Glycoconj J (2006) 23: 115–125
DOI 10.1007/s10719-006-5444-x

Search for additional influenza virus to cell interactions

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Abstract Sialyl oligosaccharides have long been considered to be the sole receptors for influenza virus. However, according to [1] some viruses are able to grow in sialic-free MDCK cells. Here we attempted to reveal a possible second, non-sialic receptor, hypothesizing the involvement of additional carbohydrate lectin recognition in influenza virus reception process, first of all in situations when a lectin of the host cell could recognize the viral carbohydrate ligand. We tested the presence of galactose- and sialic acid-binding lectins, as well as mannoside- and sulfo-*N*-acetyllactosamine-recognizing properties of MDCK and Vero cells using polyacrylamide neoglycoconjugates and antibodies. MDCK cells bind galactoside probes stronger than Vero cells, whereas Vero cells bind preferentially sialoside, mannoside and various sulfo-oligosaccharide probes. The probing of *viruses* with the neoglycoconjugates revealed specific 6′-HSO_3LacNAc (but not other sulfated oligosaccharides) binding property of A and B human strains. Affinity of 6′-HSO_3LacNAc probe was comparable with affinity of 6′-SiaLac probe but the binding was not inhibited by the sialooligosaccharide.

Keywords Galectins · Glycoconjugates · Influenza virus · Hemagglutinin · Siglecs

Abbreviations

BHK	baby hamster kidney cells
biot	biotin residue
BSA	bovine serum albumin
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
fluo	fluorescein residue
HA	hemagglutinin
MBP	mannose-binding protein
MDBK	Madin-Darby bovine kidney cells
MDCK	Madin-Darby canine kidney cells
NA	neuraminidase
OS	oligosaccharide
PAA	polyacrylamide
PBS	phosphate buffered saline
PBA	PBS containing 0.2% BSA
RBS	receptor binding site
SP	surfactant protein
Sug	mono- or oligosaccharide residue
3′SL	3′-sialyllactose
LacNAc	N-acetyllactosamine
6′SLN	6′-sialyl-*N*-acetyllactosamine
3′SLN	3′-sialyl-*N*-acetyllactosamine
Neu5Ac	α-*N*-acetylneuraminic acid
asialoGM1	Galβ1-3GalNAcβ1-3Galβ1-4Glc
Fs	GalNAcα1-3GalNAcβ
Man_3	Manα1-6(Manα1-3)Man
A_{tri}	GalNAcα1-3(Fucα1-2)Gal
A_{tetra}	GalNAcα1-3(Fucα1-2)Galβ1-4GlcNAc

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B_{tri}	Galα1-3(Fucα1-2)Gal
SiaLe^x	Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAc
7-OS	(GlcNAcβ1-2Manα1-)$_2$-3,6-Manβ1-4GlcNAcβ1-4GlcNAc
11-OS	(Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-)$_2$-3, 6-Manβ1-4GlcNAcβ1-4GlcNAc
$T_{\beta\beta}$	Galβ1-3GalNAcβ
TF	Galβ1-3GalNAcα

Introduction

Influenza virus infection is initiated by specific interactions between the viral envelope glycoprotein hemagglutinin (HA) and cell surface receptors [2–4]. Terminal sialic acid residue of glycoproteins or gangliosides is known to be the minimum binding determinant of these receptors. Virus binding also depends on the type of sialoside linkage to penultimate galactose and the structure of more distant parts of sialyloligosaccharides [2–8].

Sialyl glycoconjugates have long been considered to be the sole receptors for the influenza virus. However, a mutant virus, NWS-Mvi, grows well in MDCK cells continuously treated with exogenous neuraminidase to remove sialic acid; binding of mutant and parent reassortant viruses to MDCK cells is indistinguishable and is only partially reduced by sialidase treatment of the cells [1]. The ability to infect desialylated cells was found to be shared by recent H3N2 clinical isolates, suggesting that this might be a generic property of influenza A viruses. Thus, it was proposed that influenza virus entry is a two-stage process, whereby initial binding to surface sialic acid leads to an interaction with the second receptor necessary for the entry, and that the requirement for sialic acid can be bypassed [1] as was demonstrated recently for reovirus attachment [9,10]. Neuraminidase-treated CEM cells also can bind influenza virus [11]. Lec1 cells, which express high level of sialic acid, show that they can bind influenza virus, however, a postattachment block in infection is present in the absence of terminal carbohydrate on host cell N-linked glycoprotein, thus, sialic acid is not sufficient for infection [12]. Evidence exists that other glycoconjugates such as sulfatide are also bound by the influenza virus [13], and at the same time, MDCK cells have been shown to express sulfatide [14]. Recently, we have found that a sulfo-group in position 6 of GlcNAc moiety of 3′SLN improves binding to H5N1 and some other viruses ten-fold and more [15]. According to [16] infection of cells by influenza viruses is influenced by the abundance of the receptors on the cell surface when the affinity of HA for sialic acid is reduced. Finally, high-affinity binding of HA must be maintained for viral replication in cells expressing low levels of sialic acid [17]. Taking this into consideration we made an attempt to detect a possible second receptor, hypothesizing the participation of alternative (or additional/assistant) carbohydrate/lectin recognition in the influenza virus attachment process, being first of all in situations when a lectin of the host cell recognizes a carbohydrate ligand on the virus glycoconjugates. Using multivalent carbohydrate probes and antibodies to galectins and siglecs we tested the presence of galactose-binding and sialic acid-specific lectins on MDCK and Vero cells as well as their mannose- and sulfo-OS recognizing properties. We also took into consideration an alternative carbohydrate-mediated mechanism when lectin acquired by virion during its assembling from the host cell can recognize carbohydrates of the target cell. Madin-Darby canine kidney (MDCK) and the African green monkey kidney (Vero) cell lines are widely used for primary isolation and propagation of human influenza viruses [18–20].

