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Single-Amino-Acid Substitution in an Antigenic Site of Influenza Virus Hemagglutinin Can Alter the Specificity of Binding to Cell Membrane-Associated Gangliosides

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Antigenic variants of influenza virus A/Mem/1/71-Bel/42 (H3N1) selected with monoclonal antibodies and having single substitutions in their hemagglutinins were examined for their ability to hemagglutinate and hemolyse erythrocytes coated with different gangliosides. The majority of variants, including one with a substitution near the receptor-binding site (Asn-133 → Lys), did not differ from the parent in specificity for receptor molecules. However, a substitution in HA1 at residue 205 (Ser → Tyr), which is distant from the receptor-binding site in antigenic site D, affected hemagglutination and hemolysis of erythrocytes coated with sialyl-paraglobosides. The variant preferentially recognized *N*-acetylneuraminic acid-α2,6-galactose linkages to sialylparaglobosides, whereas the parent and other variants preferentially recognized *N*-acetylneuraminic acid-α2,3-galactose linkages. In the trimeric hemagglutinin molecule, residue 205 is located across the subunit interface from the receptor-binding site. The bulky hydrophobic tyrosine in the variant may cause a conformational change in the receptor-binding pocket on the neighboring subunit and influence receptor binding.

The hemagglutinin (HA) glycoproteins of human influenza viruses bind to the host cell's surface receptor molecules that contain sialic acids (glycoproteins [10, 18] and gangliosides [16, 17, 20]); they also induce uncoating of the virus by fusion at low pH with endosomal/lysosomal membranes in target cells (4, 6, 30, 34). The ability of influenza viruses to infect a variety of host cells is well known. Much of this versatility comes from changes in the gene during antigenic shift and drift, leading to alterations in the antigenic epitopes on HA molecules (1, 2, 9, 26, 27, 31, 32). A single-amino-acid substitution in the sialic acid-binding site at the tip of the HA molecule can alter or extend the host range of the virus by enabling it to recognize different sialic acid linkages (7, 11).

In this paper, we show that a single-amino-acid substitution (Ser-205 → Tyr) in antigenic site D (31), which is situated far from the receptor-binding pocket on the HA monomer of influenza virus A/Mem/1/71-Bel/42 (H3N1), affected the ability of the virus to recognize sialic acid linkages in receptor gangliosides bound to erythrocyte membranes.

MATERIALS AND METHODS

Monoclonal antibody production and characterization. Monoclonal antibodies directed to the HA of influenza virus A/Mem/1/71 (H3N2) were produced in BALB/c mice (28) by using SP2/O-Ag14 myeloma cells. These antibodies were classified in double-immunodiffusion tests as members of the immunoglobulin G1 (IgG1) subclass.

Variant selection and virus growth. Variants of A/Mem/1/71-Bel/42 (H3N1) were obtained by mixing equal volumes of allantoic fluid containing virus and undiluted ascitic fluid containing monoclonal antibodies and then inoculating eggs with the mixture, as previously described (28). Variants were grown in the allantoic cavity of 10-day-old embryonated hen eggs and purified as described before (19).

Nucleotide sequence analyses. Sequences were determined by the dideoxynucleotide chain-terminating procedure of Sanger et al. (12) as described before (8).

Gangliosides. I-active ganglioside, sialyllacto-*N*-*nor*-hexaosylceramide [i-active ganglioside, VI^3(NeuGc)nLc6 Cer], and sialylparagloboside containing the *N*-glycolylneuraminic acid-α2-3-galactose (NeuGcα2,3Gal) sequence IV^3(NeuGc)nLc4Cer were isolated from bovine erythrocytes (20). Sialylparagloboside containing *N*-acetylneuraminic acid-α2,3-galactose (NeuAcα2-3Gal) [IV^3(NeuAc)nLc4Cer] and NeuAcα2,6Gal [IV^6(NeuAc)nLc4Cer] were isolated from human erythrocytes and meconium, respectively, as described before (20). G_{M3}(NeuAc) and G_{M3}(NeuGc) were isolated from human liver (13) and porcine erythrocytes (23), respectively. G_{M1a}, G_{D1a}, G_{D1b}, and G_{T1b} of NeuAcα2,3Gal type were obtained from bovine brain (21). NeuAc and NeuGc in gangliosides were analyzed by gas-liquid chromatography (22). The structures of the gangliosides used are listed in Table 1.

