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Shift in oligosaccharide specificities of hemagglutinin and neuraminidase of influenza B viruses resistant to neuraminidase inhibitors

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Abstract Influenza virus neuraminidase inhibitors (NAIs), currently used as anti-influenza drugs, can lead to the appearance of drug-resistant variants. Resistance to NAIs appears due to mutations in the active site of the neuraminidase (NA) molecule that decrease the NA enzymatic activity and sometimes in the hemagglutinin (HA) that decrease its affinity for cell receptors and, therefore, reduce the requirement for NA activity in releasing mature virions from infected cells. Using a set of sialo-oligosaccharides, we evaluated changes in the receptor-binding specificity of the HA and substrate specificity of the NA of influenza B viruses that had acquired resistance to NAIs. The oligosaccharide specificity of two pairs of field influenza B viruses, namely: i) B/Memphis/20/96 and its NAI-resistant variant, B/Memphis/20-152K/96, containing mutation R152K in the NA and 5 amino acid substitutions in the HA1, and ii) B/Hong Kong/45/2005 and its NAI-resistant variant B/Hong Kong/36/2005, containing a single R371K mutation in the NA, was evaluated. Wild type viruses bound strictly to a "human type" receptor, α2-6-sialo-oligosaccharide 6`SLN, but desialylated it is approximately 8 times less efficiently than the α2-3 sialosaccharides. Both drug-resistant viruses demonstrated the ability to bind to "avian type" receptors, α2-3 sialo-oligosaccharides (such as 3`SLN), whereas their affinity for 6`SLN was noticeably reduced in comparison with corresponding wild type viruses. Thus, the development of the NAI resistance in the studied influenza B viruses was accompanied by a readjustment of HA-NA oligosaccharide specificities.

Keywords Influenza B virus · Neuraminidase · Neuraminidase inhibitor · Drug resistance · Hemagglutinin · Receptor-binding specificity · Neuraminidase substrate specificity

Abbreviations

BODIPY FL	4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid
HA	hemagglutinin
HAU	hemagglutinating unit
HARI	inhibition of hemagglutination reaction
NA	neuraminidase
NAI	neuraminidase activity inhibitor
Neu5Ac	*N*-acetylneuraminic acid
OS-PAA	oligosaccharide-polyacrylamide conjugate
RBS	receptor-binding site
3`SLN	Neu5Acα2-3Galβ1-4GlcNAc
6`SLN	Neu5Acα2-6Galβ1-4GlcNAc

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SLe^c	Neu5Acα2-3Galβ1-3GlcNAc
SLe^a	Neu5Acα2-3Galβ1-3(Fucα1-4)GlcNAc

Introduction

Two viral glycoproteins expressed on the surface of influenza virus virion interact with sialylated carbohydrate chains [1, 2]. Hemagglutinin (HA) binds to sialic acid-containing cell receptors initiating virus entry into the host cell. In contrast to HA, another virus glycoprotein, neuraminidase (NA) cleaves off the terminal sialic acid residues from cellular and viral glycans, promoting the release of virus progeny from the host cell and preventing the formation of virion aggregates at the budding site. Furthermore, NA desialylates natural inhibitors of virus binding (such as mucins) thus facilitating virus entry into target cells. The functional fitness of HA and NA to each other is a prerequisite of successful influenza virus replication [1, 3].

Balanced action of HA and NA activities is an important condition of influenza virus efficient replication [1, 3–6], but a role of the HA receptor-binding and NA substrate specificities in maintaining such a balance remains poorly studied. Virus adaptation to a new substrate (for example, embryonated chicken eggs) or to new species often causes a shift in the HA receptor-binding specificity [7, 8]. Emergence of virus variants with reduced HA binding affinity for host receptors can be also observed after virus propagation in the presence of NA inhibitors (NAI) [9]. It has been shown recently, that restoration of replication activity of avian-human reassortant viruses having HA and NA of different origin was accompanied by changing not only receptor-binding specificity of the HA [6, 10], but sometimes substrate specificity of the NA [11]. Thus, an appropriate balance of HA and NA oligosaccharide specificities may be necessary for influenza virus viability. And "HA-NA readjustment" may be needed if the balance between the HA and NA specificities was disturbed as a result of a mutation [5, 12] or a deletion [13, 14] in the glycoproteins, or as a consequence of a selection in the presence of NAIs [9, 15–17].