Materials and methods

Reagents

Label-free glycoconjugates Sug-PAA, biotinylated glycoconjugates Sug-PAA-biot, and fluorescein-labeled probes Sug-PAA-fluo were obtained from Lectinity (Moscow, Russia). 11-OS probes were synthesized as described earlier [21]; 7-OS was obtained by sequential neuraminidase and galactosidase digestion of 11-OS. Murine monoclonal antibodies to siglecs, *i.e.* Ser 4 (against siglec 1), aCD22 (against siglec 2), aCD33 (against siglec 3), anti-MAG (against siglec 4), aSiglec5 (against siglec 5), Ab7a (against siglec 7), Ab73 (against siglec 8); Ab 2.2 (against siglec 10) were kindly provided by Dr. P. Crocker (Dundee, Scotland). Rabbit polyclonal antibodies against galectin-1 and galectin-3 lacking cross-reactivity against other family members such as galectins-2, -4, -5, -7, and -8 were obtained as described in [22]. Streptavidin-peroxidase conjugate was obtained from Boehringer Mannheim (Germany). PBS tablets, BSA, FITC-labeled anti-mouse Ig and anti-rabbit Ig, sialidase from *Vibrio cholerae* were purchased from Sigma (San Diego, USA). Dulbecco medium, FBS and penicillin/streptomycin were from Invitrogene (UK).

Influenza viruses

Vero- and MDCK-variants of human influenza A viruses were obtained from the Institute of Applied Microbiology, Vienna, Austria, and were propagated as described in [23]. Virus B/Kirov/13/02 was obtained from the influenza virus repository of Ivanovsky Institute of Virology, RAMS, Moscow, Russia and propagated in MDCK cells (kindly

submitted by Dr. E. Burtseva). Virus purification was performed as previously described [24].

Cell culture

Vero (CCL-81) and MDCK (CCL-34) cell lines were obtained from the American Type Culture Collection (ATCC). For virus passages Vero and MDCK cells were adapted and further cultured as described in [23,25].

To study carbohydrate binding pattern of cells MDCK cells were cultured in a Dulbecco medium containing 10% FBS, 2 mM L-glutamine, and 100 U/ml penicillin/streptomycin and Vero cells were cultured in Dulbecco medium containing 2 mM L-glutamine and 100 U/ml penicillin/streptomycin.

Sialidase treatment

Cells harvested with Versene solution were washed 3 times with a Dulbecco medium and treated with neuraminidase (1:20 dilution) in a Dulbecco medium at 37°C for 3 h under agitation. The reaction was stopped with FBS (10 vol.%). Cells were washed three times with PBA by centrifugation at 1200 rpm/min for 2 min.

Binding of Sug-PAA-fluo probes to the cells

Cells were harvested with cold Versene solution, washed three times in cold PBA and incubated with fluorescein-labeled probes Sug-PAA-fluo (100 μg/ml) for 40 min at 4°C. Concentration of cells in flow cytometry analysis was $2 \times 10^5/100$ μl. All conjugates had the same molecular weight of 30–40 kDa and the same molar content of Sug ligand (20%) and fluorescein (1%). After washing with cold PBA fluorescence analysis was performed using a flow cytometer (Dako Galaxy, Denmark) or a FACScan (Becton-Dickinson, USA). Carbohydrate free PAA-fluo conjugate was used as a negative control.

Antibody binding assay

Cells were washed with PBA and incubated with anti-siglec mAbs or anti-galectin antibodies at 4°C for 1 h following by incubation with FITC-labeled anti-mouse Ig (1:50 dilution) or anti-rabbit Ig (1:50 dilution) conjugates, respectively, for 30 min, then washed 3 times with PBA. FACS analysis was performed as described above. Anti-mouse or anti-rabbit IgG-FITC conjugate were used as a negative control.

Probing the influenza virus with Sug-PAA-biot

96-well plates (96F Nunc-immunoplate II, Cat. N 4-42404) were coated with purified virus with the titer of 20 hemagglutinating units (50 μl/well) for 16 h at 4°C followed by washing with PBS. After that 100 μl/well of blocking solution (PBS containing 1% of BSA) was added, and then plates were kept at room temperature for 2 h and finally washed with PBS containing 0.05% Tween 20. After the addition of Sug-PAA-biot, 45 μl/well, in buffer B (PBS with 0. 5% of BSA, 0.02% of Tween 20), plates were incubated at 4°C for 1 h. Neuraminidase inhibitor, 2,3-didehydro-2,4-dideoxy-4-amino-*N*-acetyl-D-neuraminic acid, was used only in case of the use of sialylated probes. The starting concentration of Sug-PAA-biot was 50 μM on Sug; the following twofold serial dilutions were used. Plates were washed and incubated with streptavidin-peroxidase in the buffer B at 4°C for 30 min. After washing, the substrate solution was added (0.1 M sodium acetate, pH 5.0, containing 4 mM o-phenylenediamine and 0.004% H_2O_2) and the reaction was stopped with 2 M H_2SO_4 and optical density was determined at 492 nm. The controls, OD_{492} values in wells, treated by the same way but without virus, were taken.

