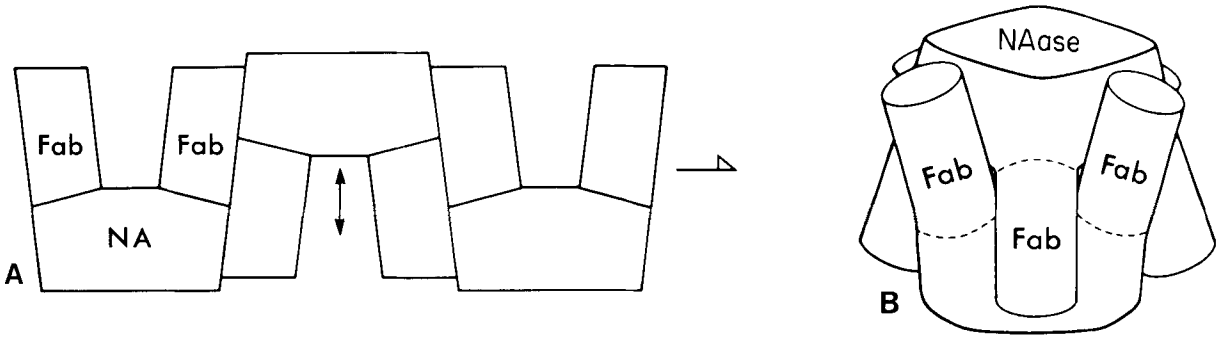


Fig. 9. Schematic views of the proposed arrangement of N9 NA–Fab complex protomers. **A:** NA-32/3 Fab protomers in the plane of the two-dimensional crystalline sheet. Lateral contact between nearest-neighbor protomers is mediated via a Fab fragment from the front side of one protomer and a Fab fragment from the rear side of its neighbor in this view. The relative position, along the vertical *c*-axis direction, of the central protomer with respect to its neighbors is not known (indicated by the vertical arrow), but the unit cell dimensions in the tetragonal plane demand that there be no relative position in which lateral contact between neighboring protomers is via NA heads alone. **B:** The proposed packing of NA–NC35 Fab complex in the *c*-axis direction of the crystals. The view is slightly inclined compared to that in (**A**).

electron imaging that cut down the effects of radiation-induced specimen movements,[40] and sophisticated methods of computer processing,[41] Bullough and Tulloch were able to extract phases from images to relatively high resolution.[42] Despite the large unit cell dimensions of these crystals ($a = b =$ 159.5 Å) and the large solvent content (approximately 70% by volume), a projection structure was determined to 7 Å resolution (Fig. 8). The N9 NA–NC41 complex, which has been solved to atomic resolution,[43] forms crystals of the same space group and is believed to have similar packing (Fig. 9).[39] Comparison of the N9 NA–NC41 and N9 NA–32/3 complexes has suggested that the 32/3 Fab may bind to the NA in the same general antigenic region as the NC41 Fab.[43] This is indeed consistent with the known serology,[44] since 32/3 can select NA escape mutants with amino acid substitutions at 329 and 370.

Crystals of five other complexes that diffract X-rays to beyond 3 Å have been obtained. These are NC41 Fab complexed with N9 NA from tern and whale viruses and with two variants of tern virus NA with sequence changes that reduce but do not abolish binding, and whale virus N9 NA with another antibody, NC10 Fab.

Fab fragments from four different monoclonal antibodies have also been complexed with influenza B virus neuraminidase (B/Lee/40) and the complexes have been crystallized. Three of the complex crystals are, so far, not suitable for X-ray diffraction studies, but the fourth (B/Lee/40 NA-B1 Fab) forms large crystals that diffract X-rays to 3 Å resolution,[45] and structure analysis is underway.

