# Human influenza virus recognition of sialyloligosaccharides

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**Abstract Sialic acids are essential components of cell-surface receptors utilized by influenza viruses. To evaluate the recognition of asialic sugar parts of the receptor, three representative strains of human influenza A and B viruses were tested for their binding of a panel of sialyloligosaccharides. The highest affinity binding carbohydrate determinants recognized by the viruses in a context of different core structures were Neu5Acα2-3Gal for the type B virus, Neu5Acα2-6Gal for the H3 subtype virus, and Neu5Acα2-6Galβ1-4GlcNAc for the H1 subtype virus. Penultimate to these determinants parts of sialyloligosaccharides studied either contributed less significantly to the binding affinity, or interfered with the binding.**

*Key words:* Influenza virus; Sialic acid; Sialyloligosaccharides; Sialyl-Lewis; Sialyl-Lewis; Protein–carbohydrate recognition

## 1. Introduction

Sialic acids are minimum essential determinants of cell surface receptors of influenza viruses recognized by the viral attachment protein hemagglutinin (HA) (for review, see [1,2]). Sialyloligosaccharide moieties of cell-surface glycoproteins and glycolipids exhibit significant structural diversity, and numerous studies indicate that the ability of sialic acid to serve as a receptor determinant of influenza virus may be influenced by the carbohydrate structure of sialylglycoprotein [1,3–5], ganglioside [6–9], or sialyloligosaccharide [10–12] to which it is attached. Particulary well documented is the ability of the viruses to differentiate between the Neu5Acα2-3Gal- and Neu5Acα2-6Gal-terminated sequences, while contribution of more distant carbohydrate parts of the receptor is poorly defined. In our previous study, we have compared the binding affinities of a variety of human influenza A and B virus strains for free αNeu5Ac and the simpliest natural receptor analogs 3′-sialyllactose (3′-SL), 6′-sialyllactose (6′-SL), and 6′-sialyl-*N*-acetyllactosamine (6′-SLN) [13]. This comparison revealed clear distinctions in the recognition of the asialic parts of these analogs by influenza viruses of different antigenic types and subtypes. In particular, subtype H1 influenza A viruses were distinctive from H3 and type B strains in their much better binding of 6′-SLN compared to 6′-SL, illuminating a possibility that the active sialo-sugar determinants for influenza viruses may be extended above the terminal Neu5Ac-Gal moiety of a sialyloligosaccharide.

In the present study, to check this possibility in more detail and to further characterize specific sialo-sugar determinants recognized by human influenza virus subtypes in different contexts, several linear and branched α2–3- and α2–6-sialyloligosaccharides were tested for their binding by H1, H3 and type B influenza viruses.

## 2. Experimental

### 2.1. Sialic acid and sialosides

Structural formulas and abbreviations of the sialosides used are shown in Table 1.

Free Neu5Ac was purchased from Serva, Germany. Methyl- and benzyl α-glycosides of Neu5Ac were obtained from Syntesome, Germany. 3-Aminopropyl glycosides of sialyl-Lewis and sialyl-Lewis were prepared as described in [14]. 3′-SL, 6′-SL, LSTa, LSTb, LSTc, MSLNH, and DSLNT from human milk and 3′-SLN, 6′-SLN, and DSGGn from human urine were isolated by subsequent ion-exchange chromatography on Dowex 1 × 2 [15], ion-pair reverse-phase HPLC [16], and normal phase HPLC [17]. Structure and purity of all compounds were estimated by $^1$H NMR (500 MHz) spectroscopy [16,18].

### 2.2. Viruses

Seed stocks of influenza viruses A/USSR/90/77 (H1N1), and B/USSR/100/83 were obtained from the Collection of viruses of the D.I. Ivanovsky Institute of Virology, Moscow. A/USSR/3/85 strain (H3N2) was kindly provided by Dr. M.A. Yakhno (D.I. Ivanovsky Institute of Virology). Viruses were grown in 10-day-old embryonated chicken eggs and were used without further purification.

### 2.3. Binding assay

The binding of the free Neu5Ac and sialosides to the RBSs of the viruses was evaluated by the ability of the ligand to compete with the binding to the solid-phase immobilized virus of the standard peroxidase-labeled fetuin preparation. The assay was performed, and the binding affinity constants ($K_d$) were calculated essentially as described previously [13,19]. To improve the reliability of results, replicate assays were done on different days, and the data were averaged. Twofold or higher differences in the values of binding affinity constants presented in the Table 1 are statistically significant.

