

# Receptor-Binding Characteristics of Monoclonal Antibody-Selected Antigenic Variants of Influenza Virus

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**Erythrocytes modified to different extents with periodate were used in hemagglutination assays to investigate the binding properties of antihemagglutinin monoclonal antibody-selected antigenic variants of X-31 influenza virus. The results allowed differentiation of groups of variants and are discussed in relation to the nature of the amino acid substitutions in the variant hemagglutinins and their molecular locations relative to the receptor-binding site.**

Entry of infectious influenza virus into susceptible cells requires an initial step in which viral hemagglutinin (HA) is bound to sialic acid residues of cell membrane receptors. The type of glycosidic linkage between the sialic acid and its adjacent galactose residue differs among species, as does the type of sialic acid itself. These factors are thought to contribute to the species specificity of individual virus strains (4, 6). The receptor-binding site of the HA is a pocket at the distal end of the molecule composed of amino acid residues which are largely conserved in various strains of the virus (10). Rogers et al. (6) have selected mutant viruses with reduced affinity for oligosaccharides containing  $\alpha$ 2,6 linkages between sialic acid and galactose and a marked increase in affinity for oligosaccharides containing  $\alpha$ 2,3 linkages, so that the mutant viruses bind better to erythrocytes derivatized with  $\alpha$ 2,3-linked than with  $\alpha$ 2,6-linked N-acetylneuraminic acid. A single amino acid substitution in the mutant HAs, glutamine for leucine at HA<sub>1</sub> position 226, is responsible for the altered specificity and confirms the location of the receptor-binding site. The perimeter of the site contains residues which have varied during the antigenic drift that has accompanied recurrent epidemics (9), but the effect of these changes on the efficiency of binding of the HA to the cell receptor is unknown.

Simple assays for differentiating HAs on the basis of their receptor-binding characteristics have been described (7, 11) and involve either chemical or enzymatic modification of the receptors on erythrocytes (RBC). The former modification, which measures the effect of periodate treatment of chicken RBC on their hemagglutination by different viruses, clearly differentiates between HAs containing leucine and those containing glutamine at HA<sub>1</sub> residue 226. Significant differences in periodate sensitivity were also demonstrated between the HAs of viruses having a leucine at this position, but since most of the viruses studied were field strains containing several amino acid changes in HA<sub>1</sub>, identification of particular residues contributing to these differences was not possible.

In the present report a number of laboratory mutants of influenza virus strain X-31 were assessed for receptor-binding activity by the periodate sensitivity test. These mutants were selected under pressure of anti-HA monoclo-

nal antibodies, and the amino acid sequences of their HAs showed only one or two changes from that of the parent virus (Table 1). The periodate sensitivity profiles of X-31 and the variants are shown in Fig. 1. The largest group, A, was centered around the parental strain with very little variability (standard error of 0.027 mM periodate). This group contained variants with amino acid changes in HA<sub>1</sub> residues 63, 128, 143, 199, and 219, and consequently these particular substitutions appear to have no influence on receptor binding. None of these residues were within the receptor pocket.

TABLE 1. Antigenic variants selected by monoclonal antibodies

Periodate sensitivity group <sup>a</sup>	Variant <sup>b</sup>	HA <sub>1</sub> residue no.	Amino acid change
A	45	63	Asp→Asn
	B12	63	Asp→Tyr
	B15	63	Asp→Tyr
	M1	128	Thr→Asn
	M14	143	Pro→Ser
	159	143	Pro→Thr
	125	199	Ser→Pro
	M3	219	Ala→Glu
B	3	144	Gly→Asp
	22	144	Gly→Asp
	24	144	Gly→Asp
	143	145	Ser→Asn
	73	145	Ser→Asn
	31	34 198	Gly→Trp Ala→Glu
C	263	158	Gly→Glu
	M4	158	Gly→Glu
	2	188	Asn→Asp
D	67	156 193	Lys→Glu Ser→Arg
	68	193	Ser→Arg
	126	189	Gln→His
E	184	135	Gly→Arg
	262	135	Gly→Arg

<sup>a</sup> See Fig. 1.

<sup>b</sup> The production and characterization of these antigenic variants is described by Daniels et al. (2).

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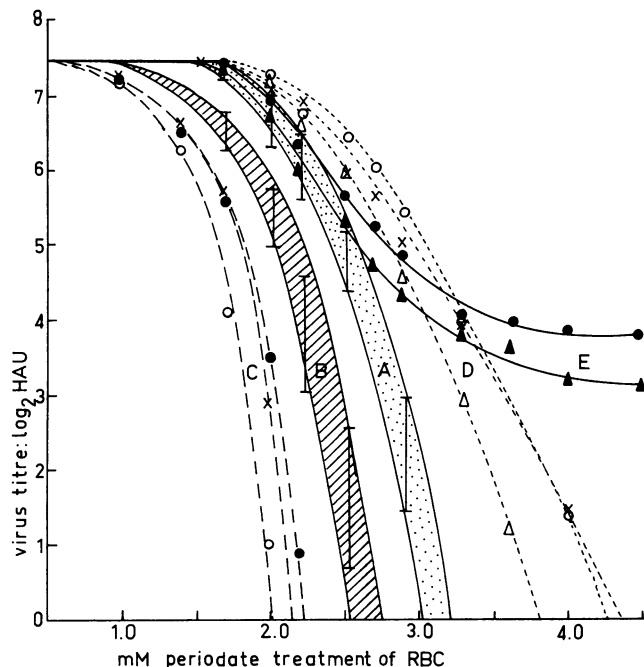


FIG. 1. Effects of treatment of RBC with various concentrations of potassium periodate on virus hemagglutination titers. Periodate treatment of RBC and titration of viruses by hemagglutination was done as described previously (8). Points on the graph are means of four replicate experiments. Symbols: Stippled area, parental group A, comprising X-31 and variants 45, B12, B16, M1, M14, 159, 125, and M3; hatched area group B, comprising variants 3, 22, 24, 179, 143, 73, 31; ---, group C, comprising variants 2 (○), M4 (×), and 263 (●); ----, group D, comprising variants 67 (×), 68 (○), and 126 (△); —, group E, comprising variants 184 (●) and 262 (▲). Bars for groups A and B represent the upper and lower values for all viruses within the group. HAU, Hemagglutination units.