NEURAMINIDASE-NC41 FAB STRUCTURE

The crystal structure of the N9-NC41 complex is reported to 2.9 Å resolution.[43]

C-α traces of antibody and antigen, shown in Figure 10, indicate that the epitope is discontinuous, being comprised of five separate peptide segments involving about 17 amino acid residues. In addition, it appears that complementarity determining region (CDR) H1 of the NC41 Fab may interact with carbohydrate covalently attached to Asn-200 of a neighboring subunit of the tetrameric antigen.[46] Carbohydrates on influenza virus antigens are usually presumed to be excluded from the antigenic repertoire as "self." However, they can be antigenic in the context of a foreign protein structure, and their involvement in antibody binding would not be surprising. Further, in the case of the influenza hemagglutinin, it has been demonstrated that carbohydrate can mask proteinaceous antigenic sites.[47]

A schematic diagram of the NA monomer is shown in Figure 11. On this, the epitope recognized by NC41 antibody covers an area on the edge of the active site crater (Fig. 3) and involves several surface loops (indicated in black). The positions of amino acid sequence changes in escape mutants, that affect binding of antibody NC41 are shown in Figure 11. Changes at positions 367 (Ser to Asn), 369 (Ala to Asp), 370 (Ser to Leu), 372 (Ser to Phe), 400 (Asn to Lys), and 432 (Lys to Asn) abolish binding of antibody NC41 to neuraminidase while changes at positions 329 (Asn to Asp) and 368 (Ile to Arg) reduce binding of NC41 antibody (44, Air and Webster, unpublished results). All of these amino acids contribute to the surface area of the antigen that is buried when NC41 antibody binds. The peptide corresponding to the epicenter of the binding site (amino acids 366–373) has been synthesized. The peptide does not bind to antibody NC41, and does not compete with NA (Webster, Air, and Laver, unpublished results).

STRUCTURE OF ESCAPE MUTANTS

How do single amino acid sequence changes in the loops abolish antibody binding? Remember, only 1 out of about 17 contact residues is altered in each

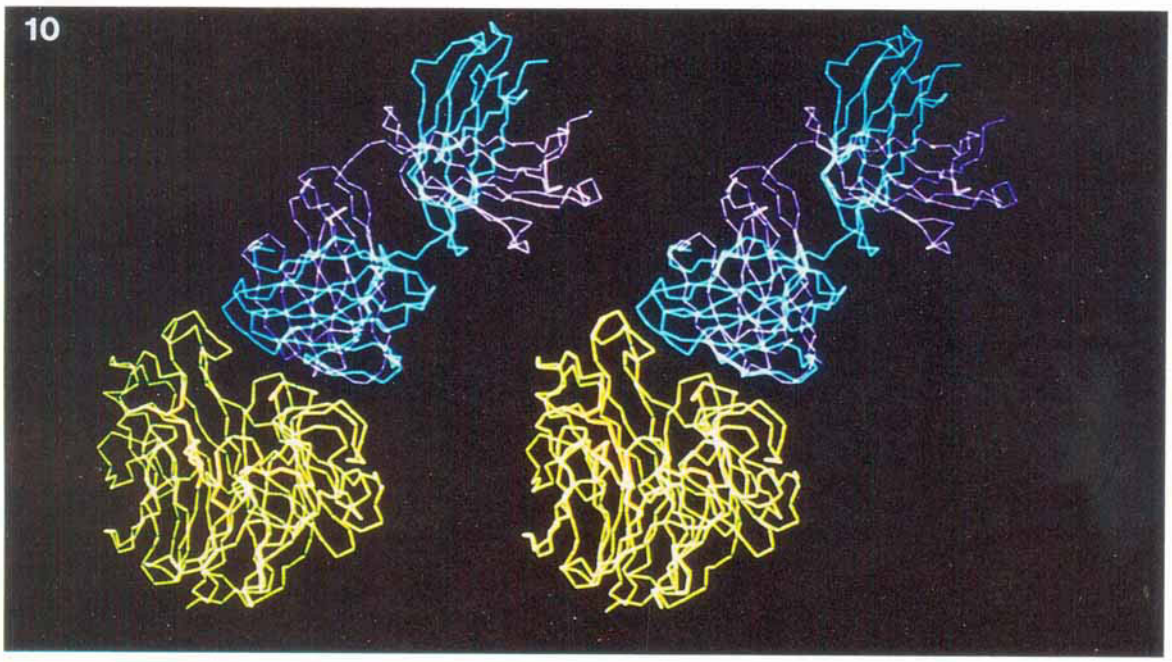


Fig. 10. Stereo image of one NA subunit complexed with one NC41 Fab. The NA is green, the antibody heavy chain purple, and the light chain blue. The enzyme active center is facing the viewer, below and to the left of the antibody binding site.