## 3. Results and discussion

In this study, three strains of the currenlty circulating in humans influenza virus types and subtypes (type A, subtype H1 and H3; type B) were tested for their binding of free Neu5Ac and a number of sialyloligosaccharides structurally identical to the terminal sequences found in natural sialylglycoproteins and gangliosides. The strains were chosen from the previously studied large virus panels taking into account their good representation of the most typical receptor-binding characterisitics of corresponding virus type and subtype [13].

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*Abbreviations:* HA, hemagglutinin; Neu5Ac, 5-*N*-acetylneuraminic acid; RBS, virus receptor binding site; TLC, thin-layer chromatography; abbreviations for the sialyloligosaccharides are given in Table 1.


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The binding affinity constants for the complexes of the viruses with Neu5Ac and sialosides determined in a competitive ligand binding assay [19] are presented in the Table 1.

Free *N*-acetylneuraminic acid in solution is a mixture of α- and β-anomers. Only the former is presented in natural oligosaccharide sequences, and the β-anomer do not bind to influenza virus HA [10,20]. By comparing the virus affinity for the free αNeu5Ac (assuming its 5% content in the mixture [10]) and for the sialosides, a contrubution of the asilic parts to the binding energy can be assessed. As can be seen from the Table 1, this contribution varies depending on both the virus and on the sialoside. The main effects are summarized below.

1. *3′-SL*, *3′-SLN*, *SLe*$^x$, and *LSTa*. Type A viruses bind these Neu5Acα2-3Gal-terminated monovalent oligosaccharides with the same affinity as free αNeu5Ac, with the exclusion of H3 revealing somewhat better binding of 3′-SLN compared to both Neu5Ac and 3′-SL. Thus, with this single exclusion, sialic acid moiety appears to be the only specific binding epitope recognized by H1 and H3 substype strains in these oligosaccharide sequences, and neither penultimate Gal, nor more distant sugar residues interact with the receptor-binding sites (RBSs) of the viruses. Since the effect of different recognition of 3′-SLN and 3′-SL by A/USSR/3/85 is only slightly above the limits of the assay reproducibility, further testing of different H3 subtype strains would be needed to specify this finding.

Type B virus does not bind free Neu5Ac, however, it binds four Neu5Acα2-3Gal-terminated sialosides with equally high affinity. Thus, opposite to the case of the type A viruses, penultimate Gal does participate in favorable specific interactions with the RBS of the type B strain. However, just like for the A strains, subsequent sugar rings are of lower, if any, importance for the binding of the sialosides by type B HA.

2. In marked contrast to both SLe$^x$ and LSTa, isomeric tetrasaccharide *SLe*$^a$ exhibits at least an order of magnitude lower affinity for the type A HAs. For the type B virus the difference is lower but significant. NMR analysis and molecular mechanics calculations on SLe$^x$ and SLe$^a$ performed in [21] suggested that these tetrasaccharides have very similar conformations except that the GlcNAc residue in SLe$^a$ flips about 180° compared to that in SLe$^x$. However, this particular steric orientation of GlcNAc in LSTa provides no measurable negative effect on binding of the sialoside by the viruses, lowering the possibility of GlcNAc moiety being responsible for the poor binding of SLe$^a$. Thus, presently we have no reasonable explanation for the different recognition of SLe$^x$ and SLe$^a$ determinants by influenza viruses.

3. In the case of H3 and type B strains, the Galβ1-4Glc- moiety of *6′-SL*, *6′-SLN*, and *LSTc* appears to participate in specific interactions with the viral hemagglutinin, the virus affinity for these sialosides being at least 10-fold higher then for αNeu5Ac. The binding is not affected substantially by substituents on the opposite sites of the Glc moiety, namely, by NAc at C-2 (6′-SLN) and by lactose at C-1 (LSTc). It seems, therefore, that the Glc residue in the Neu5Acα2-6Galβ1-4Glc-determinant is not in contact with the RBSs of H3 and type B viruses and the main contribution to the binding affinity of the sialosides compared to free Neu5Ac is from penultimate Gal. For H3 viruses, this contribution can be provided, at least partially, by energetically favorable hydrophobic and Van der Waals interactions between the 6′-methylene group of Gal and Leu in position 226 of the H3 HA [13].

