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Immunology

A carbohydrate side chain on hemagglutinins of Hong Kong influenza viruses inhibits recognition by a monoclonal antibody

(epidemiology/oligosaccharide/glycoprotein/membranes/antigenicity)

J. J. SKEHEL*, D. J. STEVENS*, R. S. DANIELS*, A. R. DOUGLAS*, M. KNOSSOW†‡, I. A. WILSON†§, AND D. C. WILEY†

*Division of Virology, National Institute for Medical Research, Mill Hill, London NW7 1AA, United Kingdom; and †Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, MA 02138

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ABSTRACT **A single amino acid substitution, Asp-63 to Asn-63, was detected in the hemagglutinin of an antigenic variant of the 1968 Hong Kong (H3) influenza virus that was selected by growth of the wild-type virus in the presence of a monoclonal antibody. The mutation generates an oligosaccharide attachment site, Asn-Cys-Thr at residues 63–65, that is glycosylated. Immunoprecipitation experiments with extracts from variant virus-infected cells prepared in the presence or absence of tunicamycin, which inhibits glycosylation, demonstrate that addition of the new oligosaccharide side chain is required to prevent reaction with the monoclonal antibody. Similar experiments with the virus of the 1969 Hong Kong influenza epidemic, A/England/878/69, which also contains a hemagglutinin glycosylated at residue 63, support this conclusion and provide evidence for the epidemiological significance of carbohydrate-mediated modifications of hemagglutinin antigenicity.**

Neutralizing antibodies against influenza virus are directed at the hemagglutinin glycoprotein, HA, of the virus membrane (1). Amino acid sequence variations resulting in altered antigenic properties of the HA accompany the recurrent epidemics of influenza respiratory disease in man. By combining data from amino acid sequence analyses of HAs of viruses that have caused epidemics (for review, see ref. 2) and from analyses of single amino acid substitutions in the HAs of antigenic variants selected by growing virus in the presence of monoclonal antibodies against the HA (3, 4), the location of regions on the three-dimensional structure of the HA to which antibodies bind have been proposed in the HA of the 1968 influenza virus (5, 6).

In these studies, it was noted (5, 6) that in some viruses of both the Hong Kong (H3) subtype and of other subtypes oligosaccharide attachment sites (Asn-X-Ser/Thr) were present in regions of the HA implicated in antibody binding in the 1968 HA, and this was subsequently observed in a study of a HA of the H1 subtype (7, 8). Since oligosaccharide chains are host specific and therefore presumably not immunogenic in an infected organism, the possibility was considered that carbohydrate might modulate the antigenic structure of the HA by masking regions of the protein from recognition by antibody.

Here direct evidence is presented that the addition of carbohydrate to the 1968 HA blocks recognition by a monoclonal antibody directed at the region near amino acid residue 63 of the HA. These observations were made for the HAs of antigenic variants of the 1968 Hong Kong virus either isolated from influenza virus-infected humans in 1969 or selected by growth of the 1968 virus, X-31, in eggs in the presence of the monoclonal antibody.

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MATERIALS AND METHODS

Viruses. The X-31 (H3N2) recombinant influenza virus (9) and the 1969 isolate A/England/878/69 (H3N2) were used. Viruses were grown in hens' eggs and antigenic variants of X-31 were isolated by mixing equal volumes of allantoic fluid containing wild-type virus and undiluted ascitic fluid containing monoclonal antibodies and using the mixture as inoculum. Variants obtained were cloned by limit dilution.

Hemagglutination-Inhibition Tests. These tests were done by standard methods (10), using anti-HA monoclonal antibodies and 1% turkey erythrocytes.

Monoclonal Antibody Production. BALB/c mice were immunized by intraperitoneal injection of 8000 HA units of purified X-31 virus. Eight weeks later they received 32,000 HA units of virus intraperitoneally and intravenously. Three days later, their spleens were removed and the cells were fused with SP2/0-Ag14 myeloma cells (11) as described by Köhler and Milstein (12, 13). Hybrid cell culture conditions were based on those described by Fazekas de St. Groth and Scheidegger (14). The antibodies produced from different cloned cells were named hemagglutinin clones (HC) and numbered according to the culture number—e.g., the antibodies used in this study for immunoprecipitation were numbered HC31 and HC100. When antibodies have been characterized by determining the sequence of the HA genes of antigenic variants that they select, they are given another number in parentheses. Thus, HC31, which selects variants with amino acid substitutions at amino acid residue 198, is finally denoted by HC31(198), and HC100, which selects variants with substitutions at amino acid residue 63, is denoted by HC100(63). The variants selected by these particular antibodies are numbered V31 and V100.