Results

Carbohydrate-binding pattern of MDCK and Vero cells

Using a panel of Sug-PAA-fluo probes, where Sug were galactoside-, sialic acid-, mannoside-, and sulfo-oligosaccharides, we inspected the carbohydrate-binding profile of MDCK and Vero cells. A number of OS, such as LacNAc, $T_{\beta\beta}$, GalNAcβ1-4GlcNAc, asialoGM1 and blood group A are known to be potent ligands for cellular lectins such as galectins [26]. Indeed, MDCK exhibited significant level of binding to LacNAc, asialoGM1, GalNAcβ1-4GlcNAc, TF, blood group A (A_{tri}, A_{tetra}) and Fucα1-2Galβ1-3GlcNAc (Le^d) oligosaccharides as shown in Figure 1. Binding of these probes to Vero cells was less pronounced. Fs disaccharide interacted intensely only with MDCK cells.

Galectin expression on MDCK and Vero cells was confirmed by antibody binding assay. Purified rabbit polyclonal antibodies, which were raised against human galectin-1 and murine galectin-3 and showed no mutual cross-reactivity [22], were used. As shown in Figure 2, the cells revealed dissimilar binding patterns with the two antibodies, Vero cells bound preferentially the antibody fraction to galectin-1, whereas MDCK - only the antibody to galectin-3. Similarly to Sug-PAA-fluo probing, the antibody staining was more intense in the case of MDCK cells than for Vero cells.

Sialic acid-dependent binding of Vero and MDCK cells was studied by using Sug-PAA-fluo probes, where Sug was sialic acid monosaccharide, 3′SL, 6′SL, Neu5Acα2-

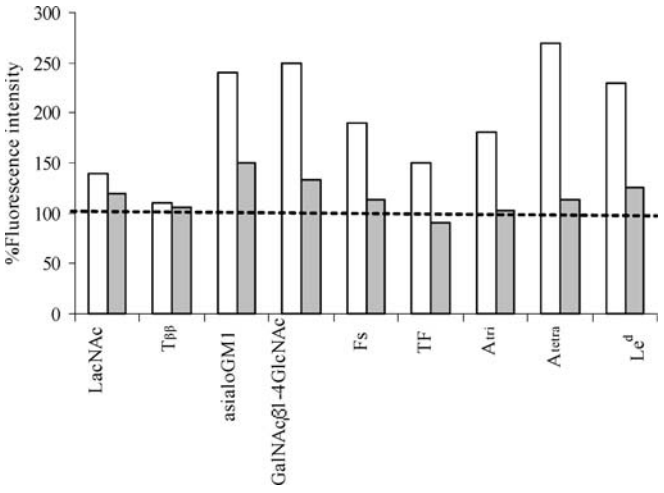

Fig. 1 Binding of galectin ligands as Sug-PAA-fluo probes to MDCK (white bars) and Vero cells (gray bars). Cells were stained with carbohydrate-free PAA-fluo as a negative control. Percentages of fluorescence intensity from cells incubated with probes relative to that of control cells are given and represent the means of three independent experiments. Dashed line indicates the level of 100% value

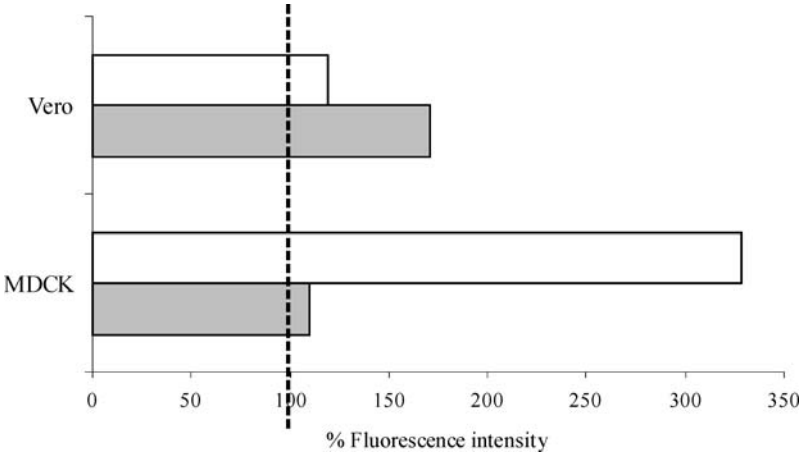

Fig. 2 Interaction of Vero and MDCK cells with rabbit polyclonal anti-galectin-1 (white bars) and anti-galectin-3 antibodies (gray bars). Cells were incubated with anti-rabbit IgG-FITC as a negative control. Percentages of fluorescence intensity from cells incubated with anti-galectin antibodies relative to that incubated with control cells are given. At least two experiments were performed for each antibody on each cell line. Dashed line indicates the level of 100% value

6GalNAcα or disialoside [Neu5Acα2-8]$_2$, respectively. We observed a lack of sialic acid binding even after the neuraminidase treatment of the cells (desialylation renders *cis*-connected siglecs accessible), only the Neu5Acα benzyl glycoside-based probe bound at a significant level (Figure 3). The same results were obtained by a solid phase assay with the corresponding biotinylated sialoside probes (data not shown).