Receptor specificity of influenza virus HA toward sialyloligosaccharides of gangliosides. The assay system is designed to estimate the ability of ganglioside-coated erythrocytes to undergo influenza virus-mediated agglutination at 4°C at pH 7.2, as well as fusion and hemolysis in low-pH medium at 37°C as described before (20). Briefly, a reaction mixture (total, 1.0 ml) containing 10% washed packed chicken erythrocytes in phosphate-buffered saline (PBS) and 70 mU of neuraminidase (*Arthrobacter ureafaciens*, protease free [Nakarai Chemicals Ltd., Kyoto] or *Clostridium perfringens* [Sigma, type VI]) was incubated at 37°C for 1 h (14, 15, 21). Neuraminidase-treated cells were washed with PBS three times and pelleted by low-speed centrifugation. Ganglioside-coated erythrocytes were prepared as follows. A reaction mixture (1.1 ml containing 1.0 ml of chicken asialo-erythrocyte suspension in PBS [10%; 6×10^8 cells] and gangliosides [3 to 15 nmol] suspended in PBS [0.1 ml] was incubated at 37°C for 30 min. The cells were washed with cold PBS three times to remove the unadsorbed gangliosides. The ganglioside-coated packed erythrocytes (2 μl) were used to make a

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TABLE 1. Structures of gangliosides[a]

Ganglioside	Structure
IV^3(NeuAc)nLc4Cer	NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-Cer
IV^3(NeuGc)nLc4Cer	NeuGcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-Cer
IV^6(NeuAc)nLc4Cer	NeuAcα2-6Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-Cer
VI^3(NeuAc)nLc6Cer, i-active ganglioside	NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-Cer
I-active ganglioside	Galα1-3Galβ1-4GlcNAcβ1 ↘ / NeuAcα2-3Galβ1-4Glcβ1 ↗ $^{6}_{3}$Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-Cer
G_{M3}(NeuAc)	NeuAcα2-3Galβ1-4Glcβ1-Cer
G_{M3}(NeuGc)	NeuGcα2-3Galβ1-4Glcβ1-Cer
G_{M2}	GalNAcβ1-4Galβ1-4Glcβ1-Cer 3 NeuAcα2
G_{M1a}	Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-Cer 3 NeuAcα2
G_{D1a}	NeuAcα2-3Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-Cer 3 NeuAcα2
G_{D1b}	Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-Cer 3 NeuAcα2-8NeuAcα2

[a] NeuAc, *N*-acetylneuraminic acid; NeuGc, *N*-glycolylneuraminic acid; GlcNAc, *N*-acetyl-D-glucosamine; GalNAc, *N*-acetyl-D-galactosamine; Gal, D-galactose; Glc, D-glucose; Cer, ceramide. Gangliosides were abbreviated according to Svennerholm (24) and recommendations of the Commission on Biochemical Nomenclature (5).

2% suspension (0.1 ml) in PBS, to which influenza virus suspension (10 μl, 2^{10} hemagglutination units [HAU]/25 μl) in PBS was added. The mixture was kept at 4°C for 5 min. Agglutination of the cells was monitored by microscopic observation.

For estimation of the virus-mediated hemolysis, 1.0 ml of the 2% suspension of ganglioside-coated erythrocytes in PBS was mixed with 50 μl of influenza viruses (2^{10} HAU) suspended in the same buffer and kept at 4°C for 5 min. Aggregates were then washed with saline once, suspended in 1.0 ml of 20 mM acetate-buffered saline (pH 5.0 to 5.8), and incubated with shaking at 37°C for 30 min. The concentration of hemoglobin in the supernatant obtained after low-speed centrifugation was estimated by measuring the A_{540}. Cell fusion of ganglioside-erythrocytes caused by influenza viruses was estimated as described previously (17).