Nucleotide Sequence Analyses. Sequences were determined by using the dideoxynucleotide (ddNTP) chain-terminating procedure (15). Each 10-μl reaction mixture contained Tris·HCl, pH 8.3, 0.05 M; magnesium chloride, 0.012 M; dithiothreitol, 0.02 M; dATP, dGTP, dCTP, and dTTP, 0.0004 M each; virus RNA, 7 μg; human placenta RNase inhibitor (Bethesda Research Laboratories), 3 units; reverse transcriptase, 5 units (Life Sciences, St. Petersburg, FL); and one of ddATP, ddCTP, ddGTP, or ddTTP, 0.00025 M. After 120 min at 42°C, products were analyzed on polyacrylamide gels containing 8% acrylamide. Reactions were primed by using 5′-^{32}P-labeled oligodeoxynucleotides prepared as described by Patel *et al.* (16) and purified by ion-exchange HPLC (Partisil SAX 10-50). The primers used, numbered according to the sequence of X-31 HA cDNA (17) were d(A-A-

Abbreviations: HA, hemagglutinin; ddNTP, dideoxynucleoside triphosphate.

‡Present address: Laboratoire de Physique, Centre Pharmaceutique, 92290 Chatenay-Malabry, France.
§Present address: Scripps Clinic and Research Foundation, La Jolla, CA 92037.

A-G-C-A-G-G-G-G) (nucleotides 5–14), d(T-G-C-T-A-C-T-G-A-G-C-T) (nucleotides 191–202), d(C-G-C-A-G-C-A-A-A-G) (nucleotides 345–354), d(G-C-A-A-A-A-G-G-G-G) (nucleotides 493–502), d(T-C-A-C-C-A-C-C-C-G) (nucleotides 623–632), and d(T-G-G-A-C-A-A-T-A-G) (nucleotides 777–786).

Immunoprecipitation of Infected-Cell Extracts. *Cell fractionation.* Six hours after infection with virus, primary chicken embryo fibroblasts were suspended in 10 mM Tris·HCl, pH 8.0/1 mM EDTA and disrupted in a Dounce homogenizer (type A, Kontes). After centrifuging at 1000 × *g* for 10 min, the supernatant was made 70% (wt/vol) in sucrose and overlayed with 3 ml of 55%, 3 ml of 35%, and 3 ml of 1% sucrose in 10 mM Tris·HCl, pH 8.0/1 mM EDTA. This discontinuous gradient was then centrifuged at 35,000 rpm for 60 min in an SW-27 Beckman rotor. The pellets obtained were resuspended in 10 mM Tris·HCl, pH 8.0, containing 100 mM NaCl, 1 mM EDTA, 0.25% gelatin, and 0.5% Nonidet P-40.

Immunoprecipitation. Staphylococcus aureus Cowan strain A cells were washed by centrifugation in 5 mM sodium phosphate, pH 7.2, containing 1% Nonidet P-40. Cell fractions were made 10% vol/vol with *S. aureus* cells and incubated at 4°C for 30 min, and the cells were removed by centrifugation at 10,000 rpm for 1 min. Monoclonal antibody preparations were mixed with supernatants for 60 min at 20°C and then the mixture was made 1% in *S. aureus* cells and incubated at 4°C for 60 min. The *S. aureus* cells were washed three times by centrifugation with 10 mM sodium phosphate, pH 7.2/1 mM EDTA/1% Triton X-100/0.1% sodium dodecyl sulfate/0.65 M NaCl and once in the same solution containing 0.15 M NaCl. The final pellet was boiled in 10 M urea/1% sodium dodecyl sulfate/0.1% 2-mercaptoethanol/1 mM Tris·H_3PO_4, pH 6.7, for 2 min and the *S. aureus* cells were then removed by centrifuging and the supernatant was analyzed by polyacrylamide gel electrophoresis as described (18).