Notably, both Vero and MDCK cells bound to Neu5Acα in benzyl glycoside spacered form, whereas binding of the cells with Neu5Acα as $OCH_2CH_2CH_2$-glycoside did not differ significantly from the background level. Interaction of Vero cells with Neu5AcαBn was three times stronger than with MDCK cells. Binding of Neu5AcαBn-PAA-fluo to both cell types was inhibited by a low molecular weight ligand Neu5Acα-OBn in a dose-dependent manner, indicating the specificity of the interaction; 50%-inhibition was reached at 10 mM conc. (data not shown).

At the same time, neither Vero nor MDCK cells interacted with monoclonal antibodies against siglecs 1, 2, 3, 4, 5, 7, 8 and 10 (data not shown). These data indicate the presence of a *non-siglec* sialic acid binding protein on the cells.

Vero cells displayed significantly higher binding with all of the three sulfated oligosaccharides than MDCK cells did (Figure 4). Apparently, the position of the sulfate group is not crucial; interaction with 6-HSO_3Galβ1-4GlcNAc (6′-HSO_3LacNAc), Galβ1-4(6-HSO_3)GlcNAc (6-HSO_3LacNAc) and 3-HSO_3Gal probes was practically equal, indicating rather a charge- than glycan-specific structure-dependent events.

Mannoside-binding potency of MDCK and Vero cells was studied with four fluorescein-labeled probes. Monomannoside-, trimannoside Manα1-6(Manβ1-3)Man- as well as the GlcNAc-terminated biantennary chain (GlcNAc-Man)$_2$-3,6-Man-GlcNAc-GlcNAc (7-OS) demonstrated binding, especially with Vero cells, whereas the corresponding biantennary Neu5Ac-terminated chain (11-OS, negative control) was indifferent to both cell types (Figure 5). These data indicate the potency of Vero cells to recognize terminal Man/GlcNAc residues.

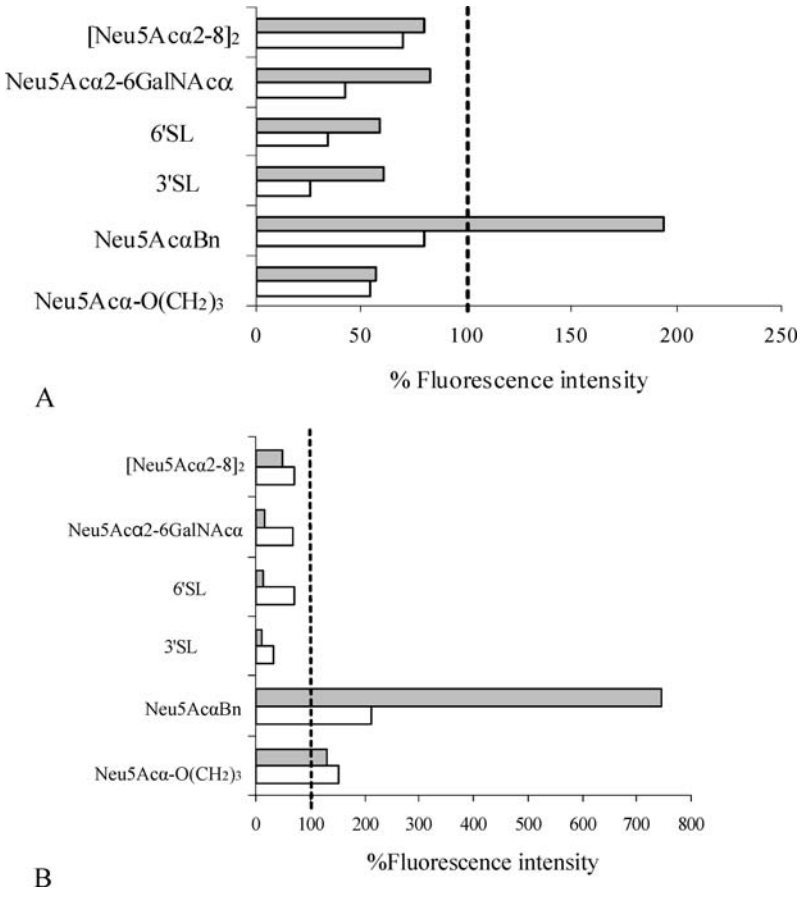

Fig. 3 Binding of sialoside-containing Sug-PAA-fluo probes to MDCK (A) and Vero (B) cells. Gray bars correspond to neuraminidase-treated cells. Percentages of fluorescence intensity from cells incubated with probes relative to that of controls are given and represent the means of three independent experiments. Cells were exposed to carbohydrate-free PAA-fluo as a negative control (100% value, dashed line)

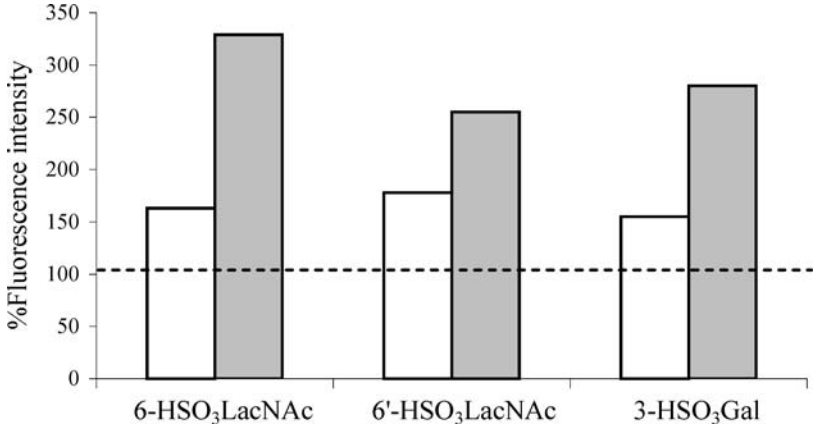

Fig. 4 Binding of sulfated Sug-PAA-fluo probes to MDCK (white bars) and Vero (gray bars). Percentages of fluorescence intensity from cells incubated with probes relative to that of controls are given and represent the means of three independent experiments. As a negative control (100% value, dashed line) cells were stained with carbohydrate-free PAA-flu