Materials and methods

Synthesis of conjugates of Neu5Acα2-3Galβ1-4GlcNAc (3`SLN), Neu5Acα2-6Galβ1-4GlcNAc (6`SLN), Neu5Acα2-3Galβ1-3GlcNAc (SLe^c), and Neu5Acα2-3Galβ1-3(Fucα1-4)GlcNAc (SLe^a) with polyacrylamide or BODIPY FL has been described elsewhere [18, 19].

Receptor-binding specificity of influenza B viruses for sialo-oligosaccharides 3`SLN, 6`SLN, SLe^c, and SLe^a, covalently coupled with a high molecular mass polyacrylamide (OS-PAA), was evaluated in a hemagglunation reaction inhibition (HARI) assay as described previously [18]. The concentration of OS-PAA at which the inhibition of agglutination is still visible was assessed and expressed as concentration of the neuraminic acid [Neu5Ac, μM]. The HA affinity for a particular oligosaccharide was expressed as a 1/[Neu5Ac, μM] value. Mean values were calculated based on three independent experiments.

NA substrate specificity towards BODIPY-labeled 3`SLN, 6`SLN, SLe^c, and SLe^a was evaluated as described earlier [19]. The method is based on a quantitative separation of neutral BODIPY-labeled product from negatively charged BODIPY-labeled uncleaved substrate, using anion exchanger microcartridges [20]. A slope of a starting linear region of the V_0 (starting velocity of enzymatic reaction) *versus* S_0 (initial concentration of substrate in reaction mixture) kinetic curve was calculated for each substrate. Substrate specificity of each virus NA was determined by comparison of V_0/S_0 values obtained for all substrates at the same conditions. The mean values of V_0/S_0 were calculated from three independent experiments.

Viruses B/Memphis/20/96 (Mem/20), B/Memphis/20–152K/96 (Mem/20–152K), B/Hong Kong/36/2005 (HK/36), and B/Hong Kong/45/2005 (HK/45) were propagated in Madin-Darby canine kidney (MDCK) cell culture and purified by ultracentrifugation [3]. Virus titers were determined in a hemagglutination reaction assay with 0.5% suspension of turkey red blood cells. Purified viruses were diluted to 8 hemagglutination units (HAU) before the receptor-binding assay. For NA substrate specificity assays, HK/45 and Mem/20 were diluted to 40 HAU, while HK/36 and Mem/20–152K ones were used at the concentration of 160 HAU.

Reverse transcription-PCR and sequence analysis have been described earlier [15]. GeneBank accession numbers are: AF129892 for HA of Mem/20; AF129921 for NA of Mem/20; EU879085 for NA of HK/36.

Results and discussion

In the present study, we investigated the HA receptor-binding and the NA substrate specificities of four clinical isolates of influenza B virus, two of which were sensitive to such NAI-drugs as zanamivir and oseltamivir (wild type viruses) while two other ones were resistant to the drugs. The Mem/20 virus and its NAI-resistant mutant Mem/20–152K containing R152K mutation (N2 numbering) in the NA active site and five amino acid substitutions in the HA1 (I121T, G141E, A168T, I198T, and E237G) were recovered from a patient before and after zanamivir treatment, respectively [17]. Compared to the wild type virus the

Mem/20–152K displayed a 10-fold and 40-fold reduction in sensitivity to zanamivir and oseltamivir, respectively [15]. The second pair of influenza B viruses was represented by HK/36 and HK/45, which were recovered from two patients. The NA of HK/36 virus contains a R371K mutation at a conserved residue of the enzyme active site. Besides this mutation, the NAs of HK/36 and HK/45 were identical as well as the HA sequences of both these viruses. Compared to HK/45 virus the HK/36 displayed a 29-fold and 407-fold reduction in sensitivity to zanamivir and oseltamivir, respectively [15]. Receptor-binding and NA substrate specificities of these two pairs of influenza B viruses for synthetic sialo-oligosaccharides 3`SLN, 6`SLN, SLe^c, and SLe^a, representing structures of naturally occurring carbohydrate chains, were evaluated.

NA substrate specificity was analyzed by the fluorescent assay with the sialo-oligosaccharides coupled to the fluorescent dye BODIPY FL. Neuraminidases of HK/45 and Mem/20 desialylated α2-3 sialoside 3`SLN (0.017 and 0.027 $min^{-1}$, respectively) approximately 8 times more efficiently than the α2–6 isomer, 6`SLN (0.002 and 0.004 min^{-1}, respectively). Both NAs discriminated the inner part of α2-3 sialylated carbohydrates (Fig. 1), cleaving off the fucose-containing tetrasaccharide SLe^a two-fold slower than non-fucosylated trisaccharide SLe^c (0.012 *vs.* 0.024 min^{-1} for HK/45 and 0.014 *vs.* 0.027 min^{-1} for Mem/20). In general, the pattern of substrate specificity for wild type influenza B viruses was similar to that for human influenza A viruses, which are also known to discriminate 3`SLN *vs.* 6`SLN and SLe^c *vs.* SLe^a [11, 19].

