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H5N1 chicken influenza viruses display a high binding affinity for Neu5Acα2-3Galβ1-4(6-HSO_3)GlcNAc-containing receptors

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Abstract

To characterize differences in the receptor-binding specificity of H5N1 chicken viruses and viruses of aquatic birds, we used a panel of synthetic polyacrylamide (PAA)-based sialylglycopolymers that carried identical terminal Neu5Acα2-3Gal fragments but varied by the structure of the next saccharide residues. A majority of duck viruses irrespective of their HA subtype, bound with the highest affinity to trisaccharide Neu5Acα2-3Galβ1-3GlcNAc, suggesting that these viruses preferentially recognize sialyloligosaccharide receptors with type 1 core (Galβ1-3GlcNAc). Substitution of 6-hydroxyl group of GlcNAc residue of tested sialylglycopolymers by 6-sulfo group had little effect on receptor binding by duck viruses. By contrast, H5N1 chicken and human viruses isolated in 1997 in Hong Kong preferred receptors with type 2 core (Galβ1-4GlcNAcβ) and bound sulfated trisaccharide Neu5Acα2-3Galβ1-4(6-HSO_3)GlcNAcβ (6-Su-3′SLN) with the extraordinary high affinity. Another chicken virus, A/FPV/Rostok/34 (H7N1), and several mammalian viruses also displayed an increased affinity for sulfated sialyloligosaccharide receptor. The binding of chicken and mammalian viruses to tracheal epithelial cells of green monkey decreased after treatment of cells with glucosamine-6-sulfatase suggesting the presence of 6-*O*-Su-3′SLN determinants in the airway epithelium. It remains to be seen whether existence of the 6-*O*-Su-3′SLN groups in the human airway epithelial cells might facilitate infection of humans with H5N1 chicken viruses.

Keywords: H5N1 chicken influenza virus; Human virus; Affinity

Introduction

Avian influenza viruses as a rule do not replicate efficiently in humans. For example, high doses of virus were found to be required for the replication of avian influenza strains in volunteers even at a limited level (Beare and Webster, 1991), and no cases of influenza virus infections were documented in workers exposed to highly pathogenic avian viruses during the 1985 poultry outbreak in the US (Bean et al., 1985). Nevertheless, chicken H5N1 viruses have been isolated from a human in 1997 (Claas et al., 1998; De Jong et al., 1997; Subbarao et al.,1998; Suarez et al., 1998; Shortridge et al., 1998). Eighteen people with influenza H5N1 infection were hospitalized and six of these patients died. Two human cases of H5N1 infection have been confirmed in Hong Kong in February 2003 (Peiris et al., 2004) The attention to influenza viruses circulating in poultry has been further strengthened after cases of human infection by H9N2 viruses in the Guangdong province of China (Guo, 1998; Guo et al., 1999) and in Hong Kong (Peiris et al., 1999).

A large body of data shows the multigenic character of influenza virus pathogenicity. All human and avian isolates from live bird markets in Hong Kong 1997 contained multiple basic amino acids at the cleavage site of the HA, a feature known to be associated with high virulence among avian influenza viruses (Claas et al., 1998; Suarez et al., 1998).

The importance of amino acid at position 627 in the PB2 protein for lethal infection was shown by the plasmid-based reverse genetics system. (Hatta et al., 2001). Moreover, this

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study suggested also that Ser-to-Ile substitution at position 227 of hemagglutinin (HA) can reduce the virulence potential of the virus. This amino acid is in the receptor-binding pocket on the distal tip of the HA and therefore may affect the ability of the virus to recognize and bind to target cells.