Opposite to the H3 and type B viruses, Galβ1-4Glc part of 6′-SL seems to interfere with the RBS of the subtype H1 virus (compare $K_d$ for 6′-SL and for free Neu5Ac). This effect correlates with the poor recognition by H1 strains of the aglycons in the methyl- and benzyl sialosides, and reflects, in our opinion, unfavorable steric interactions with the RBS of the penultimate to the glycosidic linkage methylene moieties of both synthetic and 2–6-linked natural aglycons [13].

Despite poor binding of 6′-SL, H1 strain binds 6′-SLN and LSTc stronger than any other sialoside tested. The ability to differentiate between 6′-SL and 6′-SLN was found previously to be specific property of H1 subtype human viruses [13]. However, due to Glc moiety of 6′-SLN being in the reducing state, the biological relevance of this effect was somewhat unclear. Even better binding of LSTc observed in the present study strongly suggests that the RBS of H1 subtype viruses may be specifically adapted for the recognition of the Neu5Acα2-6Galβ1-4GlcNAc-terminated oligosaccharide chains which are often encountered in glycoproteins and gangliosides.

4. *MSLNH*. Although this sialoside carries the terminal sialyl-sugar sequence well recognized in other contexts by all three strains tested, it is not bound by any of them (compare MSLNH with 6′-SLN and LSTc). It seems probable, that the conformational space available to the bulky and relatively flexible 1–6-linked substituent at GlcNAc is severily restricted in the complex.

5. In comparison with the three linear Neu5Acα2-6Gal-terminated sialosides, *LSTb* is a poorer ligand for the viruses, especially for the type B one. Since epitopes recognized by the human viruses on 2–6-linked sialosides are extended on the penultimate to Neu5Ac sugar ring (see above, item 3 and [13]), weaker binding of LSTb may result from (i) structural differences between penultimate GlcNAc and Gal, (ii) additional Gal substituent at position 3′ of GlcNAc, and (iii) lactose core.

6. The binding data for disialosides *DSLNT* and *DSGGn* seem to allow rational interpretation in terms of independent recognition by the viruses of each of two sialic acid containing epitopes with no significant interference between them.

For example, the affinities for DSLNT of H1 and type B strains are close to these for LSTa. Thus, Neu5Acα2-6GlcNAc determinant of DSLNT does not contribute to any marked extent to the binding of the disialosides by these two strains, in consistense with their poor recognition of LSTb.

H3 strain should bind both sialyl-sugar determinants of DSLNT as being represented in LSTa and LSTb, respectively. Indeed, the affinity for DSLNT is higher compared to both LSTa and LSTb.

DSGGn represents important terminal sialyloligosaccharide sequence encountered in O-linked oligosaccharides of glycoproteins and certain gangliosides. It is structurally close to the corresponding non-reducing part of DSLNT, and it is not unexpectable that the affinities of the viruses for two disialosides are rather similar. Of note are statistically significant small difference in binding of the two sialosides by H3 strains. Unlike H1 and B strains, the former reveals the ability to bind the Neu5Acα2-6-X-branches of DSLNT and DSGGn and may be more sensitive to the difference in X (GlcNAcβ1–3Galβ1–4Glc versus GalNAc).

Basing on the results presented above the following most distinct features of recognition of sialyl-sugar determinants by human influenza viruses can be noted.

Table 1
Influenza virus binding of Neu5Ac and sialosides ($K_d$, mM)