The pattern of substrate specificity for both mutant NAs was very similar to each other, but different from that for the corresponding drug-sensitive variants (Fig. 1). 3`SLN was the best substrate for the both mutant NAs, while their ability to desialylate other sialo-oligosaccharides was poorly pronounced. However, hydrolytic activity of these mutant NAs even to 3`SLN was very low (0.0004 min^{-1}), being no more than 2% comparing to their drug-sensitive counterparts. These data are in a good agreement with the earlier results on the Mem/20–152K NA activity [17]. Apparently, different NA substrate specificity patterns for drug-resistant and drug-sensitive influenza viruses result from different structures of their NAs. In the Mem/20–152K NA the Arg152, a conserved amino acid of enzyme active site forming hydrogen bond with the carbonyl oxygen of N-acetyl group at Neu5Ac [21], was substituted by Lys. Such a mutation was observed during selection of NAI-resistant variants of influenza viruses *in vivo* [9, 15]. In the HK/36 NA the Arg371, a highly conserved amino acid in all influenza virus NAs, forming tri-arginine cluster (Arg118, Arg 292, and Arg 371) of enzyme catalytic site [21], was substituted by Lys. Such a mutation had not been selected in the presence of any NAI. Thus, development of drug-resistance in the both influenza viruses was accompanied by a single amino acid substitution in the active site of the NA thus seriously affecting functional properties of the enzyme.

Sialo-oligosaccharides coupled to high molecular mass polyacrylamide (OS-PAA) were used for measuring influenza virus receptor-binding specificity. It has been shown [18, 22] that such multivalent glycoconjugates were able to inhibit the influenza virus HA-induced agglutination of erythrocytes (HARI assay) if the oligosaccharide component reflected adequately the structure of a particular HA receptor. Both wild type viruses, Mem/20 and HK/45, bound exclusively 6`SLN, a receptor for most human influenza viruses [8]. However, affinity of Mem/20 for 6`SLN was 3.5 times higher than that of HK/45 (14 μM^{-1} *vs.* 4 μM^{-1}). Such a difference may appear due to an additional glycosylation site near the receptor-binding site (RBS) of HK/45 whose HA differs from the Mem/20 HA by two glycosylation sites, at Asn196 and Asn232. It was shown that additional glycosylation sites may decrease the HA affinity for the receptor [23–25].

Receptor-binding properties of NAI-resistant variants, Mem/20–152K and HK/36, differed from the wild type viruses. HK/36 bound 6`SLN four times weaker (1 $\mu M^{-1}$) than the drug-sensitive counterpart (HK/45), while Mem/20 almost lost its 6`SLN–binding ability (<0.1 μM^{-1}). Although 3`SLN was most favorable receptor for the Mem/20–152K virus (0.4 μM^{-1}), while HK/36 was able to bind better to tetrasaccharide SLe^a (0.2 μM^{-1}), it should be noticed, that both mutants displayed low affinity towards α2-3-sialo-oligosaccharides. As a rule, a change of HA receptor-binding properties is a result of a change in HA structure [8]. The HA of Mem/20–152K differs from the HA of Mem/20 by 5 amino acid residues, most of which are located within or near the RBS (Fig. 2): Gly141, a conserved amino acid of influenza B virus HAs forming two hydrogen bonds with Neu5Ac in RBS, is substituted by Glu in the HA of the drug-resistant variant; Glu237Gly mutation is located in close proximity to conserved amino acid residues interacting with human-type receptor [25]; Ile198Thr substitution creates an additional glycosylation site at Asn196. Of note, the HA of the B/Gifu/2/73 influenza virus was shown to bind equally to α2-3 and α2-6 sialylated carbohydrates; its HA differing from HAs of α2-6-recognizing influenza B viruses by additional glycosylation site at Asn196 and two amino acid residues at 121 and 141 positions [26].