Probing of viruses with Sug-PAA-biot

In order to reveal lectins possibly acquired by virions from host cells, human influenza virus B isolate and five influenza A viruses (passaged on Vero, MDCK, or Vero followed by MDCK cells) were probed with the following biotinylated glycoconjugates: 6′-HSO_3LacNAc-PAA-biot, 6-HSO_3LacNAc-PAA-biot, 3′-HSO_3LacNAc-PAA-biot, 3-HSO_3Gal-PAA-biot, Manα-PAA-biot, Man_3-PAA-biot, 7-OS-PAA-biot, GalNAcα1-3GalNAcβ-PAA-biot, $T_{\beta\beta}$-PAA-biot, A_{tri}-PAA-biot, B_{tri}-PAA-biot, Galα1-3Galβ1-4GlcNAc-PAA-biot. A solid-phase assay had recently been devised for studying the modern influenza virus strains with a low receptor affinity [23, 24]. In contrast to other sulfated probes 6′-HSO_3LacNAc bound to both A and B viruses with affinities close to those of 6′-sialo probe (Table 1). Interaction with 6′-HSO_3LacNAc probe depends on passage history, but for all viruses tested it was strong. 6′SLN does not inhibit the sulfated probe binding.

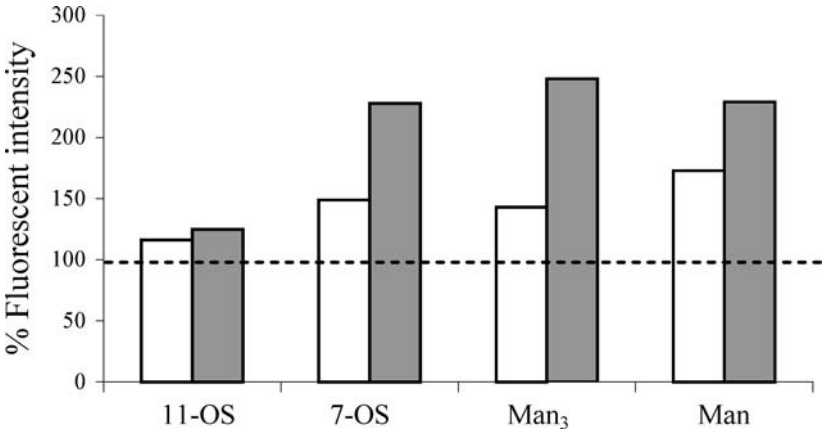

Fig. 5 Binding of mannose-containing Sug-PAA-fluo probes to MDCK (white bars) and Vero (gray bars) cells. Percentages of fluorescence intensity from cells incubated with probes relative to that of controls are given and represent the means of three independent experiments. Cells were stained with carbohydrate-free PAA-fluo as a negative control (100% value, dashed line)

Table 1 Binding of sulfated LacNAc-PAA-biot probes to influenza viruses as compared to 6′SL-PAA-biot

Virus strain, passage history (subtype)	50% binding of Sug-PAA-biot, as [Sug] μM		
	6′-HSO_3LacNAc	3′-HSO_3LacNAc	6′SL
B/Kirov/13/01, MDCK	2.0	40	0.3
A/Vienna/47/96, 4[a]Vero (H3N2)	2.0	40	1.2
A/Vienna/47/96, 3MDCK (H3N2)	3.0	>50	0.8
A/HK/1180/99, 6Vero (H3N2)	3.0	>50	1.2
A/HK/1182/99 3Vero 5MDCK (H3N2)	0.8	>50	1.2
A/HK/1182/99 3MDCK 5Vero (H3N2)	0.6	>50	1.0

[a]number of passages.

Manα, Man_3, and 7-OS (terminated with GlcNAc) probes demonstrated weak but significant binding in contrast to the five latter probes (see above), data not shown. Maximum affinity was detected in case of trimannoside, especially with MDCK-grown strain, see Figure 6.

