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Adjustment of receptor-binding and neuraminidase substrate specificties in avian–human reassortant influenza viruses

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Abstract Balanced action of hemagglutinin (HA) and neuraminidase (NA) is an important condition of influenza virus efficient replication, but a role of HA and NA specificities at oligosaccharide level in maintaining such a balance remains poorly studied. Avian virus HA binds exclusively and NA digests efficiently α2–3-sialylated carbohydrate chains, while human virus HA interacts with α2–6 chains and low-active NA cleaves both α2–3- and α2–6-sialosides. Reassortment between viruses leading to appearance of avian virus HA and human virus NA on the virion surface often resulted in decreasing the replicative potential of the formed variants because of disturbance of a functional balance between "alien" HA and NA. A restoration of the reassortant productivity happened due to the appearance of amino acid substitutions in HA and, sometimes, NA. Here, a role of NA and HA oligosaccharide specificities in a restoration of HA–NA functional balance in high-yield passage variants was studied. Post-reassortment changes in HA receptor-binding and NA substrate specificities for three reassortant/passage variant virus pairs towards 3′SiaLac, 3′SiaLacNAc, SiaLe^c, SiaLe^a, SiaLe^x, 6′SiaLac, and 6′SiaLacNAc were determined. Selection of the high-yield variants of the human-avian reassortants led either to twofold decrease in the affinity of HA for most α2–3-sialosides and the appearance of affinity for α2–6-sialosides (H3N2 reassortant), or to decreasing the HA affinity for SiaLe^c and SiaLe^a (H3N1 reassortant), or to enhancing the ability of NA to discriminate between α2–3/2–6 substrates (H4N1 reassortant). Thus, all postreassortment changes in oligosaccharide specificities of "alien" HA and NA were directed towards their adjustment to each other, but by different manner.

Keywords Sialoglycoconjugates · Influenza virus · Hemagglutinin · Neuraminidase · Oligosaccharide specificity

Abbreviations

biot	biotin
BODIPY	4,4-difluoro-5,7-dimethyl-4-bora-3a, 4a-diaza-s-indacene-3-propionic acid
BSA	bovine serum albumin
HA	hemagglutinin
HAU	hemagglutinating unit
K_d	dissociation constant
K_{aff}	affinity constant
NA	neuraminidase
Neu5Ac	N-acetylneuraminic acid
OS	oligosaccharide

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PAA	polyacrylamide
S_0	initial substrate concentration
V_0	initial rate of enzymatic hydrolysis
3′SiaLac	Neu5Acα2–3Galβ1–4Glc
3′SiaLacNAc	Neu5Acα2–3Galβ1–4GlcNAc
6′SiaLac	Neu5Acα2–6Galβ1–4Glc
6′SiaLacNAc	Neu5Acα2–6Galβ1–4GlcNAc
$SiaLe^c$	Neu5Acα2–3Galβ1–3GlcNAc
$SiaLe^a$	Neu5Acα2–3Galβ1–3 (Fucα1–4)GlcNAc
$SiaLe^x$	Neu5Acα2–3Galβ1–4 (Fucα1–3)GlcNAc
6Su-3′SiaLacNAc	Neu5Acα2–3Galβ1–4 (6-HSO_3)GlcNAc
6Su-6′SiaLacNAc	Neu5Acα2–6Galβ1–4 (6-HSO_3)GlcNAc
TN buffer	0.02 M tris-HCl, pH 7.2, with 0.1 M NaCl

Introduction

The spike glycoproteins of influenza A and B viruses, hemagglutinin (HA) and neuraminidase (NA), fulfill distinct functions during the viral reproduction cycle. Both proteins interact with sialylated carbohydrate chains. HA binds sialic acid-containing cell receptors mediating the virus absorption on the target cells and the fusion of the virus envelope with cell membrane. NA cleaves off the sialic acid residues from viral and cellular carbohydrates, preventing the formation of virion aggregates and promoting the release of virus progeny from the host cell [12, 19]. If the function of NA is impaired, sialyl residues persist at the surface of virus particles and infected cells, and they can be bound by the HA of virus particles, which leads to virus aggregation at the cell surface preventing the dissemination of infection [15, 19]. Thus, the functional fitness of HA and NA to each other is prerequisite of successful influenza virus replication.

The analysis of natural avian influenza viruses revealed that some combinations of HA and NA antigenic subtypes occurred frequently, while others were rare, or not detected at all [8]. The necessity of the functional match between the receptor-binding affinity of HA and the enzymatic activity of NA was shown to be critical for influenza virus replication [15, 29]. It has been established that the receptor-binding specificity of HA, as well as the substrate specificity of NA, is dependent on the species of origin [2, 9, 14, 18]. Thus, at least two parameters may determine functional compatibility of HA and NA in viable influenza virus, namely their activities (HA affinity for receptor–NA enzymatic activity) and their oligosaccharide specificities (HA receptor-binding specificity–NA substrate specificity). Whereas the role of activity was studied [30], the role of fine specificity has not been evaluated yet.

In order to analyze the functional match of HA and NA, in our previous studies a set of reassortant viruses, containing the *NA* gene from the human virus and the *HA* gene of different avian virus strains belonging to subtypes H2, H3, H4, H10, and H13, was obtained [7, 22]. The avian HA-human NA reassortants replicated poorly in embryonated chicken eggs due to the formation of virus aggregates: the avian-type HA, displaying high affinity for the receptor, bound sialylated carbohydrate chains of other virus particles, because human-type NA activity, which is lower than the avian-type one, was insufficient for desialylating them. After serial passages in embryonated chicken eggs non-aggregating variants, which replicated as efficiently as the parent viruses, were selected [23]. The HA of the passaged variants had acquired mutations leading to a decreased affinity for sialic acid-containing receptors and thereby lowering the tendency to virion aggregation [6, 7]. These studies revealed that the functional match between HA and NA can be restored by modulating the HA binding affinity in order to compensate for insufficient NA activity. However, our attention had been focused on the adjustment of activity of the avian-type HA to human-type NA [6, 7], whereas changes in fine oligosaccharide specificity of neither HA nor NA of the non-aggregating variants of reassortants were analyzed.