RESULTS

Nucleotide sequence analysis and amino acid substitution in the mutant HAs. Antigenic analysis established at least four antigenic epitopes on the HA molecule (28). To characterize these variants further, we deduced the amino acid sequences of their HAs from the complete sequences of the appropriate RNAs. The only sequence differences, by comparison with that of strain A/Mem/1/71-Bel/42 (H3N1), were detected in the HA1 polypeptide. All amino acid changes resulted from single substitutions; no silent base changes were detected (Table 2, Fig. 1).

Receptor-binding properties of the variant HAs. Functional receptor-binding specificities of the variants were assayed with the sialylparaglobosides IV^3(NeuAc)nLc4Cer and IV^6(NeuAc)nLc4Cer, containing the NeuAcα2,3Gal and NeuAcα2,6Gal sequences, respectively, bound to chicken asialoerythrocytes, and their abilities to agglutinate the treated cells at 4°C and induce hemolysis at 37°C were compared (Table 2). The optimum pH for the hemolysis of intact chicken erythrocytes by each variant was pH 5.2; no

TABLE 2. Receptor binding and low-pH hemolysis by antibody-selected variants of parent strain A/Mem/1/71-Bel/42

Strain	Amino acid substitution (residue no.)	Responses of sialylparagloboside-reconstituted erythrocytes to influenza viruses[a]			
		Hemagglutination titer (HAU)		Hemolysis (%)	
		IV^3(NeuAc) nLc4Cer	VI^6(NeuAc) nLc4Cer	IV^3(NeuAc) nLc4Cer	IV^6(NeuAc) nLc4Cer
Parent		128	64	80	59
V1	Ser→Tyr (205)	8	32	14	38
V2	Asn→Lys (133)	128	64	73	53
V3	Pro→Ser (143)	128	64	73	62
V4	Pro→Leu (143)	128	64	87	63
V5	Pro→Thr (143)	128	64	75	61
V6	Pro→His (143)	128	64	76	59
V7	Gly→Asp (144)	128	64	82	55
V8	Asn→Asp (188)	128	64	78	60

[a] Erythrocytes treated with neuraminidase were integrated with a sialylparagloboside [VI^3(NeuAc)nLc4Cer (NeuAcα2,3Gal sequence) or VI^6(NeuAc)nLc4Cer (NeuAcα2,6Gal sequence)]. Reaction mixtures (1.0 ml) containing chicken asialoerythrocytes (10%) and sialylparagloboside (10 nmol) suspended in PBS were incubated for 30 min at 37°C. Hemagglutination assays were performed with 0.5% chicken erythrocytes reconstituted with sialylparagloboside to contain 38 pmol/10^6 cells for the IV^3(NeuAc)nLc4Cer (NeuAc2,3Gal sequence) and 30 pmol/10^6 cells for the VI^6(NeuAc)nLc4Cer (NeuAcα2,6Gal sequence). All of the viruses agglutinated native erythrocytes but did not agglutinate neuraminidase-treated cells. Hemolysis values represent percentages of virus-mediated low-pH hemolysis, calculated by the following equation: [(A_{540} of the supernatant after treatment of sialylparaglboside-erythrocytes and each virus) − (A_{540} of the supernatant after treatment of asialoerythrocytes with each virus)/(A_{540} of the supernatant after treatment of native erythrocytes with each virus) − (A_{540} nm of supernatant after treatment of asialoerythrocytes with each virus)] × 100. The optical density of asialoerythrocytes mediated by viruses was usually <0.05.