To attach to host cells, influenza viruses bind via their HAs to sialylglycoconjugates exposed on cell surface. It has been shown that influenza A viruses isolated from avian species preferentially bind to Neu5Acα2-3Gal terminated sugar chains, although closely related human viruses demonstrate a higher binding affinity toward the Neu5Acα2-6Gal-terminated structures (Connor et al., 1994; Gambaryan et al., 1997; Matrosovich et al., 1997; Rogers and D'Souza, 1989, see also Paulson, 1985 for the review of earlier data). Moreover, H9N2 viruses from Hong Kong live bird markets, responsible for a few cases of influenza in pigs and humans, have receptor specificity, similar to that of H3N2 human viruses (Matrosovich et al., 2001). Nevertheless, human cases of H5N1 infection have demonstrated that avian viruses are capable of replicating in humans despite their Neu5Acα2-3Gal-receptor specificity (Matrosovich et al., 1999). It has been shown more recently that 2-6-linked receptors are mainly expressed on nonciliated cells and that ciliated cells express 2-3-linked sialic acid receptors in sufficient density to allow entry and replication of avian viruses (Matrosovich et al., 2004).

It is known, that avian influenza viruses may differ in respect of their fine receptor specificity. The marked distinctions were demonstrated between duck and chicken influenza viruses when recognizing gangliosides. The duck strain bound to a wide range of short- and long-chain gangliosides, although the human strains bound only to gangliosides with deca- or longer oligosaccharide moiety (Miller-Podraza et al., 2000). Chicken viruses bound more strongly than duck virus to respiratory cells of green monkey and gangliosides of monkey lung tissues with long sugar chains (Gambaryan et al., 2002). The importance of structural factors associated with more distant parts of the binding epitope was demonstrated (Gambaryan et al., 2003).

To further characterize the unusual receptor specificity of chicken H5 viruses, we investigated the attachment of viruses to receptors of cell plasma membranes and viruses binding to soluble gangliosides and receptor analogs sharing terminal Neu5Acα2-3Gal-motif but differing in the structure of inner saccharide core. A particular attention has been paid to 2–3 sialylated oligosaccharides containing additional sulfated residue.

Results and discussion

For elucidating the receptor specificity of H5N1 chicken viruses, we compared the affinity of duck and chicken viruses to plasma membrane of duck intestine and monkey trachea cells. This primate species is susceptible to human influenza viruses, O'Brien and Tauraso, 1973; therefore, we