Taking into account that the HA sequences of HK/36 and HK/45 are identical, we suppose that the shift in the specificity of HK/36 virus HA may be caused by the R371K mutation in the NA molecule. Dramatically decreased sialidase activity of HK/36 could be insufficient for a complete desialylation of 4 glycans located near RBS of

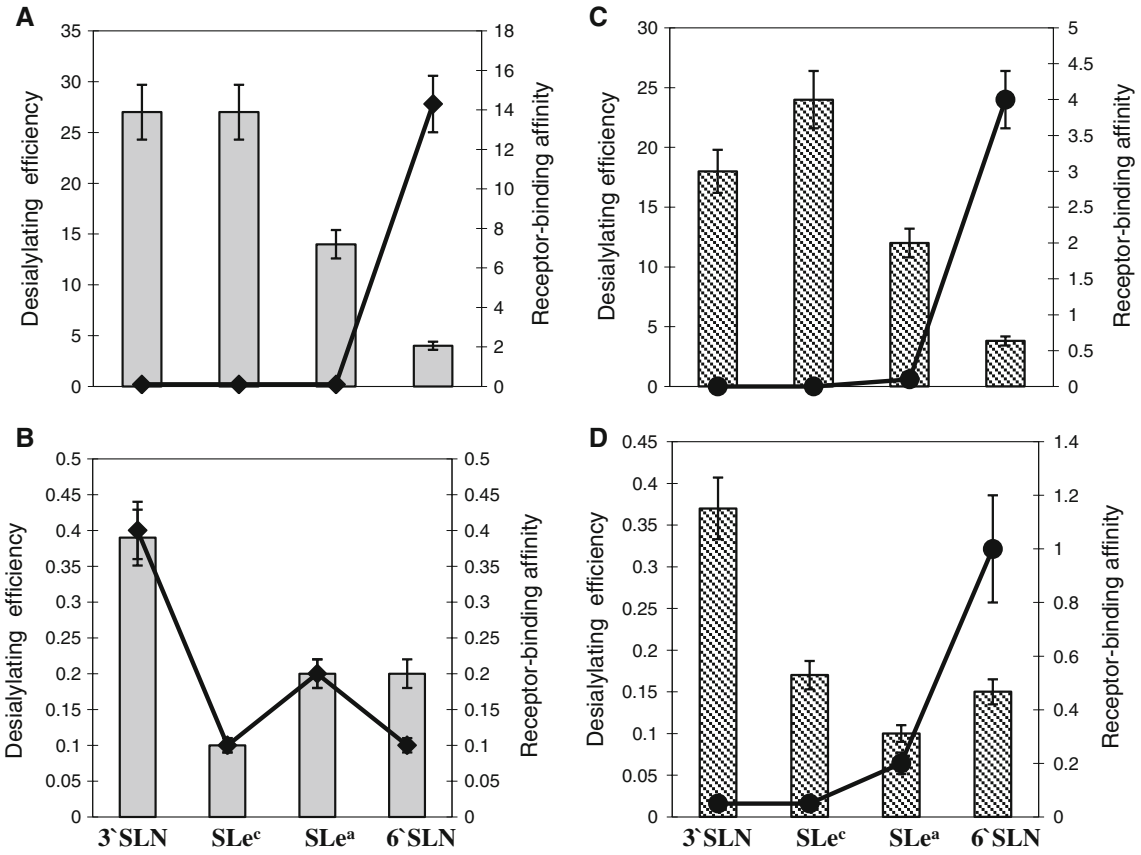

Fig. 1 Receptor-binding (*line*) and neuraminidase (*bars*) substrate specificities of Mem/20 (**A**), Mem/20–152K (**B**), HK/45 (**C**), and HK/36 (**D**) influenza viruses. Data presented for NA hydrolytic activity are mean values of $[(10^3)\times(V_0/S_0)] \pm$ SD, per minute, calculated for 30 HAU of virus. Data presented for HA affinity are mean values of 1/[Neu5Ac] ± SD, μM^{-1}

the HA (Fig. 2). Three of the four glycans are situated in or near by the regions forming RBS of influenza B virus HA [26]: 196Asn lies in the region of 190-helix, while Asn232 and Asn145 are in close proximity to 240- and 140-loops, respectively. It was shown [12, 27, 28] that sialic acid moieties on HA glycans situated in the vicinity of the RBS can reduce efficiency of the HA binding to a receptor and decrease viral dependence on the NA activity necessary for replication. Interference with haemadsorption by sialic acids of the HA carbohydrates located near the RBS was