Discussion

Influenza virus entry and penetration into the cell is very complex and only a partially understood process. Though the key event is the virus attachment to the target cell, it is necessary to take into consideration many accompanying factors, in particular virus interaction with extracellular inhibitors, such as sialoglycoproteins (mucins), antibodies to viral proteins [27], mannose-binding lectins such as conglutinin, mannoside-binding protein (MBP) and surfactant protein B (SP-D) ("β-inhibitors") [28,29]. Mannose-binding alveolar surfactant protein A (SP-A) attaches to influenza viruses via its sialic acid residues, thereby, conferring mannose-binding properties e.g. ability to attach to mannose chains of the target cells to the virus [30]. Obviously, this list of extracellular factors affecting (not necessarily as inhibitors) the virus interaction with a cell is not comprehensive, and one can assume that this group can also include other lectins capable of binding carbohydrate chains of HA and NA. There are data available which demonstrate evidence that viral neuraminidase also plays a role in viral reception [31–33], thus, it is possible to suppose that endogenous extracellular glycosidases can affect the infection process. In addition, the adhesion process is only one of many important events during virus penetration into the cell. Prior to the molecular rendezvous and specific recognition of the target cell, the virion has to pass a long way encountering various cell species having both similar and different glycosylation and lectin patterns as compared to the target cell. To be able to reach its target, the virus should avoid an interaction with non-target cells. For example, due to the high density of terminal Gal and Man residues influenza virus can be a subject of phagocytosis by macrophages, bearing the corresponding lectins. The virus should also avoid an interaction with NK-cells bearing sialylated chains, which act as a decoy for viral HA [34,35]. Indeed, the virus should not be attached firmly to non-target cells due to its HA. At the same time it is possible to consider that some "third" cells assist virus movement into the desired direction, as *e.g.* selectin-mediated leukocyte rolling is supported by endothelial cells. It was shown [36,37] that in addition to basic cell surface receptors, HIV and Herpes simplex viruses require certain additional cellular factors that are not involved in primary binding in order to infect the target cells.

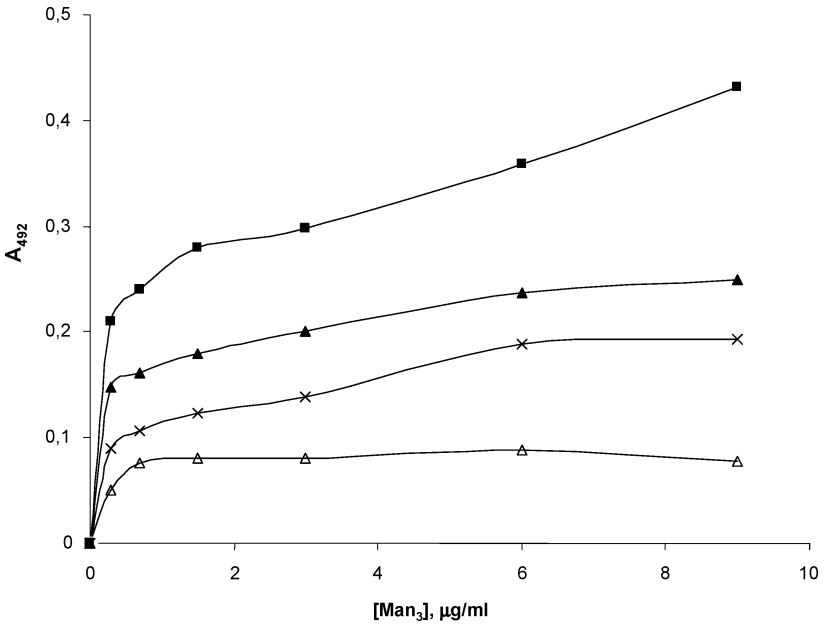

Fig. 6 Binding of Man3-PAA-biot to human influenza viruses (■—7729M, ▲—7729V, ×—7729 MV, △—B/549 V). Absorbance at 492 nm was plotted against the conjugate concentration (μg/ml). Typical data of the one from the three experiments with similar results are presented

In this investigation we attempted to resolve the issue as to whether additional carbohydrate/lectin-mediated events can be involved in the interaction of the influenza virus with host cells. In particular, the galactose-terminated chains of virion can be recognized by cell galectins; incompletely desialylated viruses—by siglecs; high-mannose N-chains—by MBP; and, finally, sulfated chains—by the corresponding protein(s). In addition, the possibility of an insertion of host cell lectins into the membrane of *de novo* assembling virion cannot be excluded. If so, it is necessary to take into account this variant of carbohydrate-mediated virus-cell interaction, when besides its own HA, the virus can also bear proteins acquired from the host cell, as for example was documented for actin [38]. The latter hypothesis can explain virus tropism to a very limited cell population: due to adsorption of specific adhesion molecules from the host cell, the virus acquires to some extent the specific adhesion properties of these cell species. Herein, we addressed the question whether MDCK and Vero cells can bind oligosaccharide chains. Additionally, we characterized the carbohydrate binding profile of viruses propagated in these cell lines owing to the "acquired cell lectins" hypothesis.

Carbohydrate-binding properties of MDCK and Vero cells

The human influenza viruses are able to grow only in a limited number of mammalian cells, mainly originating from kidneys of a different species. MDCK is the most widely used cell line for laboratory cultivation of influenza viruses; recently Vero cells (monkey originated) were shown to be a promising "substrate" [20,21,23,24]. Importantly, influenza viruses grown on these cell cultures maintain Sia2-6Gal receptor-binding specificity of original human viruses [6,20,39]. According to [1] desialylation of MDCK cells by neuraminidase does not abolish the interaction of the influenza virus NWS-Mvi with these cells. Taking into account a high HA and NA glycosylation rate, it is possible to suppose that the target cell interacts with carbohydrate chains of the virion. In other words, cell lectin(s) can serve as a trigger of the influenza virus attachment followed by the penetration into a host cell. Galectins, siglecs, mannose-binding proteins, and sulfated saccharide-binding proteins are typical animal cell membrane-associated lectins [40]. Consequently, it is necessary to cover their ligand spectrum in our lectinomic profiling. Thus, we used carbohydrate probes, namely Sug-PAA-fluo, which are capable of detecting *classes* of lectins (according to their specificity), including not yet biochemically defined proteins. Additionally, antibodies directed to *particular* galectins (galectin-1 and galectin-3) and siglecs (siglecs 1–8 and siglec 10) were used.