Viruses in group B displayed small but significant ( $P < 0.01$ ) shifts towards increased sensitivity to periodate treatment of RBC. This group contained all the variants with changes at position 144 or 145. Previously (8), viruses containing these changes displayed periodate sensitivity profiles similar to those of the parent group. However, these were field strains, containing a number of additional amino acid changes which could have modulated the individual effects of residues 144 and 145. These residues are located on a loop which projects from the HA molecule, in proximity to the receptor pocket (10), and the nature and orientation of their side chains may influence receptor binding. Also included in this group was variant 31, with a change at position 198, which is remote from the receptor pocket. However, the finding that a monoclonal antibody which recognized residue 199 failed to bind to a variant containing a single amino acid substitution at residue 226 (3) suggest that there is some interaction between these two regions of the molecule.

A more dramatic increase in sensitivity to periodate was shown by group C, comprising variants 263 and M4, which share a glycine-to-glutamic acid change in HA<sub>1</sub> residue 158, and variant 2, with an asparagine-to-aspartic acid change in HA<sub>1</sub> residue 188. These residues are both near the receptor pocket but on opposite sides of it. Since periodate oxidation is expected to result in the sequential shortening of the glycerol moiety at C-6 of sialic acid, it is difficult to envisage how residues on opposite sides of the binding site would

directly recognize this localized change. It seems more likely that one or both of the substitutions exert their effects as a result of conformational changes transmitted to the binding site or as a result of the negative charge which both substitutions add to the top surface of the HA, a region which is conspicuously devoid of negatively charged residues in the HA of X-31. Residue 158 has been implicated in receptor binding by the demonstration of altered growth characteristics of an influenza A virus isolated from swine and containing an HA with a single amino acid change in this position (1). The present result provides a direct correlation between a change at this position and receptor-binding activity. The asparagine-to-aspartic acid change at position 188 has been detected in natural isolates of the A(H<sub>3</sub>N<sub>2</sub>) subtype, and the fact that it occurs in viruses which exhibit increased or decreased periodate sensitivity (8) suggests that its effects on receptor binding are subject to modulation by other amino acids in the area.

A dramatic decrease in sensitivity to periodate was shown by viruses in group D. This group contained variants 67, 68, and 126, the HAs of which contained amino acid substitutions at residue 193 or 189. The periodate profiles of variants 67 and 68 were so similar that it is unlikely that the additional change at residue 156 in the HA of variant 67 produced any significant effect over that produced by the change at residue 193. Residues 193 and 189 are both adjacent to the receptor pocket, and both substitutions were basic, Ser to Arg and Gln to His, respectively. Their contribution to receptor activity has been suggested previously (3, 8), and the present results clearly demonstrate their involvement. The substitution Ser to Arg at position 193 places the long, flexible arginine side chain in a position where it can extend into the receptor-binding pocket, with the potential to make a number of new polar interactions with a receptor. At present, however, no such obvious statement can be made about Gln to His at position 189.

The remaining group, E, consists of variants 184 and 262, both containing a glycine to arginine change in HA<sub>1</sub> residue 135. They displayed a periodate sensitivity profile totally different from those of all the other viruses. Their sensitivity to low concentrations of periodate was similar to that of the parental group, but at higher concentrations they were resistant to further reduction in HA titer, which suggests that they were still able to bind to the fully oxidized sialic acid receptor. Residue 135 is located immediately adjacent to the receptor pocket (6), and like other amino acid substitutions which lead to reduced sensitivity to periodate, the substituted amino acid was basic (Gly to Arg).

These results show that changes in adjacent residues (e.g., 188 and 189) close to the receptor pocket can have opposite effects on receptor-binding characteristics while changes on opposite sides (e.g., 188 and 158) can result in similar receptor-binding characteristics. In some cases, the nature of the substitutions (e.g., acidic versus basic) may be more important than their respective positions.

The periodate sensitivity assay, which is simple and quick to perform, provides a useful method for detecting amino acid residues which influence receptor binding. It is, however, clearly important that the viruses to be compared be grown under identical conditions, since, for example, the set of viruses analyzed previously (8) were all slightly more sensitive to periodate treatment of RBC than those described here, and although a parental group was identified for each set and variants were detected which displayed increased or decreased periodate sensitivity, the two sets could not be compared directly.

In general, estimates of receptor binding which involve interactions with RBC do not allow distinction, particularly for mutants containing charged amino acid substitutions, between an effect on the intrinsic affinity of the receptor-binding site and an effect on the electrostatic interaction between the HA and the charged RBC surface. Similarly, the structural consequence of single amino acid substitutions, whether they contribute directly to receptor binding, cause a conformational change in the site from a position not directly in or on the boundary of the site, or indicate possible extensions of the site beyond the sialic acid-binding pocket for extended or less specific interactions with a cell surface, cannot be unambiguously determined without X-ray diffraction analysis of the mutant HAs. Nevertheless, the results obtained with the different mutants appear overall to indicate the importance of charged residues in the general region of the receptor pocket, and although this may simply be a consequence of the oxidation state of the sialic acid residues involved in this particular test, it also suggests that charged residues at these sites may influence receptor-binding affinity or specificity.

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