In the present study the H3N1, H4N1 and H3N2 avian HA-human NA influenza virus reassortants and their passage variants having amino acid substitutions in NA and HA were compared with respect to the HA receptor-binding and NA substrate specificity using a wide range of sialo-oligosaccharides. The results suggest that the changes in the *NA* along with changes in *HA* genes play a role in the restoration of the HA-NA functional match at oligosaccharide level.

Materials and methods

Materials

DEAE-Toyopearl 650S was from Tosoh (Japan). The 10-μl pipette tips with filter (Cat. # F108-96R-10), 8-strip thin-wall 0.2 ml polymerase chain reaction (PCR) tubes with caps (Cat. #435) were from Porex (USA). Black polystyrene 96-well microtiter plates (Cat. #437111) were purchased from Nunc (Denmark). Polyvinylchloride 96-well microtiter plates (Cat. #2595) were purchased from Corning. Synthesis of 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid (BODIPY)-labeled and polyacrylamide-coupled oligosaccharides was described earlier [18, 20, 21, 27].

Viruses

The generation and genotypic characterization of reassortant viruses R8/DK-RAi, R2, RCB1, PX8, and RWB1, containing *HA* gene of avian influenza virus and *NA* gene of human influenza virus, and their long-passage variants R8/DK-RAi-XII, R2-XXI, RCB1-XXI, PX8-XII, and RWB1-XII were described earlier [5–7, 22, 23]. The roman figures designate the number of egg passages used for the selection of the variant. The reassortants R2, RCB1, PX8, and RWB1 have the *HA* genes derived respectively from A/duck/Ukraine/63 (H3N8), A/duck/Czechoslovakia/56 (H4N6), A/Pintail duck/Primorie/695/76 (H2N3), and A/Pilot Whale/Maine/328/84 (H13N2) viruses, and the *NA* gene of the A/USSR/90/77 (H1N1) virus. So, the combinations of HA and NA in the reassortants were as follows: H3N1 in R2, H4N1 in RCB1, H2N1 in PX8, and H13N1 in RWB1. The reassortant R8/DK-RAi has the *HA* gene of A/duck/Ukraine/63 (H3N8) virus and the *NA* gene of A/Aichi/2/68 (H3N2) virus and, consequently, belongs to H3N2 subtype. The egg-adapted human influenza A/Aichi/2/68 (H3N2) virus was obtained from Virus Collection of the D. I. Ivanovsky Institute of Virology RAMS. The viruses were propagated in 10-old embryonated chicken eggs, concentrated and purified as described earlier [6]. Viral titers were determined in a hemagglutination reaction assay with 0.5% suspension of human red blood cells (group H (0), Rh^-). All the viruses were diluted to 16 hemagglutinating units (HAU) for the receptor-binding assays and 8 or 16 HAU for determining NA substrate specificity for the α2–3 or α2–6 sialosides, respectively.

Receptor-binding specificity assay

Receptor-binding specificity of influenza viruses towards the sialo-oligosaccharides conjugated to biotin-labeled polyacrylamide (OS-PAA-biot) was evaluated in solid phase assay with streptavidin-biotin detection system as described in our earlier publication [16] with minor modifications. Plates were coated with purified influenza virus (50 μl/well) at 4°C for 16 h followed by washing with TN buffer (0.02 M tris-HCl, pH 7.2, with 0.1 M NaCl). After that 100 μl/well of blocking solution (TN buffer containing 0.5% of bovine serum albumin (BSA)) was added, then plates were kept at room temperature for 90 min and washed with TN buffer containing 0.05% of Tween-20. After the addition of OS-PAA-biot, 45 μl/well, in the working buffer (TN buffer with 0.02% of Tween-20, 0.1% of BSA and 10 μM of neuraminidase inhibitor, 2,3-didehydro-2,4-dideoxy-4-amino-*N*-acetyl-D-neuraminic acid), plates were allowed to stay at 4°C for 1.5 h. The starting concentration of OS-PAA-biot was 20 μM on sialic acid; the following twofold serial dilutions were used. Plates were washed and incubated with streptavidin-peroxidase in the working buffer at 4°C for 1 h. After washing, 50 μl/well of substrate solution (0.1 M sodium acetate, pH 5.0, containing 4 mM *o*-phenylenediamine and 0.004% H_2O_2) was added and the reaction was stopped with 2 M H_2SO_4. Optical density was determined at 492 nm with a Multiscan plate reader (Labsystems, Finland). The K_d was determined as Neu5Ac concentration at the point $A_{max}/2$ of Scatchard plot.

Here, data on receptor-binding specificity are presented as affinity constants ($K_{aff} = 1/K_d$), calculated on the basis of results of five independent experiments.

NA substrate specificity assay

Procedure of kinetics evaluation of influenza virus NA specificity towards BODIPY-labeled oligosaccharides was described earlier [18]. Three dilutions of each OS-BODIPY substrate (10–50 μM) were prepared in 0.1 M Na-acetate buffer, pH 5.0, with 10 mM $CaCl_2$. Twelve microliters of each solution were placed in a stripped microtube and 8 μl of virus suspension per tube was added simultaneously, using a multichannel pipette. The tubes were incubated at 37°C. Every 10 min, 5-μl aliquots were taken from the strips and transferred into the stripped microtubes containing 45 μl/tube of water. The diluted reaction mixtures were heated at 70°C for 10 min and analyzed. DEAE-Toyopearl microcartridges were used for separation of BODIPY-labeled reaction product and non-reacted substrate [17]. Ten microliters of a diluted reaction mixture were placed on the microcartridge. The microcartridge was washed with water (3×150 μl), and the eluate was collected into three wells of the black plate. The microcartridge was then washed with 0.5 M sodium acetate buffer (3×150 μl), and the eluate was collected into the next three wells. Fluorescence was detected using a Victor^2 reader (Wallac, Perkin-Elmer, USA) at 480/520 nm. Summing up the values of aqueous eluates gives the yield of the formed product (I_p), whereas the same procedure for buffer eluates gives the amount of a non-reacted substrate (I_s). The yield of the enzymatic reaction and concentration of obtained product were calculated from the equations $(I_P \times 100)/(I_P + I_S)$ and $(I_P \times S_0)/(I_P + I_S)$, respectively, where S_0 is an initial molar concentration of the substrate. Analysis of each reaction mixture was performed in three replicates.