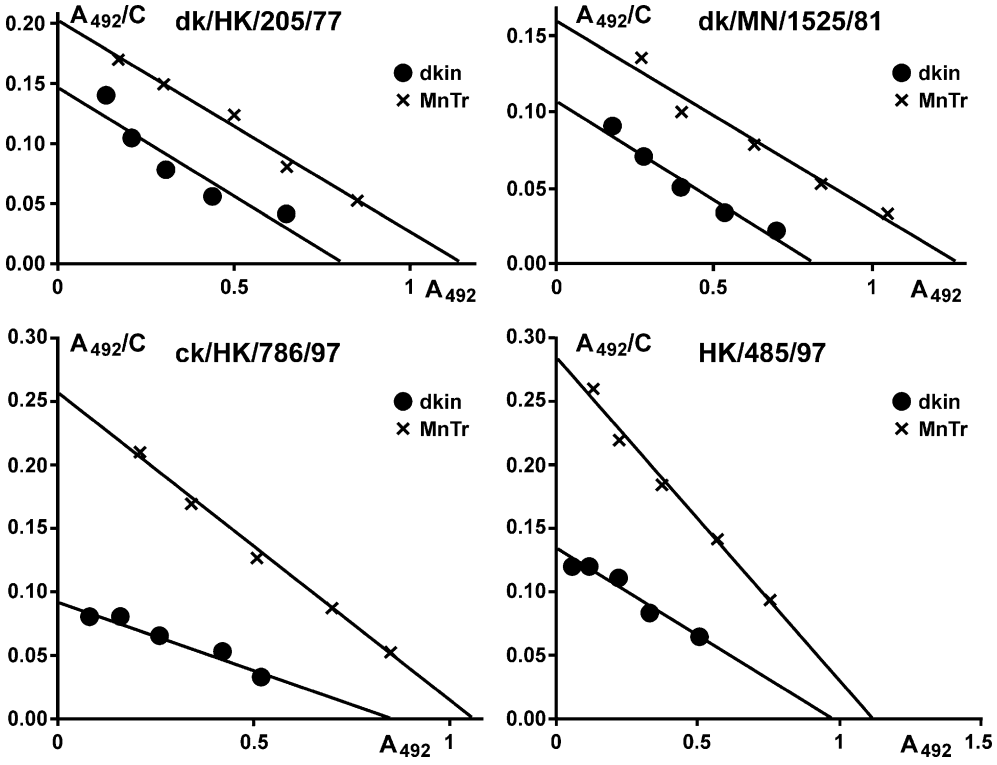

Fig. 1. Scatchard plots for the binding of Duck/HK/205/77, Duck/MN/1525/81, Chicken/HK/786/97, and HK/485/97 influenza viruses to the plasmatic membranes from epithelial cells of duck intestine (closed circles), and monkey trachea (crosses). Representative binding experiment is shown. Details of the assay are described in Materials and methods.

assumed that sialylglycoconjugate receptors on monkey target cells are similar to those on human target cells. The binding data were processed as Scatchard plots, with the slope corresponding to virus affinity for substrate. The binding of four H5 viruses to plasma membrane preparations is shown in Fig. 1. Formal analysis of the binding plots indicates that the binding affinity (slope of the plots) varies depending on the particular virus/substrate combination. The plots for binding of duck viruses to the membranes isolated from cells of duck intestine are parallel to the plots for binding to membranes of monkey trachea; this suggested that the binding affinities are equal. In contrast, chicken virus and virus isolated from human bind to monkey trachea cell membranes substantially stronger compared with duck intestine cells. Thus, receptor specificities of chicken and duck viruses are not equal and more appropriate receptors for chicken viruses do exist on monkey trachea cell membranes in compared with duck intestine cells.

For quantitative comparison of affinity of duck and chicken H5 viruses to receptors of monkey airway cells, we prepared the heavy fraction of gangliosides of monkey lung, and affinity of viruses for this preparation was measured by competitive assay. The data presented in Table 1 shows that binding affinity of chicken viruses for monkey gangliosides is essentially higher than duck viruses, in spite of similar affinity to monomeric 3′sialyllactose.

For specification of virus binding epitope, synthetic sialylglycopolymers, which differed by the structure of inner saccharide residues, were used. The saccharides were attached to soluble 30–40 kDa polyacrylamide (PAA). The structures of the tested sialooligosaccharides are presented in Table 2. 3′-*O*-Su-Le^c-PAA (without Neu5Ac) was used as negative control. None of the tested viruses bind to Neu5Acα2-6Galβ1-terminated sialylglycopolymers (data not shown). It can be seen from Table 3 that the pattern of sialylglycoconjugate binding to the virus correlates with the virus host species. The most distinct feature of duck viruses, irrespective of the subtype of the viral HA, is their high binding affinity for SiaLe^a-PAA and SiaLe^c-PAA, receptor analogs of the so-called type 1, that is, based on Galβ1-3GlcNAc core. Interestingly, GD1a ganglioside capable of high-affinity binding to duck viruses has similar core Galβ1-3GalNAc (Gambaryan et al., 2002; Matrosovich et al., 1997). Supplementation of sulfo group to position 6 of glucosamine moiety (see structures in Table 2) weakly affected the binding. In contrast, H5 chicken viruses from Hong Kong, including viruses isolated from infected patients in 1997, displayed higher affinity for Neu5Acα2-3Galβ1-4GlcNAc trisaccharide (3′SLN) and the highest affinity to its sulfo derivative, 6-*O*-Su-3′SLN. The affinity of chicken viruses to this sulfated trisaccharide was much higher than that of duck viruses (Table 3). Additional 6-sulfo group increased the binding of 3′SLN to chicken viruses receptor binding site (RBS), but weakly influenced the binding to duck RBS. It should be noted that in all the cases of very strong binding of viruses with 6-*O*-Su-3′SLN, isomeric trisaccharide 6-*O*-Su-SiaLe^c also having 6-*O*-sulfo group displayed moderate binding.