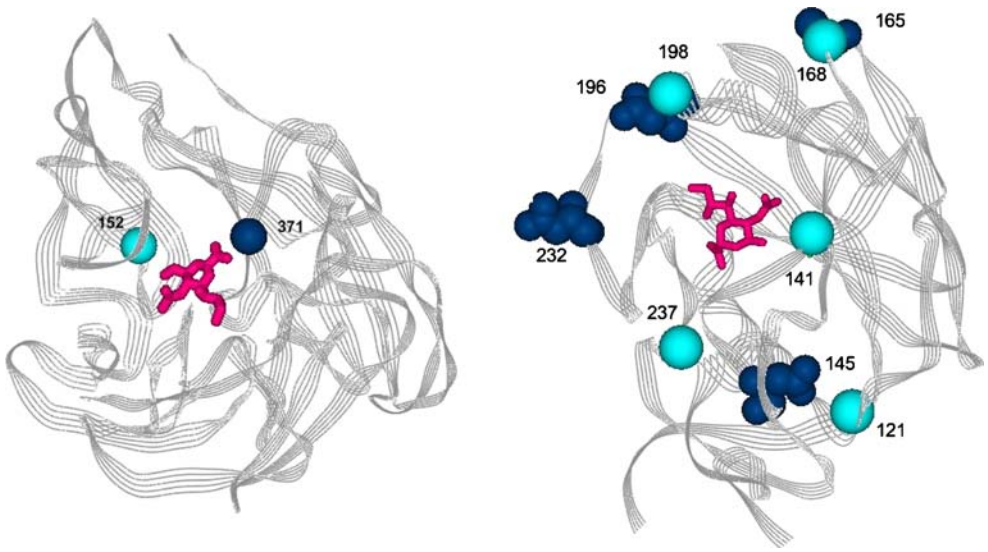

Fig. 2 Three-dimensional models of influenza B virus NA (on the left, PDB ID 1A4G) and HA (on the right, PDB ID 2RFU) molecules in complex with Neu5Ac (DS ViewerPro 5.0, Accelrys Inc. software). Positions of amino acid substitutions in drug-resistant mutants of influenza B viruses are shown by light circles (light-blue in online version) for the HA and NA of Mem/20-R152K, and dark circle (dark-blue in online version) for the HK/36 NA. Positions of potential glycosylation sites near the RBS of the HA of HK/36 and HK/45 are shown by CPK (dark-blue in online version)

observed for influenza B viruses [29]. Gubareva *et al*. [30] demonstrated that presence of terminal neuraminic acid moieties on the HA of the mutant influenza A virus, whose genome had lacked the coding capacity for NA active site, reduced the HA receptor-binding efficiency. Thus, sialylation of the HA carbohydrate chains served as a compensatory mechanism for the loss of NA activity. In addition, the nature of HA carbohydrate chains may affect the HA receptor-binding specificity [18, 23, 31, 32]. Therefore, different receptor-binding properties of HK/36 and HK/45 viruses could be explained by differences in structures and properties of their NAs.

Conclusion

It is known that the receptor-binding specificity of an influenza A virus is determined by the structure of host glycans presented on the surface of target cells as well as by natural inhibitors of receptor binding [8]. The majority of human influenza A viruses interact strictly with the trisaccharide 6`SLN [18, 31, 33], while influenza B viruses bind equally 6`SLN and 6`SL (Neu5Acα2-6Galβ1-4Glc) [31; Mochalova, Katinger *et al*. *unpublished*].

NAs of influenza A viruses isolated from various species display different ability to discriminate α2-3 and α2-6 linkages between Neu5Ac and Gal in oligosaccharides [19, 34, 35]. Human influenza A virus NAs hydrolyze α2-3-trisaccharides more efficiently than the α2-6 isomers and, furthermore, are capable of distinguishing fucosylated/non-fucosylated sialo-oligosaccharides and the structure of sialylated carbohydrate core [11, 19, 34].

Here, we studied the receptor-binding and NA substrate specificities of four influenza B viruses, two of which were sensitive and two others resistant to NAIs. The drug resistant mutants, Mem/20–152K and HK/36 acquired their resistance in the course of treating of influenza infections with NAI [15, 17]. In general, oligosaccharide specificities of the HA and NA do not match for wild type viruses: the HA receptor, 6`SLN, is not a good substrate of the influenza virus NAs and, vice versa, the α2-3-trisaccharides, preferable substrates for the influenza virus NAs, are not receptors for the corresponding HA. In fact, receptor-binding and substrate specificities have antagonistic trends in two wild type influenza B viruses studied here, as it was observed earlier for human influenza A viruses [19, 31, 36]. Such HA-NA functional specificity for oligosaccharides seems to be favorable for successful circulation of influenza viruses in human population [37]. Why influenza viruses adapted to humans maintain the α2-3-specificity of the NA along with the α2-6 one? It may be related to different activities that NA performs during the influenza virus reproduction circle: at the initial step, it desialylates α2-3-sialylated glycans inhibiting virus-cell interaction [38], whereas at the final step, it facilitates releasing of mature virions by destroying cell-surface receptors (α2-6-sialylated chains of human epithelium cells) holding virus HAs [8].