Virus NA specificity for each sialoside was calculated as the slope of the starting linear region of the V_0 against of S_0 kinetic curve; that is, as the value of V_0/S_0 at $S_0 << K_M$ [18]. Each reaction mixture was analyzed no less than three times. The data are presented as the mean value of V_0/S_0, calculated on a per-HAU basis in three independent experiments.

PCR amplification and sequencing

Viral RNA was isolated from virus-containing allantoic fluid by using the RNeasy Mini kit (Qiagen) as specified by the manufacturer. Reverse transcription of viral RNA and subsequent PCR was performed using primers specific for the *NA* gene segment (primer sequences are available upon request). PCR products were purified with the QIAquick PCR purification kit (Qiagen). The DNA template was sequenced using a DNA ABI Prism 3130 sequencer (Applied Biosystems) and BigDye Terminator v3.1 kit; DNA sequences were completed and edited by using DNASTAR sequence analysis software package (DNASTAR Inc.).

Nucleotide sequence accession numbers

The nucleotide sequence accession numbers obtained in this study have been deposited in the GenBank database under accession numbers EU643636 to EU643639.

Results

Amino acid changes in NA

The *NA* genes of N1 subtype in the low-yield reassortants R2 (H3N1), RCB1 (H4N1), PX8 (H2N1) and RWB1 (H13N1), and the *NA* genes of their non-aggregating passage variants (R2-XXI, RCB1-XXI, PX8-XIII, and RWB1-XII) were sequenced. In the NA of the passage variants PX8-XIII and RWB1-XII, the sequencing revealed no amino acid substitutions as compared to their respective initial reassortants. The amino acid changes were revealed in the NA of the passage variants R2-XXI (Asp79Val and Ser366Asn) and RCB1-XXI (Leu206Ile) as compared to their initial reassortants R2 (H3N1) and RCB1 (H4N1), respectively. In our previous report [5] the change Glu83Gly was revealed in the NA of R8/DK-RAi-XII, a passage variant of the H3N2 reassortant virus. The other four passage variants of the avian–human H3N2 reassortant contained no substitutions in NA.

The HA and NA oligosaccharide specificities of the passage variants having amino acid substitutions in the NA (Table 1), namely R8/DK-RAi-XII, R2-XXI and RCB1-XXI, as well as the corresponding initial reassortants R8/DK-RAi (H3N2), R2 (H3N1) and RCB1 (H4N1), were analyzed.

Glycoconjugates and approaches used for the evaluation of HA and NA oligosaccharide specificities

To study receptor-binding and NA substrate specificities of influenza viruses a set of synthetic sialo-oligosaccharides, most of which are typical for mammalian and avian carbohydrate chains, was used. The oligosaccharides differed by: (1) the type of linkage between Neu5Ac and Gal residues (α2–3 or α2–6, *i.e.* 3′SiaLac and 3′SiaLacNAc vs. 6′SiaLac and 6′SiaLacNAc, respectively); (2) the presence or absence of *N*-acetyl group at position 2 of Glc residue, *i.e.* SiaLacNAc vs. SiaLac; (3) the bond (1–4 or 1–3) between Gal and GlcNAc, *i.e.* 3′SiaLacNAc vs. $SiaLe^c$; (4) the presence or absence of fucose residue at GlcNAc ($SiaLe^a$ and $SiaLe^x$ are fucosylated derivatives of $SiaLe^c$ and 3′SiaLacNAc, respectively); (5) the presence or absence of sulfate substituent at GlcNAc 6-OH position in composition of 3′SiaLacNAc and 6′SiaLacNAc.

To study the influenza virus receptor-binding specificity the oligosaccharides were coupled via a spacer to high molecular mass polyacrylamide (about 1,500 kDa). The same sialo-oligosaccharides linked to the fluorescent label BODIPY were used for quantitative evaluation of NA substrate specificity [17]. Virus receptor-binding properties

Table 1 Avian–human influenza virus reassortant composition and amino acid substitutions found in HA1 and NA of non-aggregating variants of reassortants

Designation of reassortant virus	Parent viruses being the origin of		Reassortant antigenic subtype	Amino acid substitutions in the passaged variant	
	HA	NA		HA	NA
R8/Dk-RAi	A/duck/Ukraine/63 (H3N8)	A/Aichi/2/68 (H3N2)	H3N2	–	–
R8/Dk-RAi-XII				Ser205Tyr	Glu83Gly
R2	A/duck/Ukraine/63 (H3N8)	A/USSR/90/77 (H1N1)	H3N1	–	–
R2-XXI				Thr155Ser Asn248Asp Asn250Ser	Asp79Val Ser366Asn
RCB1	A/duck/Czechoslovakia/56 (H4N6)	A/USSR/90/77 (H1N1)	H4N1	–	–
RCB1-XXI				Asn160Asp Arg176Lys	Leu206Ile

were evaluated through the affinity constant ($1/K_d$) [16] and NA substrate specificity—through V_0/S_0 values (proportional to substrate specificity constant k_{kat}/K_M) [18]. The V_0/S_0 value depends on the amount of enzyme present, but when calculated for different substrates at the same enzyme concentration becomes a quantitative measure of its desialylation efficiency. Here, V_0/S_0 values were calculated on a per-HAU basis to compare oligosaccharide patterns of three pairs of initial reassortants and corresponding passaged variants. We did not analyze the absolute enzymatic activity of NA in the reassortants, because other factors than amino acid replacements in NA may affect hydrolytic activity (for example, different NA/HA ratio or different avidity of HAs in passaged variant vs. parent reassortant). The evaluation of absolute enzymatic activity needs special approaches allowing one to measure NA quantity in whole virion [10].

Functional effects of postreassortment changes occurring in HA and NA during selection were measured as difference in patterns of receptor-binding and substrate specificity of a passage variant vs. the original reassortant.