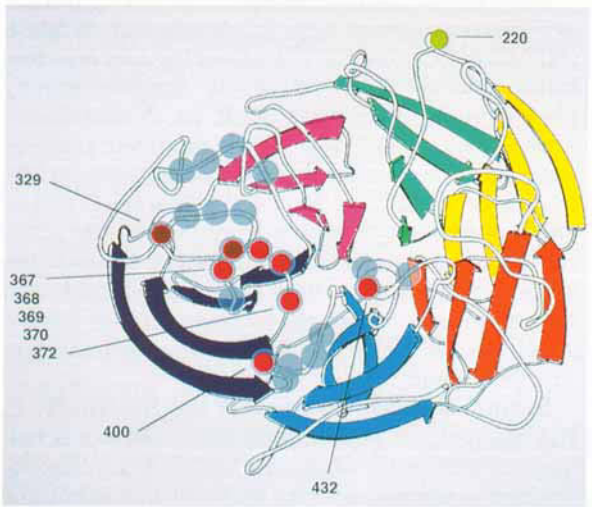
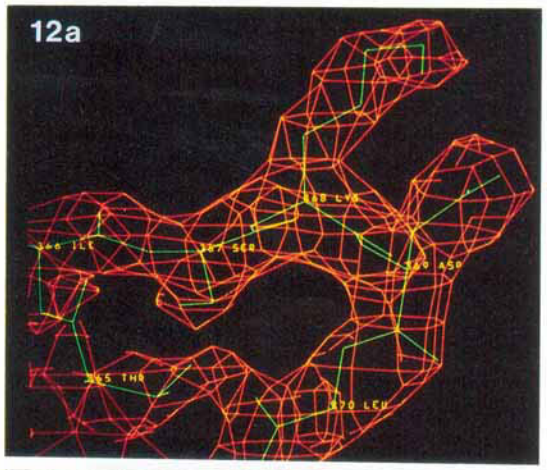


Fig. 11. Schematic diagram showing the NC41 epitope on N9 NA. The chain fold in the monomer is viewed down the 4-fold axis. This diagram has been revised from that previously published (5, and shown in Fig 3) in the loop involving amino acids 320–350. The revised structure is adapted from ref. 55. The epitope recognized by NC41 antibody involves the amino acids shown in grey[55]. N2 numbering is used. The side chains of amino acids 368–370 point towards the viewer, while that of Arg 371 (an active site residue) points away and into the catalytic site located above and to the right of Cα371. Mutations at positions 367, 369, 370, 372, 400 and 432 (shown in red) abolish the binding of NC41 antibody to neuraminidase, whereas mutations at 368 and 329 (shown in red) reduce binding. A mutation at residue 220 (outside the NC41 epitope, shown in green) has no effect on binding of NC41 to neuraminidase.



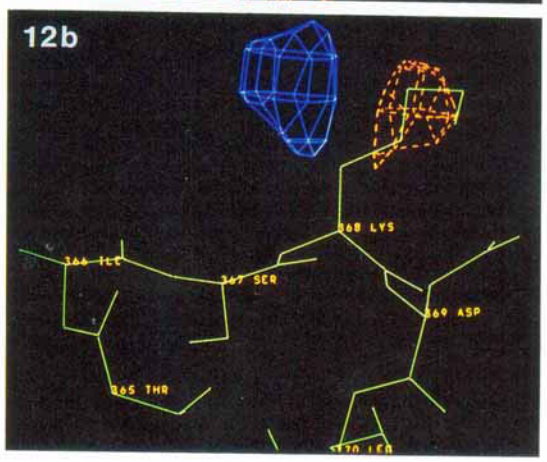


Fig. 12. An atomic model of amino acids in the neighborhood of residue 368 in neuraminidase of A/Tokyo/3/67. **a:** Wild-type NA with Lys at position 368 superimposed on an electron-density difference map. **b:** The refined atomic model of the same region in the mutant which has Glu at 368.[50]