Oligosaccharide specificities of the HA and the NA of both drug-resistant influenza B mutants studied here seem to have better match. Their NAs hydrolyzed α2-3 sialo-oligosaccharides, while their HAs displayed an ability to bind to these sialosides. In spite of the fact that NAs and HAs of these drug-resistant mutants had very low activities as a whole, they work in unison in the sense of their receptor-binding/NA substrate specificities (especially in case of Mem-20–152K). Such combination of the HA-NA oligosaccharide specificities in the drug-resistant mutants seems to reduce the negative effect of a critical mutation in the NA and helps to the mutants to survive. In fact, two ways of restoration of HA-NA functional balance destroyed during selection in the presence of NAIs have been observed. The serious damage in the active site of the Mem/20–152K mutant NA was compensated by amino acid changes in the HA and, as a result, in its receptor-binding properties. In the case of HK/36 virus, a single mutation in the NA seems to be responsible for the change in the HA receptor-binding specificity.

Thus, a readjustment of the HA-NA functional specificity for receptor-substrate provided successful replication of drug-resistant mutants in MDCK cells and, apparently, in a patient, in spite of drastic changes in the NA active site. The shift in oligosaccharide specificities of the NA and, especially, of the HA allows us to speculate that viruses acquiring resistance to NAIs have a potential for changing their cell tropism. Such a possibility requires comprehensive strategy for drug use as well as monitoring of receptor-binding and NA substrate specificities in drug-resistant viruses.

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References

1. Wagner, R., Matrosovich, M., Klenk, H.-D.: Functional balance between hemagglutinin and neuraminidase in influenza virus infections. Rev. Med. Virol. **12**, 159–166 (2002)
2. Matrosovich, M., Matrosovich, T., Gray, T., Roberts, N.A., Klenk, H.-D.: Neuraminidase is important for the initiation of influenza virus infection in human airway epithelium. J. Virol. **78**, 12665–12667 (2004)
3. Kaverin, N.V., Gambaryan, A.S., Bovin, N.V., Rudneva, I.A., Shilov, A.A., Khodova, O.M., Varich, N.L., Sinitsin, B.V., Makarova, N.V., Kropotkina, E.A.: Postreassortment changes in