Receptor-binding specificity of HA

H3 HA viruses Avian HA-human NA reassortant viruses R8/DK-RAi (H3N2) and R2 (H3N1), containing the same *H3 HA* gene from influenza virus A/duck/Ukraine/1/63 (H3N8), but different *NA* genes (Table 1), displayed, quite expectedly, the same receptor-binding properties (Fig. 1). The H3 HA viruses were found to bind most efficiently 3′SiaLac, 3′SiaLacNAc, and SiaLe^c (K_{aff} 5.0 μM^{-1}); the affinity for SiaLe^a was two times lower (2.5 μM^{-1}), while for SiaLe^x and 6Su-3′SiaLacNAc it was more than six times lower (0.8 μM^{-1}). HA of both viruses bound neither 6′SiaLac nor 6′SiaLacNAc, though weak interaction with 6Su-6′SiaLacNAc was observed (0.13 μM^{-1}).

The passage variant of the H3N2 avian–human reassortant, R8/DK-RAi-XII, had the same affinity as the initial reassortant R8/DK-RAi for 3′SiaLac, 6Su-3′SiaLacNAc and 6Su-6′SiaLacNAc (5.0, 0.8 and 0.13 μM^{-1}, respectively), whereas the binding of the passage variant to the other α2–3 sialosides (3′SiaLacNAc, SiaLe^c, SiaLe^a, and SiaLe^x) was about two times lower as compared to the initial reassortant (2.2, 1.7, 1.3, and 0.3 μM^{-1}, respectively). The passage variant R8/DK-RAi-XII also acquired a low affinity for 6′SiaLac and 6′SiaLacNAc (0.17 and 0.04 μM^{-1}, respectively), which is a characteristic feature of receptor-binding pattern of human viruses. The latter was demonstrated by the receptor-binding pattern of the egg-adapted human virus A/Aichi/2/68 (H3N2; Fig. 1).

The passage variant of the H3N1 reassortant, R2-XXI, displayed lowered affinity for SiaLe^c (3.3 μM^{-1}), SiaLe^a (1.0 μM^{-1}) and 6Su-6′SiaLacNAc (0.04 μM^{-1}) in comparison with the parent R2 virus.

H4 HA viruses The avian–human-H4N1 reassortant virus, RCB1, and its passage variant, RCB1-XXI, demonstrated a high affinity for most α2–3-sialosides 3′SiaLac, 3′SiaLacNAc, SiaLe^c, and SiaLe^a (5.0 μM^{-1}). Their interaction with SiaLe^x and 6Su-3′SiaLacNAc was one order of magnitude weaker (0.5 μM^{-1}). HA of the passaged variant displayed six-fold decreased affinity for 6Su-6′SiaLacNAc compared to the parent reassortant (0.04 vs. 0.25 μM^{-1}). The H4 HA-containing viruses did not interact with the α2–6 sialosides.

NA substrate specificity

N1 NA viruses The reassortant viruses R2 and RCB1, containing the same NA of N1 subtype originating from the human virus A/USSR/90/77 (H1N1), but different *HA* genes (H3 in R2 and H4 in RCB1) displayed identical NA substrate specificity (Table 2), which corresponded to that for the parent virus A/USSR/90/77 (H1N1) [18]. Both viruses hydrolyzed efficiently 3′SiaLac ($2.69/2.96\times10^{-3}$ min^{-1} for R2 (3 HAU)/RCB1 (1HAU)), and slightly less efficiently 3′SiaLacNAc and SiaLe^c ($2.03/2.13\times10^{-3}$ and 1.95×10^{-3} min^{-1}, respectively). The desialylation of SiaLe^x and SiaLe^a proceeded respectively 1.3 and four times slower than the hydrolysis of the corresponding non-fucosylated sialosides, 3′SiaLacNAc and SiaLe^c. The hydrolysis of 6′SiaLac and 6′SiaLacNAc proceeded ~5 and ~4 times slower than that of the α2–3-isomers.

The NA of the passage H3N1 variant, R2-XXI, hydrolyzed (Fig. 2) the trisaccharides 3′SiaLac, SiaLe^c, and 3′SiaLacNAc with a higher efficiency (1.75×10^{-3}, 1.74×10^{-3}, and 1.57×10^{-3} min^{-1}, respectively) than the NA of the initial reassortant R2. Hydrolytic activity of the NA towards fucosylated substrates, SiaLe^x (0.59×10^{-3} min^{-1}) and SiaLe^a (0.40×10^{-3} min^{-1}), was 2.7 and 4.3 times lower than that for 3′SiaLacNAc and SiaLe^c, respectively, that is, the NA of the passage variant was more sensitive for the fucosylation of α2–3-sialosides than the NA of the initial variant. R2-XXI NA hydrolyzed 6′SiaLacNAc six times slower than 3′SiaLacNAc, thus, displaying slightly increased ability to discriminate between these isomers in comparison with R2 NA.

The NA of H4N1 passage variant, RCB1-XXI, preferentially hydrolyzed 3′SiaLac (3.53×10^{-3} min^{-1}) and 3′SiaLacNAc (3.25×10^{-3} min^{-1}), and slightly slower SiaLe^c (2.55×10^{-3} min^{-1}), that is, the efficiency of hydrolysis was higher than that for the initial reassortant RCB1 NA. The RCB1-XXI NA desialylated SiaLe^x (1.91×10^{-3} min^{-1}) about twofold slower than 3′SiaLacNAc, while SiaLe^a (0.56×10^{-3} min^{-1}) 4.6 times slower than SiaLe^c. The efficiency of the 6′SiaLac (0.48×10^{-3} min^{-1}) and 6′SiaLacNAc (0.34×10^{-3} min^{-1}) hydrolysis was about one order of magnitude lower than the corresponding α2–3

sialosides. Thus, the H4N1 passage variant NA displayed an increased ability to discriminate between α2–3 and α2–6 sialyloligosaccharides as compared to the NA of the initial reassortant.

N2 NA viruses NA of H3N2 reassortant virus R8/DK-RAi hydrolyzed efficiently 3′SiaLac (3.51×10^{-3} min^{-1}) and slightly slower 3′SiaLacNAc and SiaLe^c (2.40×10^{-3} and 2.25×10^{-3} min^{-1}, respectively). Hydrolytic activity towards other sialo-oligosaccharides was considerably lower. Desialylation of SiaLe^a (0.80×10^{-3} min^{-1}) and SiaLe^x (0.67×10^{-3} min^{-1}) proceeded three and 3.5 times slower than that of 3′SiaLacNAc and SiaLe^c, respectively. In the case of α2–3/2–6 sialosides the hydrolysis efficiency ratio was ~6 (the value of V_0/S_0 was 0.56×10^{-3} min^{-1} for 6′SiaLac and 0.40×10^{-3} min^{-1} for 6′SiaLacNAc).