RESULTS

Monoclonal Antibody-Selected Variant. A variant of X-31 virus that is not neutralized by the mouse monoclonal antibody HC100(63), prepared from mice immunized with X-31 virus, was selected by growth of X-31 in the presence of HC100(63) in embryonated chicken eggs. Hemagglutination inhibition tests (Table 1) demonstrated that the variant virus does not bind HC100(63) antibody but retains its binding capacity for antibodies directed at other parts of the molecule.

Amino Acid Sequence of the Variant Virus. The complete amino acid sequence of the HA_1 chain of the HA of the variant was determined by determining the sequence of the virus RNA.

Nucleotide 264 is G in the wild type and A in the variant virus mRNAs. This results in the codon change GAC to AAC, which directs the amino acid change Asp-63 to Asn-63. Since the sequence at residues 63–65 of HA_1 was Asp-Cys-Thr, the mutation produces an oligosaccharide attachment site Asn-Cys-Thr.

Sodium dodecyl sulfate/polyacrylamide gel electrophoresis of the X-31 and variant HAs shows a decrease in mobility of the HA_1 polypeptide as a result of the single base mutation. Similarly, the mobilities of the HA_1 tryptic peptides containing amino acids 27–328 and 27–224, prepared as described before (18), also decrease (Fig. 1). The simplest explanation of these observations is that the new oligosaccharide addition site at residue 63 of the variant is glycosylated, which would be expected to shift the electrophoretic mobilities as observed.

Binding of Antibodies to the Variant HA Synthesized in the Presence or Absence of Tunicamycin. Variant virus was used to infect chicken embryo fibroblasts incubated in the presence or absence of tunicamycin, which inhibits glycosylation of the HA. The interaction of monoclonal antibodies with [^{35}S]methionine-labeled HA in cell extracts was then compared by precipitating with monoclonal antibodies and examining the resulting precipitate by sodium dodecyl sulfate/polyacrylamide gel electrophoresis. As a control a variant containing a HA with an amino acid substitution at residue 198 (remote from 63 on the structure) selected by monoclonal antibody HC31(198) was studied in parallel.

Table 1. Reactions of X-31, A/England/878/69, and monoclonal antibody-selected variants of X-31 in hemagglutination-inhibition tests

Virus	HC100 (63)	HC31 (198)	HC3 (144)	HC19 (157)	HC68 (193)
X-31	3,200	12,800	12,800	12,800	6,400
A/England/878/69	100	12,800	200	6,400	6,400
V100	100	12,800	12,800	12,800	6,400
V31	3,200	100	12,800	12,800	6,400
V3	3,200	12,800	100	12,800	6,400
V19	3,200	12,800	12,800	100	6,400
V68	3,200	12,800	12,800	12,800	100

Hemagglutination-inhibition reactions were measured by standard procedures (8). The results presented are the reciprocals of the highest dilutions of the antibodies at which hemagglutination was inhibited.

In Fig. 2, lane b shows that glycosylated variant V100 HA (no tunicamycin) is not precipitated by HC100(63) monoclonal antibodies as expected because V100 was selected for its ability to grow in the presence of that antibody. However, lane a shows that in the presence of tunicamycin the unglycosylated form of the HA of variant V100 is precipitated by the HC100(63) antibody. Thus, the binding of monoclonal antibody HC100(63) to the antigenic variant V100 is unimpaired in the absence of the oligosaccharide at 63, indicating that the escape of the variant virus from neutralization by

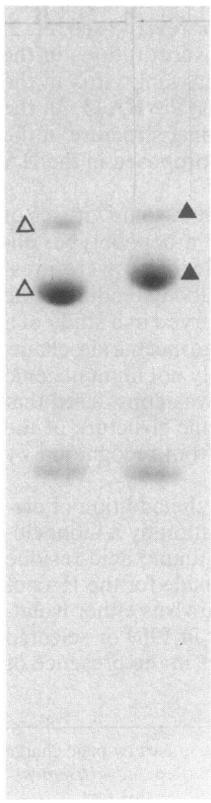

FIG. 1. Soluble tryptic glycopolypeptides of X-31 and V100 HAs released from viruses incubated at pH 5.0 as described (18). Polyacrylamide gels were as described in ref. 19 and electrophoresis was at 7.5 V/cm for 16 hr. From left to right, the lanes contain X-31 and V100 glycopolypeptides. The components of altered mobility—namely, the glycopolypeptides containing amino acids 27–328 and 27–224—are indicated by arrowheads. The equivalent components of X-31 HA are indicated by open symbols.