| Compound | Virus | | |
|---|---|---|---|
| | A/USSR/90/77 (H1) | A/USSR/3/85 (H3) | B/USSR/100/83 (B) |
| αNeu5Ac | 0.4 | 1.3 | >5* |
| Neu5AcαMe | 2.3 | 0.45 | 1.2 |
| Neu5AcαBn | 1.3 | 0.07 | 1 |
| **Neu5Acα2-3Gal-terminated monosialosides** | | | |
| Neu5Acα2-3Galβ1-4Glc (3'-SL) | 0.3 | 1.1 | 0.11 |
| Neu5Acα2-3Galβ1-4GlcNAc (3'-SLN) | 0.3 | 0.5 | 0.07 |
| Neu5Acα2-3Galβ1-4GlcNAcβ-O-R** (SLe$^x$) (Fucα1-3) | 0.4 | 1.3 | 0.13 |
| Neu5Acα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc (LSTa) | 0.3 | 1.3 | 0.17 |
| Neu5Acα2-3Galβ1-3GlcNAcβ-O-R** (SLe$^a$) (Fucα1-4) | >5 | >10 | 0.7 |
| **Neu5Acα2-6Gal(GlcNAc)-terminated monosialosides** | | | |
| Neu5Acα2-6Galβ4Glc (6'-SL) | 1.5 | 0.1 | 0.3 |
| Neu5Acα2-6Galβ1-4GlcNAc (6'-SLN) | 0.1 | 0.15 | 0.3 |
| Neu5Acα2-6Galβ1-4GlcNAcβ1-3Galβ1-4Glc (LSTc) | 0.03 | 0.15 | 0.3 |
| Neu5Acα2-6Galβ1-4GlcNAcβ1-6 (Galβ1-3GlcNAcβ1-3Galβ1-4Glc (MSLNH) | >5 | >5 | >5 |
| Neu5Acα2-6 (Galβ1-3GlcNAcβ1-3Galβ1-4Glc (LSTb) | >2 | 0.5 | >5 |
| **Disialosides** | | | |
| Neu5Acα2-6 (Neu5Acα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc (DSLNT) | 0.15 | 0.1 | 0.15 |
| Neu5Acα2-6 (Neu5Acα2-3Galβ1-3GalNAc (DSGGn) | 0.15 | 0.25 | 0.07 |

*For compounds which were not inhibitory at the highest concentration tested, only lower limit of dissociation constant could be estimated.
**R = $CH_2CH_2CH_2NH_2$.

(i) In Neu5Acα2-3Gal-terminated oligosaccharides Neu5Ac moiety is the only specific binding epitope recognized by influenza A strains.

(ii) In Neu5Acα2-6Gal-X-terminated sequences, specific binding epitope for H3 virus extends to the penultimate Gal, while that one for H1 strain includes Neu5Ac and the GlcNAc moiety of Neu5Acα2-6Galβ1-4GlcNAc.

(iii) Type B virus binds Neu5Ac only in context of underlying galactose with the preference for the Neu5Acα2-3Gal epitope over Neu5Acα2-6Gal one.

(iv) Penultimate to the mentioned above specific determinants, as well as more distant parts of sialyloligosaccharides either contribute less significantly to the binding affinity, or interfere with the binding of the specific determinant.

These features are consistent with the data on the receptor-binding properties of influenza viruses obtained in previous studies on virus binding to desialylated/specifically resialylated erythrocytes [1,4,5] and glycoproteins [3]. In the first case, human influenza A virus strains of H1, H2, and H3 subtypes were found to be distinct from the avian strains of the same subtype by their better binding to Neu5Acα2-6Galβ1-4GlcNAc-terminated sequences as compared to Neu5Acα2-3Galβ1–3GalNAc-terminated ones. Our results seem to explain this effect for H1 and H3 subtypes in more details. Namely, unlike for the asialic part of the latter sequence, HAs of both human virus subtypes provide for favorable interactions with the *N*-acetyllactoseaminic moiety of Neu5Acα2-6Galβ1-4GlcNAc. However, H1 and H3 HAs recognize the different domains of the aglycon. It may be speculated that this difference occured spontaneously at the early stage of adaptation of the animal H1 and H3 HAs to the cell-surface receptors of the human host.

Our results are less consistent with the data on virus binding to gangliosides adsorbed on TLC-plates [8,9]. For example, in a recent study [22], various type B virus strains were reported to prefer Neu5Acα2-6Gal determinants exposed on gangliosides over Neu5Acα2-3Gal ones, while opposite specificity was revealed for the binding of sialyloligosaccharides in solution (this study and [13,19]). Possible reasons for this inconsistense might be different exposure of the sugar determinants in the two cases, steric accessibility of the sialo-sugar groups in gangliosides being affected by the proximity of the ceramide moieties and/or by specific presentation of the ganglioside on the surface of TLC-plate.