Table 1
Concentrations of 50% inhibition of HRP-F binding with H5 influenza viruses by 3′sialyllactose (Neu5Acα2-3Galβ1-4Glc) and heavy fraction of gangliosides of monkey lung (GML) (μM SA)[a]

Viruses	Inhibitors	
	3′SL	GML
Duck/HK/205/77	200 ± 110	300 ± 150
Duck/HK/698/79	200 ± 100	300 ± 130
Duck/MN/1525/81	200 ± 90	200 ± 90
Duck/HK/308/78	300 ± 150	100 ± 40
Chicken/HK/220/97	500 ± 220	50 ± 20
Chicken/HK/786/97	300 ± 130	20 ± 8
A/HK/481/97	200 ± 110	10 ± 4
A/HK/485/97	300 ± 140	5 ± 2

[a] Mean data and standard deviations calculated for three replicate experiments.

Table 2
Structures of oligosaccharides attached to polyacrylamide

Structure	Abbreviation
3′-*O*-Su-Galβ1-3GlcNAcβ	3′-*O*-Su-Le^c
Neu5Acα2-6Galβ1-4GlcNAcβ	6′SLN
Neu5Acα2-6Galβ1-4-(6-*O*-Su)GlcNAcβ	6-*O*-Su-6′SLN
Neu5Acα2-3Galβ1-4GlcNAcβ	3′SLN
Neu5Acα2-3Galβ1-4-(6-*O*-Su)GlcNAcβ	6-*O*-Su-3′SLN
Neu5Acα2-3Galβ1-3GlcNAcβ	SiaLe^c
Neu5Acα2-3Galβ1-3-(6-*O*-Su)GlcNAcβ	6-*O*-Su-SiaLe^c
Neu5Acα2-3Galβ1-3(Fucα1-4)GlcNAcβ	SiaLe^a
Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAcβ	SiaLe^x
Neu5Acα2-3Galβ1-4(Fucα1-3)(6-*O*-Su)GlcNAcβ	6-*O*-Su-SiaLe^x

The putative disposition of 6-*O*-Su-3′SLN and 6-*O*-Su-SiaLe^c in RBS of H5 hemagglutinin of A/Duck/Singapore/3/97 virus based on X-ray structure of H5 hemagglutinin (Ha et al., 2001) is shown in Fig. 2. 6-Sulfo group of 6-*O*-Su-SiaLe^c is directed in water and thus is incapable of interacting with RBS. In case of 6-*O*-Su-3′SLN, the sulfo group is localized closely to positively charged amino group of Lys193 (H3 numbering), a conserved residue for all H5 sequences currently deposited in Genbank. Probably, the favorable interaction between this amino acid and negatively charged sulfo group is possible in HA RBS of chicken but not duck viruses.

The high-affinity receptor determinant of H7 chicken virus A/FPV/Rostok/34 is 6-*O*-Su-SiaLe^x, that is, fucosylated 6-*O*-Su-3′SLN. The increased affinity to sulfated sialosugar receptors was observed also for three swine viruses and Equine/Miami/1/63. The preference of sulfated compound for reception by all tested viruses allows speculating about the availability of such determinants on the host airway epithelial cells, chicken, and mammalian.

It can be supposed that monkey trachea bears sulfo receptors binding chicken but not duck viruses. To test this hypothesis, we compared the binding of viruses to native plasma membrane of monkey trachea cells with binding to the membrane after treatment with glucosamine-6-*O*-sulfa-