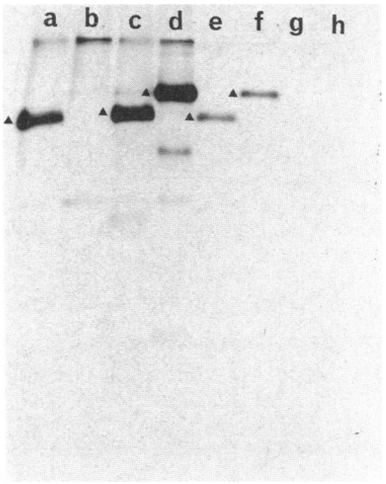

FIG. 2. Immunoprecipitation of X-31 and V100 HAs in membrane fractions prepared from infected chicken embryo fibroblasts incubated with or without tunicamycin. Where appropriate, cells were incubated in medium containing tunicamycin at 1 μg/ml from 1 hr before virus infection. Membranes from [^{35}S]methionine-labeled cells were prepared 6 hr after infection and HAs were precipitated with antibodies. The gel lanes shown in the autoradiogram contained, from left to right, extracts from cells infected with a, V100 with tunicamycin; b, V100 without tunicamycin; c, V31 with tunicamycin; and d, V31 without tunicamycin, and precipitated with HC100(63) antibodies. Lanes e–h contained extracts from cells infected with e, V100 with tunicamycin; f, V100 without tunicamycin; g, V31 with tunicamycin; and h, V31 without tunicamycin, precipitated with HC31(198) antibodies. Electrophoresis conditions were as described for Fig. 1. The precipitated HA polypeptides are indicated by arrowheads.

HC100(63) was dependent on the presence of the oligosaccharide added to residue 63.

Three control experiments were done: (*i*) Lanes e and f of Fig. 2 demonstrate that the HA of variant virus V100 synthesized either in the presence or in the absence of tunicamycin was precipitated by HC31(198), an antibody that recognizes amino acids near 198 at a site that is remote from any carbohydrate side chains and from the amino acid 63 region. Thus, these control antibodies bind to both glycosylated and unglycosylated V100 HA. (*ii*) A control variant virus V31, selected by monoclonal antibody HC31(198), has residue 198 changed from Ala to Glu (4). Lanes g and h of Fig. 2 demonstrate that the HA of this control variant is not precipitated by the monoclonal antibody HC31(198) used for variant selection, whether the HA was synthesized in the presence (g) or absence (h) of tunicamycin. Thus, in this case the amino acid change Ala to Glu at position 198 is recognized directly, the state of glycosylation of the HA being immaterial. (*iii*) The HA of the control variant virus V31 synthesized in the presence or absence of tunicamycin is precipitated by HC100(63) antibodies (lanes c and d of Fig. 2). This indicates that HC100(63) antibodies recognize the wild-type region of this HA near amino acid residue 63 independently of whether or not the HA is glycosylated.

In summary, Fig. 2 demonstrates that only the HA of variant V100 that has a novel glycosylation site at amino acid residue 63 interacts differently with a specific monoclonal antibody, HC100(63), depending upon the state of antigen glycosylation.

A/England/878/69(H3) Hong Kong Variant. In influenza epidemics in 1969, viruses were isolated that were antigenically similar but distinguishable from the initial Hong Kong virus of 1968. The prototype of these viruses is A/England/878/69 and the amino acid sequence of its HA was reported by Sleigh *et al.* (20). There are four amino acid sequence differences between the HA_1 polypeptide chains of X-31 and A/England/878/69: substitutions of Asn for Asp at residue 63, Asp for Asn at residue 81, Asp for Gly at residue 144, and Val for Ile at residue 182. As in V100 described above, the substitution at residue 63 introduces a site for glycosylation, and to assess the effect of this sequence change on the antigenicity of A/England/878/69, we analyzed its HA in hemagglutination inhibition and immunoprecipitation reactions with monoclonal antibodies. The results in Table 1 indicated that the amino acid sequence changes at residues 63 and 144 both influence the antigenicity of A/England/878/69 HA since the virus fails to react with either HC100(63), which recognizes residue 63, or HC3(144), which recognizes residue 144. Other antigenic regions of X-31 and A/England/878/69 appear from this test to be similar, although the sequence change at position 81 may also be antigenically significant.