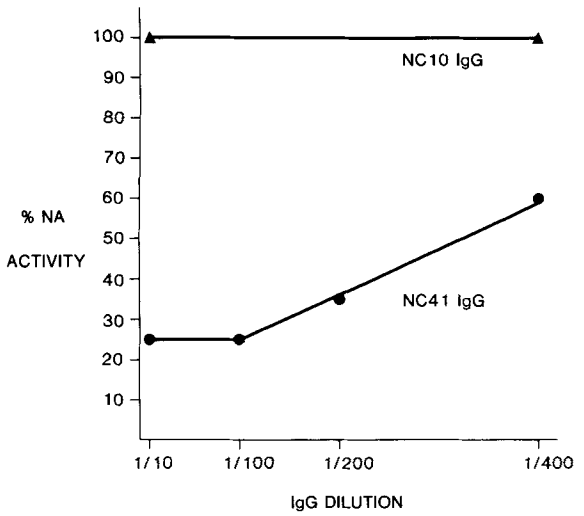


Fig. 13. NA inhibition curves for monoclonal antibodies NC41 and NC10 on N9 neuraminidase using *N*-acetylneuraminyl lactose (MW 500) as substrate. Both antibodies inhibited N9 NA activity almost totally (95% inhibition) when a large substrate, fetuin (MW 50,000) was used.

mutant. One possible mechanism is that a single sequence change can drastically alter the conformation of the whole epitope, so that it is no longer recognized by antibody. The structure of HA escape mutants,[48] and similar experiments with NA mutants, suggests, however, that this is unlikely.

The structure of an escape mutant of influenza virus N2 NA (A/Tokyo/3/67), which was selected with monoclonal antibody S10/1[49], has been determined.[50] Residue 368, which is lysine in the wild-type, changed to glutamic acid in the mutant. This change abolished S10/1 antibody binding. The difference Fourier map (Fig. 12) shows difference peaks resulting from a shift of electrons from the lysine side chain to a new position of glutamic acid in the mutant. Lysine-368 in the wild-type A/Tokyo/3/67 neuraminidase structure is linked by a salt bridge with aspartic acid-369, and the altered position of glutamic acid-368 in the mutant may result from a charge repulsion by Asp-369. No other differences between the structures are indicated in the difference electron-density map.

This experiment shows that the S10/1 epitope does indeed include amino acid residue 368, and that the change in the side chain has sufficed to effectively abolish binding of the antibody to the epitope. The single amino acid substitution of lysine to glutamic acid in the mutant introduces two free charged acid groups (Glu-368 and Asp-389) in the interface of the antibody and the antigen. The change in the epitope structure in this case is substantial, being two electronic charges plus attending shape changes. In some cases, however, escape mutants that have lost the capacity to bind antibody show more subtle chemical changes than are observed here, such as arginine for lysine.[33,49] The same change (Arg to Lys) was found to lower the affinity of lysozyme to HyHEL-5 by a factor of 1000, suggesting that factors other than charge, such as specific hydrogen bond arrangment, also play a significant role in binding antibody to antigen.[51]

A survey of single amino acid changes in escape mutants of influenza antigens[32,33,44,52,53] does not show any preference for size or character of the replacing or the replaced residue in the antigen, and indicates that even very subtle changes abolish the binding of an antibody to an antigen. The structure has been reported of only one other viral escape mutant, an influenza hemagglutinin.[48] In this case, the substitution of an aspartic acid for a glycine residue also caused only local structural alterations on the surface of the molecule.

Although the possibility of some escape mutants influencing antibody binding at a distance cannot be ruled out, the two influenza virus surface antigens studied so far both show that local structural changes suffice to effectively abolish antibody binding to an antigen.