Table 3
Binding affinity (K_{ass}, mM^{-1}) of virus with sialylglycoconjugates*

Viruses		Sugar determinants							
		3′-*O*-Su-Le^c	SiaLe^c	6-*O*-Su-SiaLe^c	3′SLN	6-*O*-Su-3′SLN	SiaLe^x	6-*O*-Su-SiaLe^x	SiaLe^a
Duck/France/46/82	H1N1	<5	200	200	100	100	20	20	200
Pintail/Primorje/695/76	H2N3	<5	100	100	100	100	100	200	2000
Duck/Buryatia/652/88	H3N8	<5	200	200	100	100	20	20	400
Duck/Buryatiya/1905/00	H4N6	<5	100	100	50	50	10	10	15
Mallard/Primorie/3/82	H9N2	<5	150	200	100	150	10	40	150
Duck/Hoshimun/014/78	H5N3	<5	100	100	100	150	20	50	200
Duck/HK/205/77	H5N3	<5	200	200	200	200	40	100	500
Duck/HK/698/79	H5N3	<5	100	100	200	200	50	100	500
Duck/MN/1525/81	H5N1	<5	100	100	100	100	10	20	100
Duck/HK/308/78	H5N3	<5	50	50	100	100	10	20	150
Chicken/HK/220/97	H5N1	<5	100	500	400	4000	15	50	100
Chicken/HK/728/97	H5N1	<5	100	500	300	3000	10	50	100
Chicken/HK/786/97	H5N1	<5	300	1000	1000	10,000	10	500	500
Chicken/HK/915/97	H5N1	<5	300	1000	300	5000	10	50	1000
A/HK/481/97	H5N1	<5	100	500	500	5000	10	50	250
A/HK/485/97	H5N1	<5	400	2000	2000	20,000	10	100	1000
FPV/Rostok/34	H7N1	<5	100	100	300	600	500	3000	1000
Swine/Iowa/31	H1N1	<5	30	50	50	20	50	200	400
Swine/Finistere/82	H1N1	<5	100	100	100	100	50	500	1000
Swine/Kazakhstan/48/82	H3N6	<5	100	100	100	300	20	300	500
Equine/Miami/1/63	H3N8	<5	100	500	300	2000	50	500	50

* The data were averaged from three sets of experiments. Standard errors did not exceed 50% of the mean values.

tase, the enzyme capable of specific destroying of the 6-*O*-Su-3′SLN. The Scatchard plots for duck, chicken, and equine influenza viruses binding to membrane preparations before and after sulfatase treatment are shown in Fig. 3. The treatment did not influence duck virus binding, but definitely decreased the binding of chicken and equine viruses. Similar experiments with three other *O*-sulfatases (type H-2 from *Helix pomatia*, type VI from *Aerobacter aerogenes*, type IV from *Patella vulgate*), incapable of splitting off the sulfate group in composition of 6-*O*-Su-3′SLN structure, demonstrated the lack of effect on further membrane binding with viruses (data not shown), this confirming the specificity of the observed effect. Sensitivity of the receptor to glucosamine-6-*O*-sulfatase treatment allows suggesting the existence of 6-*O*-Su-3′SLN motif in epithelial cells of monkey respiratory tract.

It has been shown that sulfotransferases are expressed in human airway epithelial cells and their product 6-*O*-Su-SLN was detected in human airway mucin (Lamblin et al., 2001; Lo-Guidice et al., 1994; Mawhinney et al., 1992) and seems