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## References

[1] Paulson, J.C. (1985) in: The Receptors, Vol. 2 (Conn, M., Ed.), pp. 131–219, Academic Press, Orlando, FL.
[2] Wiley, D.C. and Skehel, J.J. (1987) Annu. Rev. Biochem. 56, 365–394.
[3] Suzuki, Y., Nagao, Y., Kato, H., Suzuki, T., Matsumoto, M. and Murayama, J. (1987) Biochim. Biophys. Acta 903, 417–424.
[4] Rogers, G.N. and D'Souza, B.L. (1989) Virology 173, 317–322.
[5] Connor, R.J., Kawaoka, Y., Webster, R.G. and Paulson, J.C. (1994) Virology 205, 17–23.
[6] Bergelson, L.D., Bukrinskaya, A.G., Prokazova, N.V., Shaposhnikova, G.I., Kocharov, S.V., Shevchenko, V.P., Kornilaeva, G.V. and Fomina-Ageeva, E.V. (1982) Eur. J. Biochem. 128, 467–474.
[7] Suzuki, Y., Nagao, Y., Kato, H., Matsumoto, M., Nerome, K., Nakajima, K. and Nobusawa, E. (1986) J. Biol. Chem. 261, 17057–17061.
[8] Suzuki, Y., Nakao, T., Ito, T., Watanabe, N., Toda, Y., Xu, G., Suzuki, T., Kobayashi, T., Kimura, Y., Yamada, A., Sugawara, K., Nishimura, H., Kitame, F., Nakamura, K., Deya, E., Kiso, N. and Hasegawa, A. (1992) Virology 189, 121–131.
[9] Müthing, J., Unland, F., Heitmann, D., Orlich, M., Hanisch, F.-G., Peter-Katalinic, J., Knäuper, V., Tschesche, H., Kelm, S., Schauer, R. and Lehmann, J. (1993) Glycoconjugate J. 10, 120–126.
[10] Pritchett, T.J., Brossmer, R, Rose, U. and Paulson, J.C. (1987) Virology 160, 502–506.
[11] Sauter, N.K., Bednarsky, M.D., Wursburg, B.A., Hanson, J.E., Whitesides, G.M., Skehel, J.J. and Wiley, D.C. (1989) Biochemistry 28, 8388–8396.
[12] Sabesan, S., Duus, J.O., Neira, S., Domaille, P., Kelm, S., Paulson, J.C. and Bock, K. (1992) J. Am. Chem. Soc. 114, 8363–8396.
[13] Matrosovich, M.N., Gambaryan, A.S., Tuzikov, A.B., Byramova, N.E., Mochalova, L.V., Golbraikh, A.A., Shenderovich, M.D., Finne, J. and Bovin, N.V. (1993) Virology 196, 111–121.
[14] Nifant'ev, N.E., Tsvetkov, Y.E., Shashkov, A.S., Tuzikov, A.B., Maslennikov, I.V., Popova, I.S. and Bovin, N.V. (1994) Bioorgan. Khim. 20, 552–555.
[15] Parkkinen, J. and Finne, J. (1987) Methods Enzymol. 138, 289–300.
[16] Grönberg, G., Lipnius, P., Lundgren, T., Erlansson, K., Lindh, F. and Nilsson, B. (1989) Carbohydr. Res. 191, 261–278.
[17] Bergh, M.L.E., Koppen, P. and van den Eijnden, D.H. (1981) Carbohydr. Res. 94, 225–229.
[18] Haverkamp, J., van Halbeek, H., Dorland, L., Vliegenthart, J.F.G., Pfeil, R. and Shauer, R. (1982) Eur. J. Biochem. 122, 305–315.
[19] Gambaryan, A.S. and Matrosovich, M.N. (1992) J. Virol. Methods 39, 111–123.
[20] Weis, W., Brown, J.H., Cusack, S., Paulson, J.C., Skehel, J.J. and Wiley, D.C. (1988) Nature 333, 426–431.
[21] Ichikawa, Y., Lin, Y.-C., Dumas, D.P., Shen, G.-J., Garcia-Junceda, E., Williams, M.A., Bayer, R., Ketcham, C., Walker, L.E., Paulson, J.C. and Wong, C.-H. (1992) J. Am. Chem. Soc. 114, 9283–9298.
[22] Xu, G., Suzuki, T., Tahara, H., Kiso, M., Hasegawa, A. and Suzuki, Y. (1994) J. Biochem. 115, 202–207.