MECHANISM OF INHIBITION OF NA ACTIVITY BY ANTIBODY

Many monoclonal antibodies to the NA inhibit enzyme activity toward large substrates such as fetuin (MW 50,000), presumably by blocking access of this large molecule to the active site. Enzyme activity toward small substrates such as *N*-acetylneuraminyl lactose (MW 500) is not inhibited at all by some of these monoclonal antibodies, even at the highest concentration that can be obtained (Fig. 13). Other antibodies, however, do inhibit enzyme activity toward the small substrate. NC41 antibody belongs to this category. Both the intact antibody and Fab fragments reduce the enzyme activity toward NANL by 75% (Fig. 13). What is the mechanism of this inhibition?

In the published structure of the N9 NA–NC41 Fab complex[43] it appears that the active site is still accessible to small substrates, and that therefore inhibition of enzyme activity by NC41 Fab is not due to steric hindrance. The crystal structure of the complex of NC41 Fab and NA[43] shows a very small shift in the position of an active site residue, Arg-371, which is known to be critical for enzyme activity since mutation to Lys resulted in loss of enzyme activity.[18] It is possible that this shift causes the lower enzyme activity. However, it is also possible that the enzyme-active site may need to be mobile in order to carry out its catalytic function and that binding of NC41 antibody freezes the NA in an unfavorable configuration.

ISOSTERIC BINDING OF NC41 ANTIBODY TO A MUTANT NEURAMINIDASE

The NC41 Fab fragment crystallizes when complexed with the monoclonal variant of N9 known as

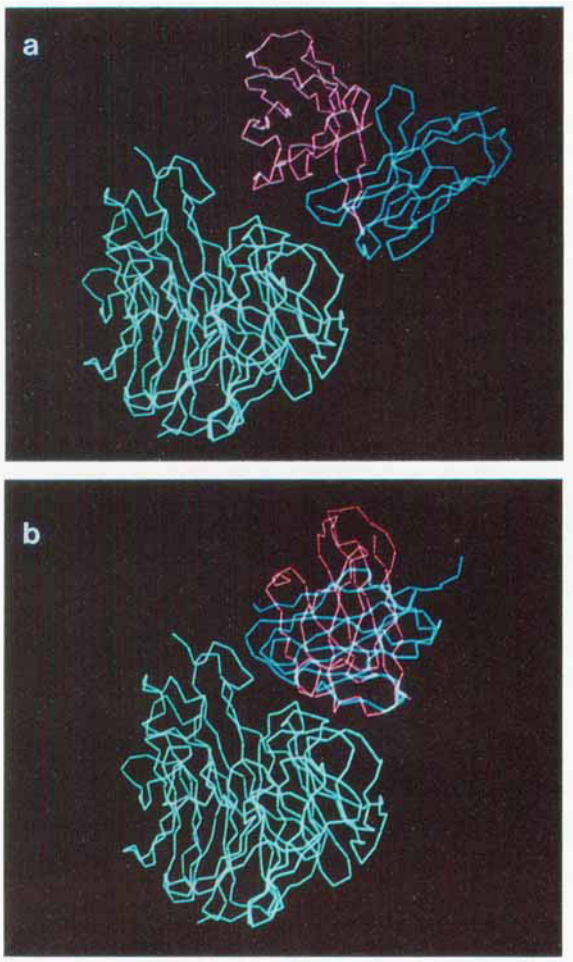


Fig. 14. C_α chain tracings of the complexes between (**a**) whale virus NA and antibody NC10 Fab and (**b**) tern virus NA and antibody NC41 Fab. Only the V-module is shown; in the case of the NC10 complex the C module is not visible in the electron-density map.[46] The two antibodies recognize much the same region on the NA, but are in very different orientations. Picture kindly provided by Bill Tulip.

Fig. 15. Crystals of N9 NA heads from the whale influenza virus complexed with Fab fragments of monoclonal antibody NC41. The crystals were grown from 1.7 M potassium phosphate pH 6.8, and photographed using back lighting and an arrangement of colored filters. The crystals grew to approximately 1 mm on an edge.

Ox2, Asn-329 to Asp.[54] Residue 329 is in the region of N9 NA where the structure has been revised,[55] and it appears to be at the edge of the epitope for NC41. These crystals are isomorphous with crystals of wild-type complex and diffraction data from them imply that the binding of NC41 to wild-type and variant neuraminidase is isosteric, although the binding affinity for the variant is less than for wild-type.[44,54] When high-resolution data are made available, it may be possible to explain why the change from Asn to Asp reduces binding of antibody NC41.