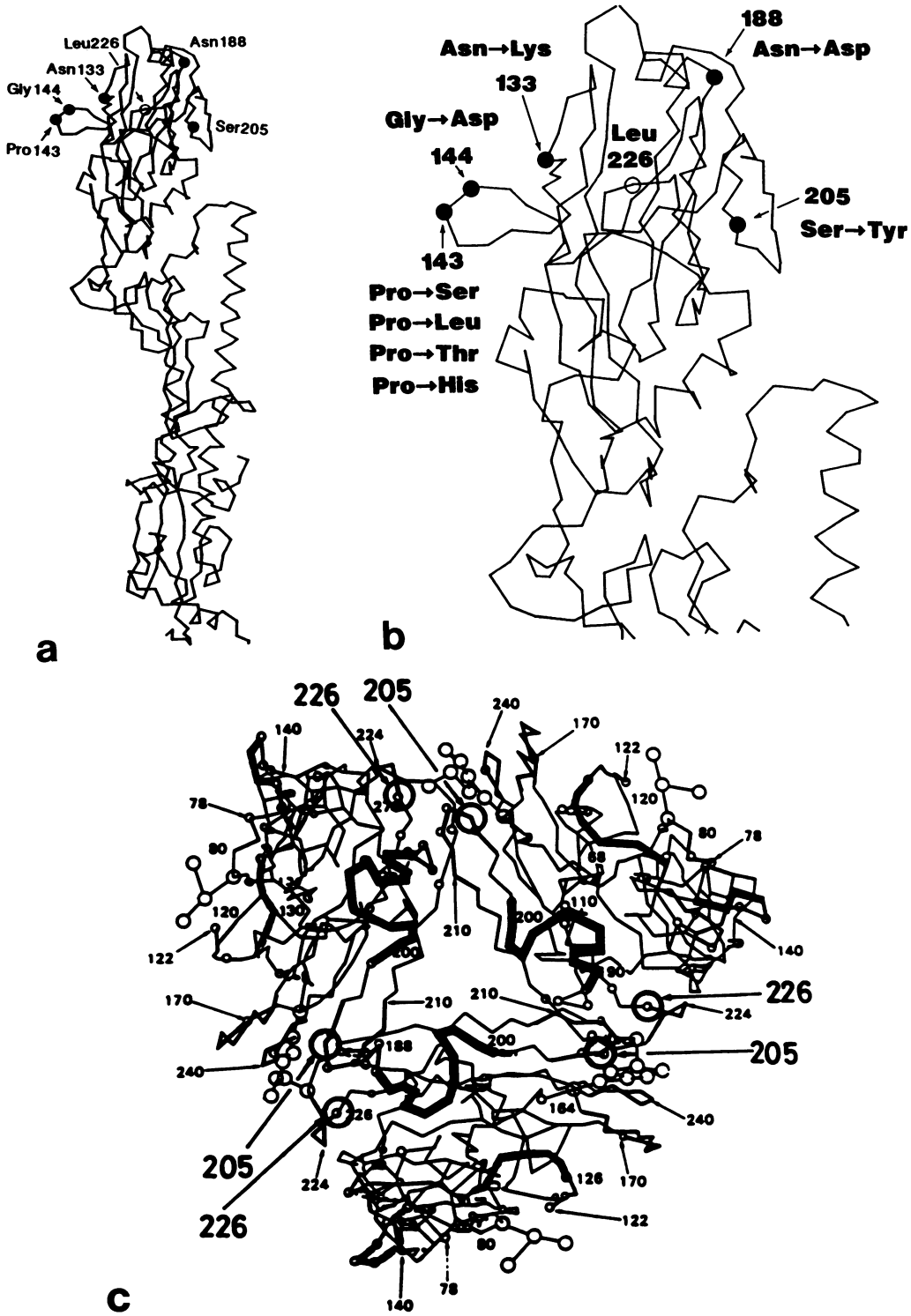

FIG. 1. Location of amino acid substitution in influenza virus strain A/Memphis/1/71-Bel/42 (H3N1) variants V1 to V8. (a) Standard view of the HA monomer as determined by Wilson et al. (30). (b) Expanded view of the distal tip of the HA monomer labeled as in panel a. The distances from α-carbon to α-carbon between Ser-205 and the residues at position 226, located in the receptor-binding pocket of the H3 trimer structure, are as follows: Ser-205 (A) to Leu-226 (A), 30; Ser-205 (A) to Leu-226 (B), 14. The monomers are arbitrarily labeled A, B, and C. (c) Schematic drawing of the α-carbon backbone of residues 48 to 280 (HA1) of the HA trimer from influenza virus A/X-31. The view is down the trimer axis, viewed from the outside towards the viral membrane (30). Ser-205 is located far from Leu-226 in the receptor-binding pocket on the distal tip of the HA (30), but is very close to Leu-226 in the neighboring HA subunit (14). Changing Ser-205 to Tyr alters the receptor-binding specificity to the sialosyl linkage NeuAcα2,3Gal to NeuAcα2,6Gal.