As for the NA of the passage variant R8/DK-RAi-XII, it demonstrated a lower efficiency of hydrolysis of 3′SiaLac, 3′SiaLacNAc and SiaLe^c than the NA of the initial reassortant, although 3′SiaLac still was the preferential substrate

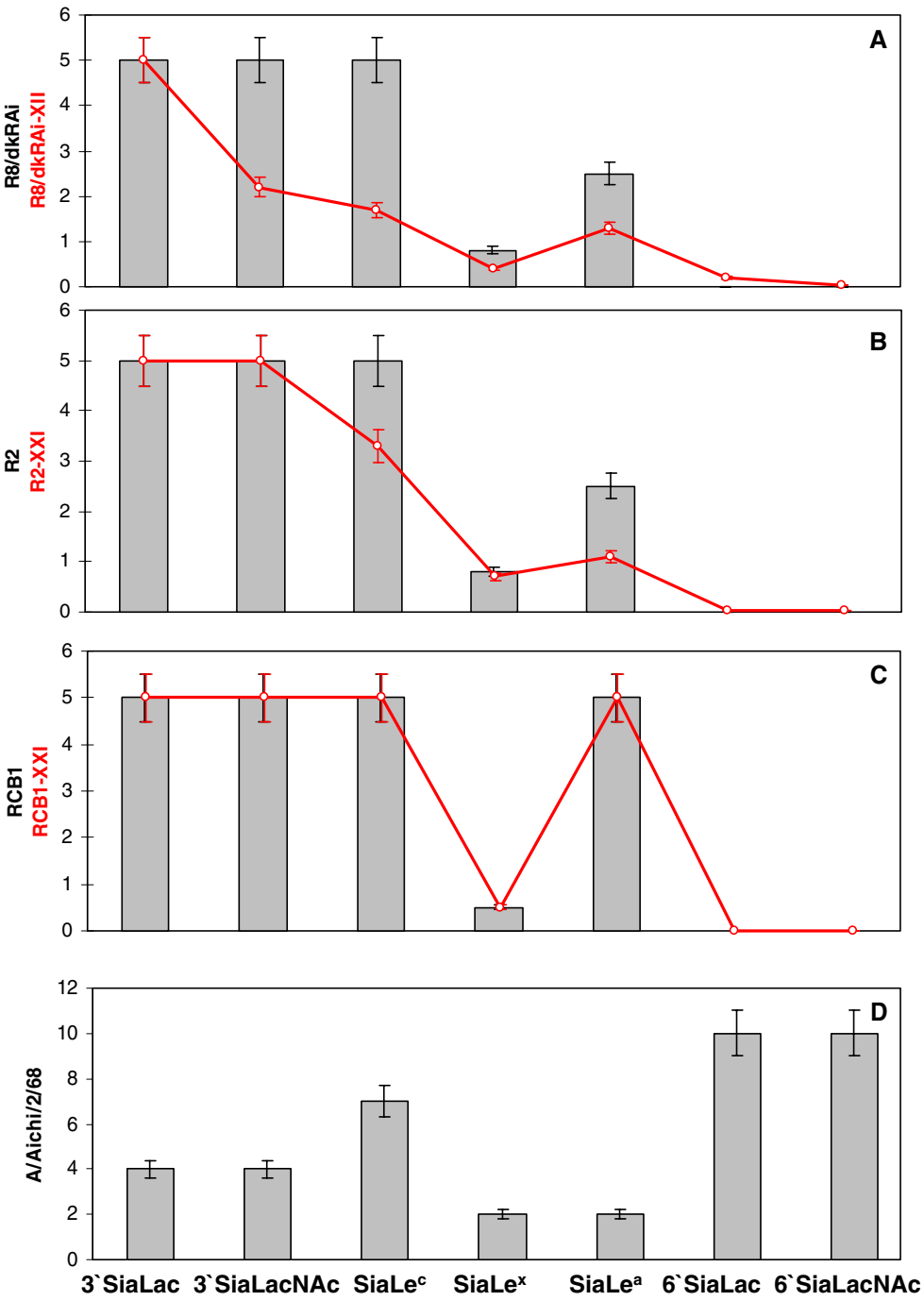

Fig. 1 Receptor-binding specificity of reassortant influenza viruses (*columns*) and their passaged variants (*line*). Data presented for the H3N2 reassortant R8/DK-RAi and its passage variant R8/DK-RAi-XII (**A**), H3N1 reassortant R2 and its passage variant R2-XXI (**B**), H4N1 reassortant RCB1 and its passage variant RCB1-XXI (**C**), and egg-adapted human influenza virus A/Aichi/2/68 (H3N2) (**D**) are mean values of K_{aff} ($1/K_d$) ± SD, μM^{-1}

Table 2 Substrate specificity of A/USSR/90/77 virus NA in reassortant viruses

BODIPY-labeled oligosaccharide	R2[a]	RCB1[a]
3′SiaLac	2.69±0.21	2.96±0.25
3′SiaLacNAc	2.03±0.20	2.13±0.18
SiaLe^c	1.95±0.12	1.95±0.15
SiaLe^x	1.32±0.13	1.59±0.16
SiaLe^a	0.54±0.05	0.50±0.05
6′SiaLac	0.57±0.05	0.53±0.05
6′SiaLacNAc	0.45±0.05	0.55±0.05

Values of $(V_0/S_0)\times 10^3$ are the mean of at least three independent experiments ± standard deviation.
[a] Virus dilutions in the reaction mixtures corresponded to 1 HAU for RCB1 and 3 HAU for R2.