influenza A virus hemagglutinin restoring HA-NA functional match. Virology **244**, 315–321 (1998)
4. Baigent, S.J., McCauley, J.W.: Glycosylation of haemagglutinin and stalk-lenth of neuraminidase combine to regulate the growth of avian influenza viruses in tissue culture. Virus Res. **79**, 177–185 (2001)
5. Mitnual, L.J., Matrosovich, M.N., Castrussi, M.R., Tuzikov, A.B., Bovin, N.V., Kobasa, D., Kawaoka, Y.: Balanced hemagglutinin and neuraminidase activities are critical for efficient replication of influenza A virus. J. Virol. **74**, 6015–6020 (2000)
6. Rudneva, I.A., ll'yushina, N.A., Shilov, A.A., Varich, N.L., Sinitsyn, B.V., Kropotkina, E.A., Kaverin, N.V.: Functional interaction of the influenza virus glycoproteins. Mol. Biol. (Mosk.) **37**, 31–36 (2003)
7. Gimsa, U., Grötzinger, I., Gimsa, J.: Two evolutionary strategies of influenza viruses to escape host non-specific inhibitors: alteration of hemagglutinin or neuraminidase specificity. Virus Res. **42**, 127–135 (1996)
8. Matrosovich, M.N., Klenk, H.-D., Kawaoka, Y.: Receptor specificity, host-range, and pathogenicity of influenza viruses. In: Kawaoka, Y. (ed.) Influenza virology: Current topics. Caister Academic, Norfolk (2006)
9. Gubareva, L.V., Hyden, F.G.: M_2 and neuraminidase inhibitors: anti-influenza activity, mechanisms of resistance, and clinical effectiveness. In: Kawaoka, Y. (ed.) Influenza virology: Current topics. Caister Academic, Norfolk (2006)
10. Ilyushina, N.A., Rudneva, I.A., Shilov, A.A., Klenk, H.-D., Kaverin, N.V.: Postreassortment changes in a model system: HA-NA adjustment in an H3N2 avian-human reassortant influenza virus. Arch. Virol. **150**, 1327–1338 (2005). doi:10.1007/s00705-005-0490-4
11. Shtyrya, Y., Mochalova, L., Voznova, G., Rudneva, I., Shilov, A., Kaverin, N., Bovin, N.: Adjustment of receptor-binding and neuraminidase substrate specificities in avian-human reassortant influenza viruses. Glycoconj. J. **26**, 99–109 (2009)
12. Wagner, R., Wolf, T., Herwig, A., Pleschka, S., Klenk, H.-D.: Interdependence of hemagglutinin glycosilation and neuraminidase as regulators of influenza growth: a study by reverse genetics. J. Virol. **74**, 6316–6323 (2000)
13. Hughes, M., Matrosovich, M., Rodgers, M., McGregor, M., Kawaoka, Y.: Influenza A viruses lacking sialidase activity can undergo multiple cycles of replication in cell culture, eggs, or mice. J. Virol. **74**, 5206–5212 (2000)
14. Gulati, S., Smith, D.F., Air, J.M.: Deletions of neuraminidase and resistance to oseltamivir may be a consequence of restricted receptor specificity in recent H3N2 influenza viruses. Virology J. **6**, 22 (2009). doi:10.1186/1743-422X-6-22
15. Sheu, T.G., Deyde, V.M., Okomo-Adhiambo, M., Garten, R.J., Xu, X., Bright, R.A., Butler, E.N., Wallis, T.R., Klimov, A.I., Gubareva, L.V.: Surveillance for neuraminidase inhibitor resistance among human influenza A and B viruses circulating worldwide from 2004 to 2008. Antimicrob. Agents Chemother. **52**, 3284–3292 (2008). doi:10.1128/AAC.00555-08
16. Dharan, N.L., Gubareva, L.V., Meyer, J.J., Okomo-Adhiambo, M., *et al.*: Infections with oseltamivir-resistant influenza A(H1N1) virus in the United States. JAMA (2009). doi:10.1001/jama.2009.294
17. Gubareva, L.V., Matrosovich, M.N., Brenner, M.K., Bethell, R.C., Webster, R.G.: Evidence for zanamivir resistance in an immunocompromised child infected with influenza B virus. J. Infect. Dis. **178**, 1257–1262 (1998)
18. Mochalova, L., Gambaryan, A., Romanova, J., Tuzikov, A., Chinarev, A., Katinger, D., Katinger, H., Egorov, A., Bovin, N.: Receptor-binding properties of modern human influenza viruses primarily isolated in Vero and MDCK cells and chicken embryonated eggs. Virology **313**, 473–480 (2003)
19. Mochalova, L., Kurova, V., Styrya, Y., Korchagina, E., Gambaryan, A., Belyanchikov, I., Bovin, N.: Oligosaccharide specificity of influenza H1N1 virus neuraminidases. Arch. Virol. **152**, 2047–2057 (2007)
20. Mochalova, L.V., Korchagina, E.Y., Kurova, V.S., Styria, J.A., Gambaryan, A.S., Bovin, N.V.: Fluorescent assay for studying the substrate specificity of neuraminidase. Analyt. Biochem. **34**, 190–193 (2005)
21. Janakiraman, M.N., White, C.L., Laver, W.G., Air, G.M., Luo, M.: Structure of influenza virus neuraminidase B/Lee/40 complexed with sialic acid and dehydro analog at 1.8 Å resolution: implications for the catalytic mechanism. Biochem. **33**, 8172–8179 (1994)
22. Matrosovich, M.N., Mochalova, L.V., Marinina, V.P., Byramova, N.E., Bovin, N.V.: Synthetic polymeric sialoside inhibitors of influenza virus receptor-binding activity. FEBS Lett. **272**, 209–212 (1990)
23. Gambarayn, A.S., Marinina, V.P., Tuzikov, A.B., Bovin, N.V., Rudneva, I.A., Sinitsin, B.V., Shilov, A.A., Matrosovich, M.N.: Effect of host-dependent glycosylation of hemagglutinin on receptor-binding properties of H1N1 human influenza A virus grown in MDCK cells and in embryonated eggs. Virology **247**, 170–177 (1998)
24. Gambaryan, A., Robertson, J.S., Matrosovich, M.N.: Effect of egg-adaptation on the receptor-binding properties of human influenza A and B viruses. Virology **258**, 232–239 (1999)
25. Xu, G., Horiike, G., Suzuki, T., Miyamoto, D., Kumihashi, H., Suzuki, Y.: A novel strain, B/Gifu/2/73, differs from other influenza B viruses in the receptor binding specificities toward sialo-sugar chain linkage. Biochem. Biophys. Res. Communs **224**, 815–818 (1996)
26. Wang, Q., Tian, X., Chen, X., Ma, J.: Structural basis for receptor specificity of influenza B virus hemagglutinin. Proc. Nat. Acad. Sci. **104**, 16874–16879 (2007)
27. Ohuchi, M., Ohuchi, R., Feldmann, A., Klenk, H.-D.: Regulation of receptor binding affinity of influenza virus hemagglutinin by its carbohydrate moiety. J. Virol. **71**, 8377–8384 (1997)
28. Mishin, V.P., Novikov, D., Hayden, F.G., Gubareva, L.V.: Effect of hemagglutinin glycosylation on influenza virus susceptibility to neuraminidase inhibitor. J. Virol. **79**, 12416–12424 (2005)
29. Luo, C., Nobusawa, E., Nakajima, K.: An analysis of the role of neuraminidase in the receptor-binding activity of influenza B virus: the inhibitory effect of zanamivir on haemadsorption. J. Gen. Virol. **80**, 2969–2976 (1999)
30. Gubareva, L.V., Nedyalkova, M.S., Novikov, D.V., Murti, K.G., Hoffmann, E., Hayden, F.G.: A release-competent influenza A virus mutant lacking the coding capacity for the neuraminidase active site. J. Gen. Virol. **83**, 2683–2692 (2002)
31. Gambaryan, A.S., Tuzikov, A.B., Piskarev, V.E., Yamnikova, S.S., Lvov, D.K., Robertson, J.S., Bovin, N.V., Matrosovich, M.N.: Specification of receptor-binding phenotypes of influenza virus isolates from different hosts using synthetic sialylglycopolym**ers**: non-egg-adapted human H1 and H3 influenza A and influenza B viruses share a common high binding affinity for 6`-sialyl (N-acetyllactosamine). Virology **232**, 345–350 (1997)
32. Romanova, J., Katinger, D., Ferko, B., Voglauer, R., Mochalova, L., Bovin, N., Lim, W., Katinger, H., Egorov, A.: Distinct host range of influenza H3N2 virus isolates in Vero and MDCK cells is determined by cell specific glycosylation pattern. Virology **307**, 90–97 (2003)
33. Kumari, K., Gulati, S., Smith, D.F., Gulati, U., Cummings, R.D., Air, G.M.: Receptor-binding specificity of recent human H3N2 influenza viruses. Virology J. **4**, 42 (2007). doi:10.1186/1743-422X-4-42
34. Kobasa, D., Kodihalli, S., Luo, M., Castrucci, M.R., Donatelli, I., Suzuki, Y., Suzuki, T., Kawaoka, Y.: Amino acid resides