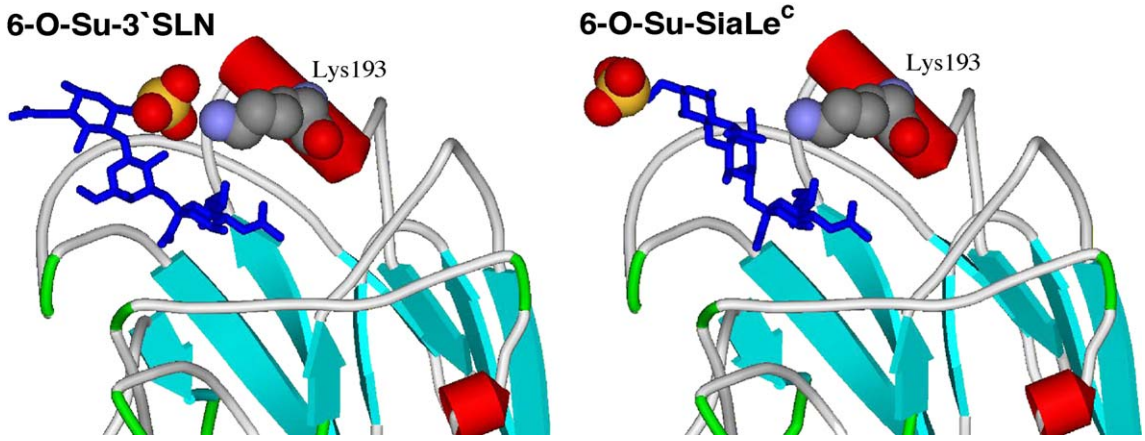

Fig. 2. Putative disposition of the sulfo group (spacefill mode) of 6-*O*-Su-3′SLN and 6-*O*-Su-SiaLe^c in the receptor-binding site of the H5 HA. The analogs were fitted into the RBS of H5 HA complexed with LSTa (1JSN structure, Brookhaven Protein Databank, Ha et al., 2001) by superimposing sialic acid residue of the sulfated analog over the sialic acid residue of LSTa. The molecular model of 6-*O*-Su-SiaLe^c was built based on LSTa (1JSN); molecular model of 6-*O*-Su-3′SLN was built based on the atomic coordinates of 3′sialyllactose complexed with X31 HA (PDB, 1HGG, Sauter et al., 1992). Lys193 of HA is represented in spacefill mode. The modeling was performed and figures were generated using Discovery Studio ViewerPro.

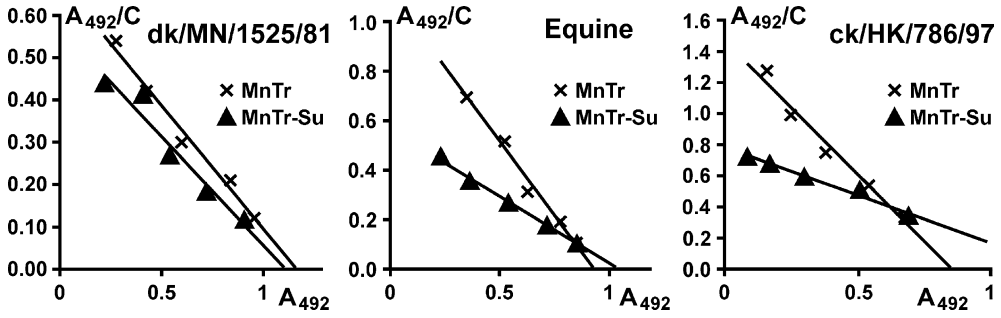

Fig. 3. Scatchard plots for the binding of Duck/MN/1525/81, Equine/Miamy/1/63, and Chicken/HK/786/97 influenza viruses to mock-treated (crosses) or glucosamine-6-*O*-sulfatase-treated (closed triangles) plasmatic membranes from monkey trachea epithelial cells. Representative binding experiment is shown.

to be presented in composition of membrane glycoproteins. Interestingly, the analogous structures containing 2-6 bound sialic acid in combination with 6-*O*-sulfo group are met considerably more rare (Lamblin et al., 2001; Lo-Guidice et al., 1994; Mawhinney et al., 1992).

Taking into account that human airway epithelium harbors 2-3-linked sialic acids on ciliated cells (Matrosovich et al., 2004) and the existence of glucosamine-6-*O*-sulfotransferases in human airway epithelial cells (Lamblin et al., 2001), one can suggest that human membrane glycoproteins could also carry such determinants. We speculate that the existence of these groups in human airway epithelium might enhance binding of chicken viruses to airway epithelial cells and thus facilitate infection of humans by H5 chicken viruses.

Materials and methods

Sialylglycopolymers, water-soluble conjugates of oligosaccharide (20% mol) with polyacrylamide m.w. 30–40 kDa, were synthesized as described (Bovin et al., 1993). Oligosaccharide content and polymer m.w. are the same for all the studied glycoconjugates. Oligosaccharides containing sulfo group in position 6 of hexosamine residue were synthesized as described in Pazynina et al. (2003). Glucosamine-6-*O*-sulfatase (#GKGAG-5010) was purchased from Glyco (USA). Sulfatases from *H. pomatia* (type H-2) (#S 9751), from *A. aerogenes* (type VI) (#S 1629), and from *P. vulgate* (type IV) (#S8629) were purchased from Sigma.

Viruses

H5 influenza A viruses were from the repository of St. Jude Children's Research Hospital (Memphis, TN, USA). Highly pathogenic H5N1 viruses were inactivated by treatment with β-propiolactone. Other viruses were from the virus repository of the D.I. Ivanovsky Institute of Virology (Moscow, Russia). The viruses were grown in 9-day-old embryonated chicken eggs. The allantoic and culture fluids were clarified by low-speed centrifugation; the viruses were pelleted by high-speed centrifugation, resuspended in 0.1 M NaCl, 0.02 M Tris buffer (pH 7.2) containing 50% glycerol, and stored at −20 °C.