TABLE 3. Hemolysis mediated by variant V1 on erythrocytes coated with different sialylparaglobosides

Desialylated chicken erythrocytes used to coat	Virus-mediated hemolysis (% of control)	
	V1 (Ser-205→Tyr)	Parent virus
Sialylparaglobisides		
G_{M3}(NeuAc)	39	42
G_{M2}(NeuAc)	0	0
G_{M3}(NeuGc)	0	0
G_{M1a}(NeuAc)	0	0
G_{D1a}(NeuAc)	42	47
G_{D1b}(NeuAc)	0	0
IV^3(NeuAc)nLc4Cer (NeuAcα2,3Gal)	29	80
IV^6(NeuAc)nLc4Cer (NeuAcα2,6Gal)	63	57
IV^3(NeuGc)nLc4Cer (NeuGcα2,3Gal)	0	0
i-active ganglioside [VI^3(NeuAc)nLc6Cer, NeuAcα2,3Gal]	42	63
I-active ganglioside (NeuAcα2,3Gal)	49	73
Native erythrocytes (control)	100	100

differences were detected in the pH dependence of virus-mediated hemolysis between the variants (V1 to V8) and the parent A/Mem/1/71-Bel/42. However, the V1 variant was much less able to hemagglutinate and hemolyse erythrocytes coated with IV^3(NeuAc)nLc4Cer than were other variants or the parent virus. Variants V2 to V8 did not differ appreciably from the parent virus in their ability to hemagglutinate and hemolyse the asialoerythrocytes coated with sialylparaglobosides. These results indicate that a single-amino-acid substitution at residue 205 in HA1 (Ser → Tyr) can alter recognition of the membrane-associated receptor ganglioside by influenza viruses.

Specificity of the recognition of the variant V1 for sialyloligosaccharides. The hemolytic activity of variant V1 and of the parent virus was assayed on erythrocytes coated with different gangliosides (Table 3). The NeuAcα2,6Gal sequence-containing sialylparagloboside, IV^6(NeuAc)nLc4 Cer, was the most active ganglioside in restoring hemolysis by the variant V1. By contrast, hemolysis of the asialoerythrocytes reconstituted with IV^3(NeuAc)nLc4Cer, which had the highest activity relative to the parent virus, was much lower than that observed with IV^6(NeuAc)nLc4Cer. The other variants and the parent virus showed maximum hemolysis of erythrocytes coated with IV^3(NeuAc)nLc4Cer, followed in order by IV^6(NeuAc)nLc4Cer, I-active ganglioside, i-active ganglioside [VI^3(NeuAc)nLc6Cer], G_{M3}(NeuAc), and G_{D1a}(NeuAc). The responsiveness of all the variants tested with erythrocytes coated with NeuGc-bearing gangliosides was substantially lower than that of erythrocytes coated with gangliosides that bore NeuAc. Gangliosides (G_{M1a}, G_{M2}, and G_{D1b}) whose sialic acids were joined to the "inner" galactose of the ganglio-series oligosaccharide core by α2,3 linkage also showed very low responses to all of the viruses.