contributing to the substrate specificity of the influenza A virus neuraminidase. J. Virol. **73**, 6743–6751 (1999)
35. Shtyrya, Y., Mochalova, L., Gambarayn, A., Korchagina, E., Xu, X., Klimov, A., Bovin, N.: Neuraminidases of H9N2 influenza viruses isolated from different hosts display various substrate specificity. In: Options for the control of influenza VI, pp. 64–65. International Medical, Atlanta (2008). Toronto, Ontario, Canada, 17–23 June 2007
36. Barros Jr., J.F., Alviano, D.S., Silva, M.H., Wigg, M.D., Alviano, C.S., Schauer, R., Couceiro, J.N.S.S.: Characterization of sialidase from an influenza A (H3N2) virus strain: kinetic parameters and substrate specificity. Intervirology **46**, 199–206 (2003). doi:10.1159/000072428
37. Gulati, U., Wu, W., Gulati, S., Kumari, K., Waner, J.L., Air, J.M.: Mismatched hemagglutinin and neuraminidase specificities in recent human H3N2 influenza viruses. Virology **339**, 12–20 (2005). doi:10.1016/j.virol.2005.05.009
38. Matrosovich, M., Klenk, H.-D.: Natural and synthetic sialic acid-containing inhibitors of influenza virus receptor-binding. Rev. Med. Virol. **13**, 85–97 (2003). doi:10.1002/rmv.372