(2.45×10^{-3} min^{-1}), and 3′SiaLacNAc and SiaLe^c were hydrolyzed slightly slower (1.89×10^{-3} and 1.60×10^{-3} min^{-1}, respectively). Desialylation efficiencies for SiaLe^x and SiaLe^a (0.75×10^{-3} and 0.80×10^{-3} min^{-1}) were about twofold lower than those for the corresponding non-fucosylated trisaccharides, and about fivefold lower for 6′SiaLac and 6′SiaLacNAc (0.4×10^{-3} min^{-1} for the both) than for α2–3 isomers.

Discussion

HA and NA oligosaccharide specificities in duck and human influenza viruses

Receptors for influenza A and B viruses are carbohydrate chains that contain terminal sialic acid (Sia) moieties linked

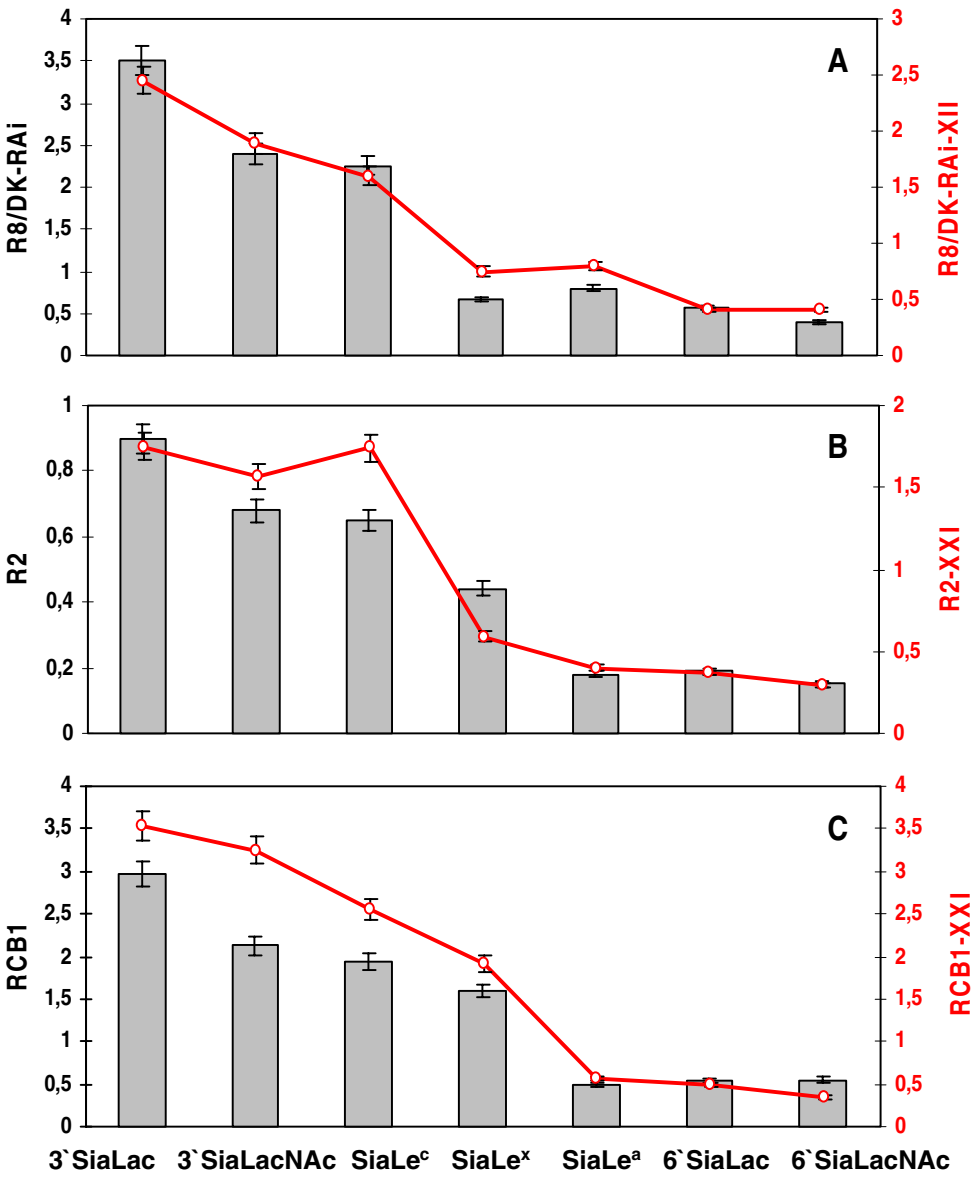

Fig. 2 Neuraminidase substrate specificity of reassortant influenza viruses (*columns*) and their passaged variants (*line*). Data presented for the H3N2 reassortant R8/DK-RAi and its passage variant R8/DK-RAi-XII (**A**), H3N1 reassortant R2 and its passage variant R2-XXI (**B**), and H4N1 reassortant RCB1 and its passage variant RCB1-XXI (**C**) are mean values of $[10^3\times(V_0/S_0)]$ ± SD, per minute, calculated for 1 HAU of virus

to adjacent residue via either α2–6 or α2–3-linkage (reviewed in [14]); majority of avian influenza viruses bind exclusively α2–3 sialylated chains, whereas human viruses exhibit a strict receptor preference for trisaccharide motif 6`SiaLacNAc [4, 11, 16].

Influenza virus NA cleaves off the terminal Sia residues from (1) the cellular receptors, (2) natural inhibitors of virus adhesion (mucins), and (3) HA and NA glycoproteins in composition of newly assembled virions [13, 30]. All influenza virus NAs digest preferably α2–3 sialylated oligosaccharides, but differ greatly in ability to discriminate between α2–3/2–6 and fucosylated/non-fucosylated sialo-oligosaccharides [1, 9, 18, 24]. So, duck virus isolates hydrolyze tetrasaccharide SiaLe^a two times slower than fucose-free trisaccharide SiaLe^c, while the α2–6 sialosides —more than 50 times slower than α2–3 ones. The human influenza virus NAs desialylate α2–3-trisaccharides about five times more efficiently than α2–6 isomers and fucosylated derivatives; also, they display an ability to discriminate the structure of the inner core [18, 24]. It is worth to notice that all avian virus NAs display much higher enzymatic activity than human viruses do [10].