STRUCTURE OF A SECOND N9 NA–FAB COMPLEX

The N9 neuraminidase used here was isolated from an influenza virus found in a whale in Maine in 1984. It differs from the neuraminidase of the virus from terns (isolated in Australia in 1975 and used in the NC41 complex study) by 14 amino acids,[56] mostly on the underside of the globular neuraminidase head.[5] The NC10 antibody was raised against the tern virus neuraminidase,[44] but binds an epitope where the tern and whale virus neuraminidases are identical in sequence. Crystals of tern virus N9 NA–NC10 Fab have also been grown, but are disordered and unsuitable for structure analysis.

The structural data reported are not yet of high quality,[46] but an α-carbon chain trace has been made for the V region of Fab NC10. No electron density is seen for the C-region of the Fab. The general shape of the tetrameric N9–NC10 Fab protomer is similar to that of the NC41 complex (and other complexes examined by electron microscopy.[39] Packing of the protomers is as described for N9 NA–NC35 Fab crystals (Fig. 9),[39] which share the same space group and similar cell dimensions with the N9–NC10 complex crystal. Two neuraminidase heads make close contact via the membrane-prox-

imal surface. Two sets of Fab fragments interdigitate, interacting through the V-modules of the Fab. Crystal growth does not require the participation of the disordered C-module.

The epitope recognized by NC10 antibody is, to a large degree, common to that seen by the NC41 antibody.[44] In the structure,[46] contacts with surface loops around residues 330, 342, 369, 401, and 432 appear possible. These observations are consistent with studies showing lack of binding of NC10 to neuraminidase variants with sequence changes at positions 329, 369, 370, and 432.[44]

Although the two antibodies NC10 and NC41 are binding to the same region of the NA surface, the arrangements of the two antibodies on that surface are strikingly different, each being rotated about 90° from the position of the other (Fig. 14). Thus, whereas of all the NC41 CDRs, L1 is most remote from the antigen, L2 and H1 of NC10 are most distant from the epitope. Since the antibodies NC10 and NC41 have very different amino acid sequences, belonging to different families of heavy chains and different classes of light chains, this difference in binding configuration of the variable CDR's is not surprising. It will be interesting to compare the interactions of NA with each of the antibodies when high-resolution structural data are available.

CONCLUSIONS AND FUTURE PROSPECTS

Neuraminidase molecules from two influenza virus A subtypes and from influenza B virus have been crystallized and the three-dimensional structures of the type A NAs have been determined to medium resolution, while that of type B is underway. Site-specific mutagenesis experiments have been used to partially define the role of some active site residues and future experiments using suitable vectors aim to obtain sufficient mutant NAs for crystallization and structure determinations. Refinement of these structures and knowledge of how the catalytic site functions will then be used in attempts to design inhibitors specific for the viral enzyme that can be used to control influenza in people or domestic animals. Influenza virus neuraminidase is also being used in experiments aiming to understand the structure of epitopes on protein molecules and the way in which an antibody is able to recognize one out of an almost infinite variety of epitopes in nature. Seven crystalline complexes, involving NA with Fab fragments, are currently being analyzed. These are NC41 Fab complexed with N9 NA from tern and whale (Fig. 15) viruses and with two variants of tern virus NA with sequence changes which reduce but do not abolish binding. The other three complexes are N9 NA–NC10 Fab, N9 NA–32/3 Fab (thin crystals only), and type B influenza virus NA complexed with B1 Fab. Furthermore a number of escape mutants affecting the epitope recognized by NC41 antibody have been crystallized and their refined structures will tell us how single sequence changes are able to abolish antibody binding.

Thus, studies on the influenza virus NA are yielding a wealth of information about its structure, enzymic activity, and antigenic properties, and ways in which the enzyme interacts with substrates, inhibitors, and antibodies. Moreover, the work is being done on a component of an important human pathogen, and therefore some of the results obtained with this protein, while yielding information of great general interest, may also be directly applicable to alleviating a widespread and potentially devastating human disease.

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