Binding of Antibodies to A/England/878/69 HA Synthesized in the Presence or Absence of Tunicamycin. The antigenic importance of glycosylation at position 63 was investigated in experiments similar to the experiment described in Fig. 2 involving immunoprecipitation by monoclonal antibody HC100(63) of A/England/878/69 HA synthesized in cells incubated with or without tunicamycin. The results show (Fig. 3) that monoclonal antibody HC100(63) precipitates unglycosylated HA in extracts of tunicamycin-treated cells (lane 7) but does not react with glycosylated HA produced in the absence of tunicamycin (lane 5). Extracts from X-31-infected

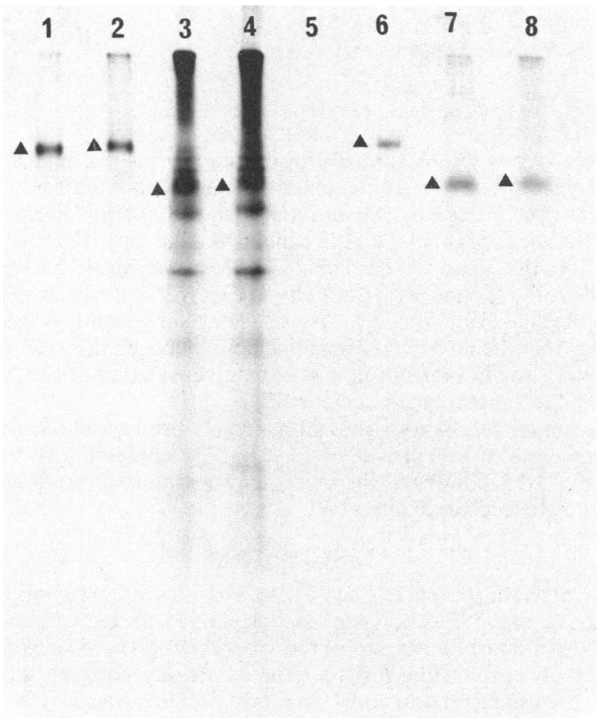

FIG. 3. Immunoprecipitation of X-31 and A/England/878/69 HAs by HC100(63) antibodies. The HA polypeptides in membrane fractions prepared from virus-infected cells incubated with or without tunicamycin at 1 μg/ml (lanes 1–4) were precipitated with HC100(63) antibodies (lanes 5–8). Lanes 1 and 3 of the autoradiograph contain, respectively, membrane fractions of A/England/878/69-infected cells incubated without and with tunicamycin; lanes 2 and 4 contain, respectively, membrane fractions of X-31-infected cells incubated without and with tunicamycin; lanes 5 and 7 contain extracts in lanes 1 and 3 after precipitation with HC100(63); and lanes 6 and 8 contain extracts in lanes 2 and 4 after precipitation with HC100(63). Electrophoresis was as in Fig. 1. The HA polypeptides are indicated by arrowheads. Lanes 3 and 4 were overloaded to compensate for the low yields in cells incubated with tunicamycin.