According to a number of studies, a balance of HA and NA activities is essential for influenza virus functioning [7, 15, 30]. At the same time, the role of the HA receptor-binding and NA substrate fine specificities in maintaining such a balance remains poorly understood. This factor may be important in influenza virus viability and transmission. It is well known that the oligosaccharide specificity of HA is a result of influenza virus adaptation to the host species possessing host-specific carbohydrates on cell surface and natural inhibitors of influenza virus adhesion [14, 26]. The fine oligosaccharide specificity of NAs of various influenza viruses has not been studied in such detail as that of HAs.

Does the restoration of high yield in avian–human reassortants result from the adjustment of HA and NA oligosaccharide specificities?

Here, substrate specificity of NA and receptor-binding specificity of HA of six variants of influenza A viruses bearing HA from an avian virus and NA from a human virus (Table 1) have been studied. The reproduction level of the reassortants R2 (H3N1), RCB1 (H4N1) and R8/DK-RAi (H3N2) in chicken embryos was decreased as compared to the parent viruses [5, 22]. Viruses R2-XXI, R8/DK-RAi-XII, and RCB1-XXI are the variants of the corresponding reassortants that have restored the original reproducibility as the result of selection in the course of serial passaging in the embryonated chicken eggs [5, 23]. It was demonstrated earlier that reconstitution of virus replication activity was accompanied by mutations in the HA of passage variants, substitutions being located in close proximity to the HA receptor-binding site and leading, as a rule, to a decrease of the virus affinity towards α2–3 sialosaccharides [5, 7]. It should be noted that most of the passage variants, which restored high reproduction level, had amino acid substitutions in HA, and only a minor part of them in NA ([5] and this report). We have selected for analysis only the variants where mutation(s) in NA protein appeared during passaging. All the selected variants have also replacements in HA. The changes in the NA substrate specificity during the passaging of the reassortants had not been studied before. Here, we compared the oligosaccharide specificity of HA and NA in reassortants and their passage variants in order to understand the character of changes in substrate specificity of the human influenza virus NA that occurred during its adjustment to the avian influenza virus HA, and, oppositely, in the receptor-binding properties of the avian-type HA when combined with the human-type NA. Since the low reproducibility of the reassortants having HA and NA of different origin is considered to be a result of disbalanced functioning of these two sialo-recognizing proteins, it seems logical to suppose that the reconstitution of virus replication activity in passage variants is a result of the balance restoration. Here, functional compatibility of HA and NA was evaluated on the oligosaccharide level, through measuring the receptor-binding and substrate specificities of reassortant/passage variant virus pairs.

Avian–human H3N2 reassortant

Oligosaccharide binding patterns of the reassortant virus R8/DK-RAi and its passaging variant differed significantly. The HA of R8/DK-RAi-XII acquired the ability to interact with α2–6 sialosides, while its affinity for most α2–3 ones decreased in comparison with the parent reassortant. This is an evident adjustment of receptor-binding specificity of avian-type HA to human virus NA. The change seems to be the result of Ser205Tyr replacement, which has occurred in HA of the passage variant. These results are in agreement with the data of Suzuki *et al.* [25], where the same substitution at residue 205 (Ser→Tyr) in H3 HA resulted in preferential binding α2–6 sialo-containing carbohydrate chains instead of α2–3 ones. The appearance of bulky Tyr in the polypeptide loop linking the two β-strands directed to the 194 and 225–227 residues (Fig. 3) and on the subunit interface is likely to disturb the architecture of the HA receptor-binding site. The result suggests that the post-reassortment amino acid change in HA not only restored the functional HA-NA balance by lowering the affinity of HA to the receptor, but also shifted the specificity of HA receptor recognition towards the human virus pattern.

NA substrate specificity profiles of these two viruses were similar, but not identical. There is an obvious decrease in the ability of R8/DK-RAi-XII virus NA to differentiate

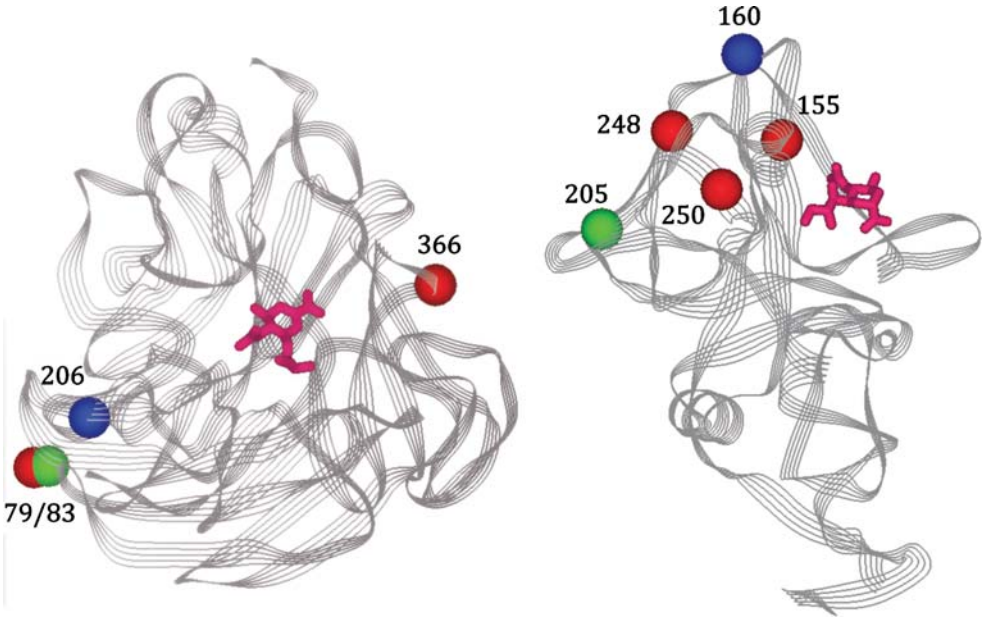

Fig. 3 Three-dimensional models of N1 NA (*on the left*, PDB ID 2HTY) and H3 HA (*on the right*, PDB ID 1HGI) complexed with Neu5Ac (DS ViewerPro 5.0, Accelrys Inc. software). The amino acid substitutions that appeared in HA and NA of the passaged variants R8/DK-RAi-XII (subtype H3N2), R2-XXI (subtype H3N1) and RCB1-XXI (subtype H4N1) are shown by *circles* (*green*, *red*, and *blue*, respectively, in online version)

sialosides with α2–3 linkage. A single substitution, Glu83Gly (the numbering of A/Aichi/2/68 NA), has occurred in the passaged variant NA. Glu83 is a conservative amino acid in human and pig influenza N2 viruses, whereas Gly at this position is found in viruses isolated from birds. So, the mutation in position 83 of R8/DK-RAi-XII NA is a replacement of the amino acid typical for NA from human H3N2 isolates to that found in N2 NA of avian viruses. The 83 residue is situated in the region of the stalk and head joining (Fig. 3). It has been demonstrated that this is a phylogenetically important protein region, but its biological role is still unclear [3].