Flow cytometry analysis using galectin-binding carbohydrate probes demonstrated significant binding of asialoGM1 oligosaccharide and several related probes to MDCK cells, whereas only weak staining of Vero was observed under the same conditions, thus intimating only a low level of presentation of accessible galactose-binding lectins on Vero cells. Using polyclonal antibodies to galectin-1 and galectin-3 we tested their interaction with the cells and observed the significant binding of anti-galectin-1 (but not anti-galectin-3) to MDCK, whereas Vero cells demonstrated weak binding only with anti-galectin-3 antibodies. It should be noted that in [41] galectin-3, former name L-29, was shown to be one of the

major cytosolic proteins of MDCK cells which undergoes apical secretion. Evidently, rebinding after secretion is not a major route for this protein, but it establishes galactoside-binding potency of MDCK cells evidenced both from carbohydrate probes and antibodies, whereas Vero cells differ in this respect. Fittingly for an interaction, the presence of multiple galactose-terminated chains on the influenza virus HA and NA is a well-known fact: due to the action of its own sialidase influenza virion has an unusually high density of Gal residues when compared to other mammalian surface glycoproteins, where most of the Gal residues are masked by sialic acids. Based on the results it is of interest to further characterize MDCK cell galactose-specific lectins that are able to interact with virion glycoproteins, and how relevant this event is for virus reception. Thus, our data on galactoside-binding ability of MDCK cells is in agreement with data on infection of desialylated MDCK cells [1], however this phenomenon seems to be rather exceptional, MDCK-specific, than general. It is even challenging to understand, whether galectin/galactoside interaction may play a role during the infection process, taking into account the presence of galectins in bronchial and tracheal epithelium [42, 43]. It should be also noted that several galectins demonstrate elevated binding to sulfated galactosides, and, at the same time, as mentioned above, influenza virus envelop, especially HA is extensively sulfated.

The next widespread group of animal lectins is the siglec family. Eleven characterized human siglecs are known to bind either 3′SL, or 6′SL, or a disialic fragment [44]. We did not raise evidence for presence of siglecs on MDCK and Vero glycocytochemically using the 3′SL-PAA-fluo, 6′SL-PAA-fluo, and [Neu5Acα2-8]$_2$-PAA-fluo probes, as well as with help of monoclonal antibodies. Siglecs are often presented on the cell membrane in masked state, *i.e.* they are masked by endogenous carbohydrate chains [45], which become available for carbohydrate probes only after neuraminidase action. In our case the treatment of cells with neuraminidase did not increase the interaction with the mentioned probes, except for the Neu5AcBn-PAA-fluo. Notably, this probe is a potent *group-specific* binder for most of siglecs, whereas the mentioned three sialooligosaccharide probes are not [46]. Thus, the question concerning the expression of siglecs or other Sia-binding proteins on MDCK and Vero cells warrants further study. Do siglecs in principle take part in an interaction with influenza viruses, normally lacking sialylated chains? Supposedly, in rare situations when due to low activity of viral NA the virion maintains a notable amount of sialylated chains, a situation is established with concomitant decrease in HA affinity [31,32], and one can speculate then that in order to retain cell-binding ability a virus requires the second, additional receptor.

The tandem-repeat-type mannose receptor of macrophages is involved in infection of macrophages by influenza viruses, the infection being inhibited by yeast polysaccharide mannan [47]. It was shown [47] that this lectin is not expressed on MDCK cells, but MDCK cells have the 36 kDa vesicular-membrane protein (VIP36), an intracellular lectin recognizing high-mannose type glycans [48]. Notably, this lectin indeed localized also to the plasma membrane of MDCK cells [49]. In our flow cytometry experiments mannoside probes demonstrated binding with both Vero (more pronounced) and MDCK cells. Thus, high-mannose chains of modern highly glycosylated H3N2 influenza viruses may affect their binding to MDCK and Vero cells. This fact probably could explain the ability of certain viruses to infect low sialylated cells. It should be noted that hemagglutinin of Vero cell-grown viruses is more heavily mannosylated as compared to MDCK viruses derived from the same isolate [23]. Effective participation of lectin/mannoside recognition system in viral adhesion remains to be fully investigated.

A number of proteins, such as CD44, CD54, I-type lectins, and scavenger receptor, recognize sulfated oligo- or polysaccharides and other anionic glycans; the macrophage mannose receptor is known to have a second binding site specific to sulfated saccharides [50]. At the same time HA of influenza virus grown in MDCK and other kidney cells carries sulfated *N*-acetyllactosamine-type carbohydrate N-glycan chains, namely as 3-HSO_3Gal- and 6-HSO_3GlcNAc-motifs [51], potential partners for sulfo-OS-binding cell lectins. As shown in [52], the glycoproteins contain sulfate in composition of their glycans, additionally, sulfated mucins of host cells are strongly associated with MDBK-grown virus (no corresponding MDCK and Vero data). In our study, we found that sulfated probes bind to Vero cells, whereas the interaction with MDCK was weak. All probes tested, namely possessing 6-HSO_3Gal-, 6-HSO_3GlcNAc- and 3-HSO_3Gal-motifs, yielded practically the same level of binding, intimating rather a charge-dependent unspecific binding than an interaction with a specific lectin.