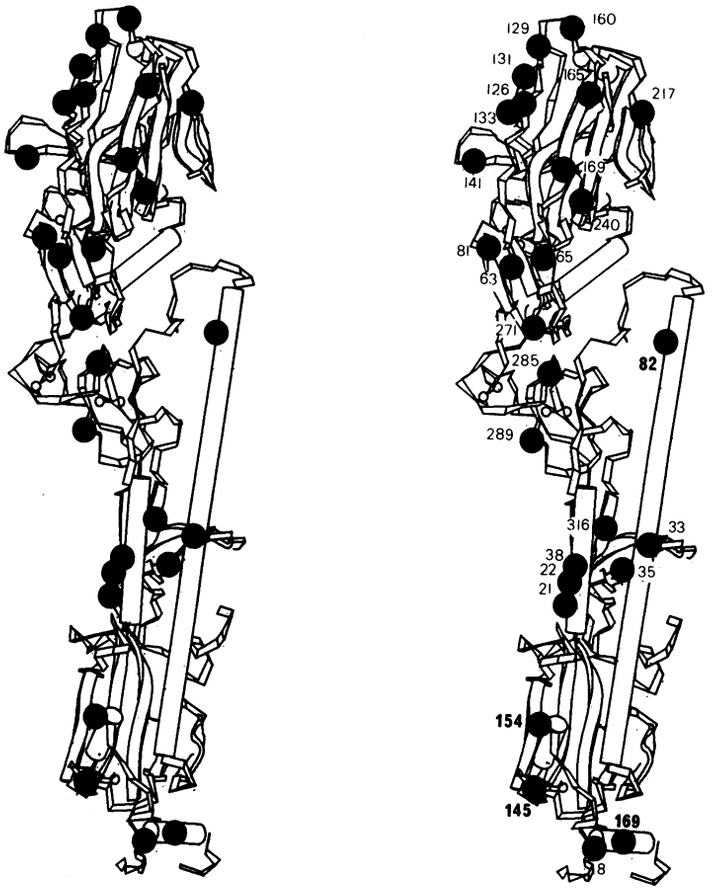

FIG. 4. Potential Asn-linked oligosaccharide attachment sites (●) on the HAs of 30 influenza A and B viruses are shown positioned on the structure of the HA from the 1968 strain X-31 (A/AICHI/68). Many of these sites (boldface numbers) are in regions shown to be involved in antibody binding by HAs of the H3 (compare to figure 2d of ref. 5, figure 1b of ref. 6, and see ref. 21) and H1 subtypes (compare to figure 3a of ref. 6).

cells were used as controls for these experiments, and X-31 HA produced in cells incubated with or without tunicamycin was precipitated by the antibody (lanes 8 and 6). These results are consistent with the conclusion that, as in the case of V100, the HA of A/England/878/69 is specifically prevented from reacting with antibody HC100(63) by the carbohydrate side chain linked to Asn-63. They support the proposition that the differences in antigenicity between the 1968 and 1969 virus HAs result in part from glycosylation of the Asn residue substituted at residue 63.

Similar results (not shown) have also been observed with the virus strain prevalent in the 1975 epidemic, A/Victoria/3/75, which has the same Asn substitution, resulting in an oligosaccharide site at 63.

DISCUSSION

Evidence is presented that oligosaccharides attached at position 63 of the HA_1 polypeptide chains of a selected variant of X-31 influenza virus and of the virus A/England/878/69 are directly responsible for the reduced affinity between these viruses and a monoclonal antibody produced against X-31 virus. These are experimental demonstrations of earlier suggestions (5–8) that the glycosylation of oligosaccharide attachment sites in regions of the HA implicated in antibody binding could have a role in antigenic variation.

Similar modifications of antigenicity may occur in other regions of the HAs of epidemiologically important viruses. Fig. 4 shows the potential Asn-linked oligosaccharide attachment sites in the HAs of 30 influenza A and B viruses whose sequences are available. Many of these on the distal globular domain of the HA structure are in regions shown to be involved in antibody binding by HAs of either the H3 subtype (compare figure 2d of ref. 5, figure 1b of ref. 6, and see ref. 21) or the H1 subtype (compare figure 3a of ref. 7).

A detailed example of the possible structural basis for antigenic modification of the HA by a carbohydrate chain can be made for one of these sites of glycosylation. Fig. 5*A* shows the coordinates of the oligosaccharide attached at residue 165 in the X-31 (1968) HA structure; it is clear that this oligosaccharide chain would block access of antibodies to the surface of the molecule between residues 166 and 168. Accordingly no variation either in strains isolated from patients between 1968 and 1982 or in variants selected by monoclonal antibodies has been observed in this region (165–168) of HAs of the Hong Kong (H3 subtype) viruses (4, 5, 21, 22). In contrast, the HA of A/PR/8/34 of the H1 subtype has no glycosylation sites in this region and an analysis of monoclonal antibody-selected antigenic variants has shown that amino acids 165, 166, and 167 can be recognized by monoclonal antibodies (Fig. 5*B*) (7). It therefore seems reasonable to conclude from this example and the information presented here that the presence of oligosaccharide side chains can modify the antigenic structure of a HA by effectively masking regions of the molecule that may be antigenic in the HAs of viruses that lack the appropriate glycosylation site.