Avian–human H3N1 reassortant

Compared to the parent R2 reassortant, the HA of R2-XXI virus affinity was lowered for SiaLe^c and SiaLe^a, i.e for sialosides with 1–3 backbone**,** and for 6Su-6′SiaLacNAc as well. It is difficult to reveal which of the three mutations (Thr155Ser, Asn248Asp or Asn250Ser) has affected the HA receptor-binding specificity, as all the residues are situated not far from the receptor-binding site (Fig. 3).

Significant changes in NA substrate specificity were observed for the passage variant R2-XXI (Fig. 2): (1) the appearance of tolerance towards the structure of carbohydrate chain backbone of α2–3 substrates, and (2) an increase of ability to discriminate fucosylated vs. non-fucosylated sialosides (especially SiaLe^x/3′SiaLacNAc) and 6′SiaLacNAc vs. 3′SiaLacNAc. Such changes bring to the human virus NA substrate specificity profile the features of the avian virus NA.

Adjustment of the NA of N1 human virus to the HA of H3 avian virus is associated with two mutations in NA: Asp79Val and Ser366Asn (numbering by N1 NA of virus A/USSR/90/77). Asp is found in position 79 of human H1N1 influenza virus NAs, while Ala and, sometimes, Val are located at this position in N1 NAs of avian isolates. Interestingly, the residue 79 of N1 NA subtype coincides with residue 83 in N2 subtype (according to the alignment). Thus, a mutation of the same type leading to the increase of the positive charge of the protein molecule takes place in NA of the passage variants of H3N1 and H3N2 reassortants (R2-XXI and R8/DK-RAi-XII, respectively). Taking into account that a single mutation at the position 83 of N2 NA had only a minor effect on the oligosaccharide specificity, one can suppose that significant changes of substrate specificity having appeared in high-yield virus variant N1 NA are not associated with the change at position 79, but result from the other mutation. The 366 residue is Asn for most NA of the avian isolates; conversely, Ser is preferentially found in this position in human viruses**.** The residue at this position is exposed on protein surface near the catalytic center (Fig. 3) and is a part of the loop forming the hemadsorption site (HB-site) of avian virus NAs [28]. There are literature data indicating that HB-site promotes high activity of avian virus NA towards α2–3 sialosides [10]. Thus, an increase of the ratio of desialylating activity/HAU observed for the high-yield virus variant may be also a result of the replacement at position 366 of NA.

Avian-human H4N1 reassortant

A single change in the oligosaccharide specificity of HA, a six-fold decrease of affinity towards 6Su-6′SiaLacNAc, was observed for the passage variant RCB1-XXI in comparison with the reassortant RCB1. Taking into account that the Asn160Asp substitution leads to an increase of negative

charge in the HA of the passage variant, and that the residue 160 is situated on protein surface not far from the receptor-binding pocket (Fig. 3), such a change in the properties of HA is explicable.

In RCB1-XXI more notable changes took place in the oligosaccharide specificity of NA than in the specificity of HA. The ratio of hydrolytic activity of the passage variant NA increased from 3.9 to 9.6 for α2–3/α2–6 SiaLacNAc and from 5.6 to 7.3 for α2–3/α2–6 SiaLac in comparison with the parent reassortant. Such a difference in desialylation of substrates with different linkage type between Neu5Ac and Gal is not characteristic for human influenza viruses, but it is found for influenza viruses of pigs and poultry [9, 18, 24]. Adjustment of human virus N1 NA to avian virus H4 HA was associated with mutation Leu206Ile. The 206 residue is located on the interface between neighboring subunits in NA tetramer (Fig. 3). Leu206 and adjacent region are absolutely conservative for NAs of N1 subtype, this indicating to the importance of this region for the enzyme function. Obviously, in this case the human virus NA of H4N1 reassortant was adjusted to the avian-type HA. It cannot be excluded that the conservative receptor-binding properties of H4 HA limit the host range of the HA subtype: influenza viruses of subtype H4 have been found almost exclusively in waterfowl.

Conclusion

Several common features are typical for all reassortants and their passaged variants studied. During serial passaging of viruses having 'avian' HA and 'human' NA**,** the amino acids which are characteristic for NAs of human influenza viruses are changed to those typical for avian virus NAs. Oligosaccharide specificity of NA becomes changed in the same way. Conversely, substitutions in 'avian' HA observed during adjustment to 'human' NA lead to an increase of negative charge of HA surface near receptor-binding site, this providing a decrease of HA affinity for avian-like receptor. Consequently, the changes in oligosaccharide specificity of both HA and NA resulting from a selection during serial passaging of reassortants are directed to functional adjustment of alien NA and HA, though the patterns of change are different for particular cases. Thus, here, three different variants of HA and NA functional balance readjustment were observed for the three reassortant viruses during their passaging: (1) in the case of H3N2 subtype the HA receptor-binding specificity became the most changed; (2) NA substrate specificity changed more notably in the case of H4N1 subtype, whereas the changes in HA oligosaccharide specificity profile were small; and (3) both proteins became affected in the case of H3N1 subtype virus. Taking into consideration that the mutations in HA protein take place most often during passaging, whereas NA is changed rather rarely, it can be supposed that the first correlation model is the most widespread in nature.

Summing up, the changes in human NA and avian HA during passaging of reassortant viruses take place in concert with respect to their oligosaccharide specificities. Apparently, one of the factors determining a functional balance between HA and NA in the viable virus, is the compatibility of receptor-binding specificity of HA and substrate specificity of NA.

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