DISCUSSION

Antigenic variants of A/Mem/1/71-Bel/42 (H3N1) with single substitutions in their HA molecules (Table 2) were examined for their ability to hemagglutinate and hemolyse erythrocytes coated with different gangliosides. X-ray crystallographic studies (29) showed that the receptor-binding site of the HA consists of amino acids 98, 134 to 138, 153, 155, 183, 190, 194, 195, and 224 to 228. Antigenic variant 2, which has a substitution at residue 133 (Asn → Lys), is located near the receptor-binding site but had no influence on the binding of different gangliosides or on hemolysis. Conversely, variant 1, with a substitution at residue 205 (Ser → Tyr), located in antigenic site D, showed decreased ability to agglutinate and hemolyse erythrocytes coated with sialylparaglobosides (Table 2). Sialylparaglobosides [IV^3(NeuAc)nLc4Cer and IV^6(NeuAc)nLc4Cer] have been identified as the common receptor molecules (i.e., those associated with the highest fusion and hemolytic activity toward ganglioside-reconstituted asialo chicken erythrocytes) for antigenically different influenza viruses: A/PR/8/34 (H1N1), A/Japan/305/57 (H2N2), A/Aichi/2/68 (H3N2), A/X-31 (H3N2), and B/Lee/40 (20). Variant 1 (Ser-205 → Tyr) was selective for NeuAcα2,6Gal linkages in sialylparagloboside, whereas the parental strain A/Mem/1/71-Bel/42 (H3N1) and the other variants were selective for NeuAcα2,3Gal linkages. These studies indicate that a single-amino-acid substitution in an antigenic site may alter binding specificity in the microdomain (NeuAcα2,3Gal and NeuAcα2,6Gal) of the receptor-sialylglycoconjugate molecules.

Rogers et al. (11) reported that amino acid 226 in HA1 was leucine in influenza virus A/X-31 (H3N2), which preferentially bound NeuAcα2,6Gal linkages, glutamine for variant HAs that bound the NeuAcα2,3Gal linkage, and methionine for the variant that bound both linkages. It is possible that substitution of Ser-205 in the parent HA to a bulky hydrophobic amino acid (Tyr) causes a conformational change of the receptor-binding pocket in the neighboring subunit of the HA trimer. The loop containing Leu-226 may be perturbed by the presence of Tyr-205 (Fig. 1).

Six single-amino-acid differences at positions 2, 31, 78, 158, and 182 in HA1 and 132 in HA2 occurred between the HAs of A/X-31 and A/Mem/1/71-Bel/42 (Table 4). Although the amino acid at position 226 in the HA of both viruses is leucine, X-31 (H3N2) HA preferentially bound to sialylparagloboside containing NeuAcα2,6Gal [IV^6(NeuAc)nLc4Cer] (20), and A/Mem/1/71-Bel/42 (H3N1) bound preferentially to NeuAcα2,3Gal [IV^3(NeuAc)nLc4Cer]. These results indicate that amino acid 226 in the receptor-binding pocket of the HA molecule may not be the sole residue in the receptor-binding pocket that directly regulates recognition of the sialic acid-Gal linkages in the receptor molecules. The different recognition specificity for the NeuAc-Gal sequence between A/X-31 and A/Mem/1/71-Bel/42 may be caused by the single or multiple changes in amino acids listed in Table 4.

It was reported that an amino acid residue not immediately in the sialic acid-binding site of the HA can influence binding specificity (3, 25). Yewdell et al. (33) reported that the mutation of an amino acid (Ile-224 → Met) located at the trimer interface a considerable distance away from the receptor-binding site can increase the agglutinability of neur-

TABLE 4. Amino acid differences between the HAs of influenza virus strains A/X-31 and A/Memphis/1/71-Bel/42

HA subunit	Amino acid residue no.	Amino acid residue	
		A/X-31	A/Memphis/1/71-Bel/42
HA1	2	Asp	Tyr
	31	Asp	Asn
	78	Val	Gly
	158	Gly	Glu
	182	Ile	Val
HA2	132	Glu	Asp

aminidase-treated erythrocytes. This report supports the notion that amino acid substitutions in antigenic sites on the HA affect the conformation of the receptor-binding pocket located in the neighboring subunit of the HA trimer, leading to changes in receptor-binding specificity toward NeuAcα 2,3Gal and NeuAcα2,6Gal sequences in cell membrane-associated gangliosides.

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