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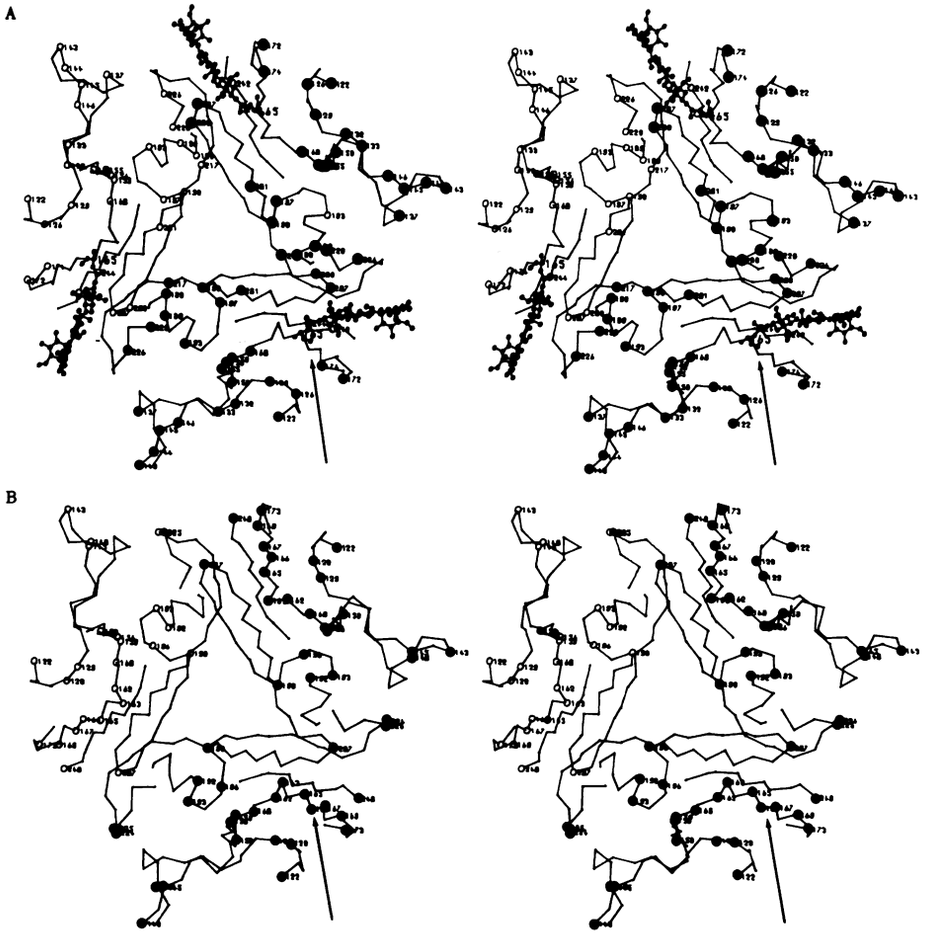

Fig. 5. The oligosaccharide attached at Asn-165 of the HA from the 1968 (X-31) strain blocks access of antibodies to residues 166–168, consequently no variation in either natural strains isolated between 1968 and 1982 or variants selected by monoclonal antibodies is seen in the 165–168 region. (*A*) Coordinates of the oligosaccharide attached at 165 are shown on a portion of the surface of the 1968 HA_1. Amino acid substitutions observed between 1968 and 1982 have occurred at positions shown by ●. (*B*) In contrast, monoclonal antibody-related variants of the 1934 strain A/PR/8/34, which has no oligosaccharide at 165, show amino acid substitutions (●) in the 165–168 region. The position of substitutions in the 1934 HA (7) are shown on a schematic diagram of the 1968 structure [the structures are expected to be similar on the basis of amino acid sequence homology (5, 7)].

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