Preparation of plasma membranes

Freshly killed mallard ducks (*Anas platyrhynchos*) were purchased from a live bird market. Tracheal tissues of African green monkey (*Cercopithecus aethiops*) were provided by the Department of Standardization and Control of poliomyelitis vaccine, M.P. Chumakov Institute of Polyomielitis (Moscow, Russia). Cells from epithelial tissues and plasma membranes from these cells were prepared as described previously (Gambaryan et al., 1998). Briefly, epithelial cells were scratched off, washed by repetitive centrifugation in 50% percoll, and disrupted in a Dounce homogenizer. Nuclei and cellular debris were removed by low-speed centrifugation, and plasma membranes were pelleted by high-speed centrifugation. The pellets were suspended in distilled water and stored in aliquots at −20 °C.

Preparation of gangliosides

Total lipids were extracted from freshly prepared cells with 20 volumes of chloroform/methanol solution (2:1) (light fraction) followed by extraction with 20 volumes of ethanol/water solution (4:1) (heavy fraction). Each extraction was performed at 60 °C over the course of 2 h. Gangliosides were isolated from lipid extracts by two consecutive partitions in chloroform/methanol/water (Folch et al., 1957).

Treatment of membranes by glucosamine-6-O-sulfatase

Plasma membranes of green monkey trachea (0.1 mg of protein) were suspended in 80 μl H_2O and disrupted by the forcing through the syringe needle. Twenty microliters of 5× buffer for glucosamine-6-*O*-sulfatase (from the Manufacturer) was added. Sample was divided into two equal parts. One microliter of glucosamine-6-*O*-sulfatase was added to one part; the other part was used as a control. Both parts were incubated for 3 h at 37 °C. After incubation the samples were

dissolved in 5 ml PBS, dispersed by ultrasound treatment, and used for coating of 96-well plates. Treatment by sulfatases from *H. pomatia* (type H-2), *P. vulgate* (type IV), and from *A. aerogenes* (type VI) incapable of splitting off the sulfate group in composition of 6-*O*-Su-3′SLN structure (this being confirmed experimentally: HPLC showed no change in 6-*O*-Su-3′SLN-O$(CH_2)_3NH_2$ when incubated with mentioned enzymes) was performed the same way.

Virus attachment to plasma membranes of cells was assayed by the microplate adsorption method as previously described (Gambaryan et al., 1999). In brief, the wells of 96-well polyvinyl chloride microplates (Costar, USA) were coated with membrane fraction, and blocked by incubation with 0.1% solution of bovine serum albumin (BSA) in PBS at 4 °C for 2 h. The BSA solution was discarded, and 0.1 ml of serial twofold dilutions of the virus in solution of 0.1% BSA, 0.003% Tween-20, and 10 μM of neuraminidase inhibitor, 4-amino-4-deoxy-Neu5Ac-en (a gift of Prof. M. von Itzstein), in PBS were incubated in the wells of the plate for 2 h at 4 °C. After washings, the bound viruses were quantified with the help of peroxidase-labeled fetuin (HRP-F) and *o*-phenylenediamine, the absorbance at 492 nm was measured, and the data were converted to Scatchard plots (A_{492} vs. A_{492}/C), where concentration of the viruses was expressed in hemagglutination units.

The affinity of the influenza viruses for soluble receptor analogs was evaluated in a competitive assay based on inhibition of binding by the solid-phase immobilized virus of the standard preparation HRP-F (Gambaryan and Matrosovich, 1992). The data were expressed in terms of binding constants (K_{ass}) formally equivalent to the association constants of virus/receptor analog complexes. For the calculation of the constants, concentration of the sialic acid residues in solution was used. Each set of experiments for the figures and the tables presented here was repeated three or four times with similar results. The table data were averaged from the same set of experiments. Figure presents one experiment from the set, that is, representative experiment.

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