Summarizing the cell probing experiments, the two studied cell lines have different carbohydrate-binding patterns. MDCK cells bind galactoside probes stronger than Vero cells, whereas Vero cells bind preferentially sialoside, mannoside and sulfo-OS probes.

Carbohydrate-binding properties of the influenza virus

Influenza virus membrane contains only one lectin, HA. Additionally, as mentioned above, host cell lectins theoretically might be acquired during viral particle formation, either membrane lectins or multisubunit soluble molecules, – in the latter case due to strong interaction with viral glycans. What pattern of carbohydrate-binding activity can one anticipate for the influenza virus? Firstly, the well-known interaction with 6′SLN due to classical carbohydrate-binding

site of HA. Secondly, HA is shown to have additional Sia-binding motif [53], the role and fine specificity of this site remaining unclear. It may be speculated that the second Sia-binding motif is actually able to bind other negatively charged chains, for example sulfated molecules. Thirdly, NA known to have hemagglutinating properties separated from enzyme activity functionally and in respect of a.a. region responsible for this phenomenon ([54] and references herein, [55]). Additionally, the active site of NA might be considered as a weak galactoside-binding—indeed, NA is able to discriminate oligosaccharide substrates and to bind asialo part, as for example documented for *Trypanosoma* trans-sialidase [56]. It can not be excluded that HA or NA possess unknown yet additional lectin properties. Finally, due to the acquisition of host cell lectins, the influenza virus might interact with a broader spectrum of carbohydrates. As shown in the present work, MDCK and Vero cells exhibit sialoside-, galactoside-, mannoside-, and also sulfo-OS-binding properties. This is a reason why we tested MDCK- and Vero-grown viruses, with respect to their carbohydrate-recognizing properties. Although binding of mannoside- and galactoside- (including typical galectin ligands) probes was demonstrated, the interaction was shown to be basically weak and it is significant only in some strains. In contrast to other probes the binding with 6′-HSO_3LacNAc is typical for all the strains tested—both A and B, the affinity was of the same order of magnitude as in the case of the sialylated probe, 6′SL-PAA-biot, in the same conditions (viruses originating from the same isolate were compared). The binding of 6′-HSO_3LacNAc probe to influenza *viruses* is independent of the nature of the host cells (MDCK or Vero), this conclusion emerging from an experiment where host *cells* were stained with this probe: only Vero cells (but not MDCK) have demonstrated significant affinity to the probe, thus it is not likely that viruses acquire 6′-HSO_3LacNAc-binding lectin from the host cell. Interaction of viruses with 6′-HSO_3LacNAc is specific; indeed, isomeric disaccharide probes 6-HSO_3LacNAc and 3′-HSO_3LacNAc as well as monosaccharide probe 3-HSO_3Gal did not bind viruses. In the case of selectin/ligand interaction, the substitution of Sia in $SiaLe^x$ to sulfate in position 3 of Gal moiety did not impair selectin-binding potency [57]. In our case the structural similarity of 6′-HSO_3LacNAc and 6′SiaLacNAc (6′SLN) is obvious. However, we do not expect that a sulfated probe is able to bind to the primary Sia-binding site of HA because this interaction has not been inhibited with 6′SLN and has no structurally (in terms of HA/OS complex) based rationale. It is also known that trachea tissues express heavily sulfated mucins [58], that together with sulfatide-binding properties as mentioned above [13,14], and our data on 6′-HSO_3LacNAc-binding properties of influenza virus provides reason to speculate that sulfated sugars (on cell) and corresponding lectins (on virus) are involved or can be involved in the infection process *in vivo*. The nature of 6′-HSO_3LacNAc-binding molecule remains to be clarified in future research, as currently a little is known about cell proteins binding the sulfated probes. However, the data obtained will stimulate further analysis of the sulfated chains' involvement in processes of influenza virus motility towards and eventually binding the host cells. It should be noted that all the Galβ-terminated probes, *i.e.* galectin receptors, did not bind either MDCK or Vero-grown influenza viruses. Besides sialooligosaccharides and 6′-HSO_3LacNAc only trimannoside as Man_3-PAA-biot demonstrated notable binding with the viruses.

In summary, we have obtained new data on the presence of galactose-binding molecules on the surface of MDCK cells. We also revealed the 6′-HSO_3LacNAc binding ability of human influenza A and B viruses. In the current study, the functional investigation of detected molecules and their actual participation in virus/cell interaction was not addressed. What is the role of the host cell *lectinome* [59] towards the influenza virus, especially in formation of new dangerous strains, what carbohydrate chains and what particular viral glycoprotein(s) interact with the host cells, and what is the role of the sulfated oligosaccharide recognition by a virus, this is only a selection of the questions, which remain to be answered for a better understanding of the influenza virus entry, adhesion onto host cells and the ensuing events.

Acknowledgments We would like to thank Dr. P.R. Crocker for the mAbs to siglecs, Dr. G.V. Pazynina, Dr. E.Y. Korchagina and Dr. S.D. Shiyan for the synthesis of the oligosaccharides and glycoconjugates, G.P. Voznova for technical assistance, and Dr. E. Burtseva for submitted virus sample.

This work was partially supported by the grants from the Russian Foundation for Basic Research 01-04-49253, 04-04-49669, 04-04-49689, Russian Academy of Sciences program "Physico-chemical biology", and Consortium for Functional Glycomics (NIH grant No 5 U54 GM62116–03).

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