MISCELLANEOUS

The Effect of Losing Glycosylation Sites Near the Receptor-Binding Region on the Receptor Phenotype of the Human Influenza Virus H1N1

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Abstract—The receptor properties of influenza virus A/USSR/90/77 isolates are studied. The isolates are peculiar for losing glycosylation sites at the Asn131 receptor-binding region (GS 131) after passaging in mice and at the Asn158 region (GS 158) after cultivation in the presence of mouse serum. The loss of each carbohydrate residue increases the influenza virus affinity for carbohydrate chains with the terminal group Neu5Aca2-6Gal and reduces its affinity for Neu5Aca2-3Gal receptors. The effect is more pronounced in the GS158-depleted virus. Upon substitution of asparagine by aspartic acid, the electrostatic component of virus binding to the receptor is altered because of the increased negative charge on hemagglutinin. The virus receptor phenotype changes depending on the cultivation conditions. The isolate adapted to mice has higher affinity to mouse lung cell receptors, while the virus propagated in chick embryos in the presence of inhibitors has higher affinity to allantoic membrane cells.

Key words: influenza viruses, glycosylation sites, receptors

INTRODUCTION

The primary receptor determinant of the influenza virus (Flu) is sialic acid. Naturally occurring sialosides are distinguished by the type of bond between the sialic acid and the next element of the oligosaccharide chain, galactose. This can be either sialyl (α2-3)galactose (Neu5Acα2-3Gal) or sialyl(α2-6)galactose (Neu5Acα2-6Gal). Avian virus isolates usually have higher affinity for the first sialoside type, and those of the human Flu, for the second [1-4]. The ability to bind sialic acid is determined by the structure of the deepest part of the hemagglutinin receptor-binding site (RBS) [5, 6]. Interaction with neighboring residues of the oligosaccharide chain is mediated by amino acid residues at the rim and more distant residues around the active center [6-8]. Analysis of individual amino acid substitutions within and near the RBS revealed several mechanisms of modulating receptor specificity: (i) amino acid substitutions at positions 225, 226, 186, and 190 alter the receptor specificity to the Neu5Acα2-3Gal or Neu5Acα2-6Gal bonds between the sialic acid and galactose [7]; (ii) loss of glycosylation sites (GS) in the upper left corner of the RBS (see Fig. 1) facilitates binding to 3'-sialosides, while a GS loss rightward of the RBS increases the affinity for 6'-sialosides [7, 8]; (iii) substitutions leading to an increase of the positive charge facilitate virus binding to negatively charged sialosides and the negatively charged cell. The latter results in higher affinity for all sialosides regardless of the bond type. Increasing the negative charge has the opposite effect [7, 9, 10].

The second and third substitution types occur frequently when the virus propagation conditions somehow change, for instance, in the presence of inhibitors [11-13], after transition to a different host [7, 14] or during postreassortment when the hemagglutinin and neuraminidase activity balance is altered [10].

In earlier studies, we showed that adaptation of influenza virus isolate A/USSR/90/77 (USSR/90) to MDCK cell culture and mouse lungs leads to the loss of GS at Asn131 (GS 131) caused by an Asn→Asp substitution at this site [14]. Probably, the crucial factor here is replacement of chick embryo cell receptors with mammalian ones. In case of passaging the virus in mice, selective factors may be lectins and mucins of mouse lungs. It is to be emphasized that adaptation to a cell culture does not cause such the effect, even though the same mutation does regularly occur and GS 131 is lost. Adaptation of the same isolate to chicken eggs in the presence of mouse serum leads to Asp substitution for Asn158, which also causes a GS
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loss [11, 12]. Here the host cell remains the same, but the selective factor is the inhibiting effect of lectins and sialyl-containing macroglobulins of the serum. Interestingly, mutations leading to the GS131 and GS158 loss are structurally similar and located in close neighborhood in the upper right corner of the RBS. Both mutations enhance the virus ability to agglutinate mouse and sheep erythrocytes and reduce its sensitivity to mammalian serum inhibitors in hemagglutination inhibition reactions [11]. In earlier studies, we showed that the GS131-depleted Flu exhibits a higher affinity for Neu5Aca2-6Gal receptors and a lower affinity for certain 3'-sialyl glycoconjugates [8].

This work is aimed at studying the receptor-binding properties of the GS158-depleted virus.

EXPERIMENTAL

Reagents. N-Acetylneuraminic acid, 3'-sialyllactose, fetuin, and horseradish peroxidase were from Serva. Sialyl-containing polymers were synthesized as described in literature [15]. Equine and bovine serum macroglobulins and human glycophorin were isolated following a published protocol [8].

Viruses. The A/USSR/90/77 isolate (H1N1) was obtained from the strain collection of the Ivanovsky Institute of Virology. Isolates adapted to mice (further referred to as 131*) and to the inhibiting effect of the mouse serum (158*) were obtained as described elsewhere [11, 14]. Viruses were passaged in the 9-day chick embryo allantoic cavity, purified and stored at -20°C in 60% glycerol buffer containing 0.02 M Tris-HCl pH 7.4, 0.1 M NaCl, and 0.02% sodium azide [7].

Virus affinity for receptor analogs in solution. Association rate constants of the virus in reactions with sialosides were determined in a competitive inhibition reaction with fetuin–horseradish peroxidase conjugate (FP) as described in literature [16]. To study the correlation between virus affinity for fetuin and the ionic strength, the primary FP buffer was made without NaCl and different amounts of 5 M NaCl were added individually in reactions, after which the association constant ($K_{ass}$) was measured.

Mouse respiratory tract mucins. Mouse lungs with intact trachea were mounted on a 1-ml plastic tip filled with PBS. The lungs were rinsed with the buffer several times, and the sialic acid content was determined in the rinsing liquid [17].

Cell membranes. In order to isolate epithelial cells, allantoic membranes of 10-day chick embryos were passed through a 0.5 mm clearance of a Dounce homogenizer, and mouse lungs were forced through nylon gauze. Cell suspensions were washed with PBS, layered over a 50% Percoll solution, and centrifuged at low speed to pellet erythrocytes. The cells were further disintegrated by sonication and spun down at 1000 g. The supernatant was centrifuged at 40,000 g for 2 h to pellet membranes.

Virus affinity for total cell membrane sialoglycoconjugates. Cell membranes (10 μg/ml in protein) were suspended in PBS, ultrasound-disintegrated, and applied in 0.1 ml aliquots onto a 96-well plate. After 2 h the plates were water-rinsed and nonspecific binding was blocked with 0.1% BSA for 1 h. The virus was then suspended in 50 μl of PBS buffer containing 2 mg/ml BSA and 2 μM 4-amino-2-deoxy-2,3-dehydro-N-acetylnucleoside, applied to each well and incubated for 2 h at 4°C. The amount of virus conjugate was measured as described elsewhere [8] in 492 nm light absorption units ($A_{492}$). The data obtained were represented as Scatchard plots, with the line slope corresponding to the virus affinity for substrate.

RESULTS AND DISCUSSION

Three isolates of influenza virus H1N1 were studied: A/USSR/90/77 and its two mutants, 131* and 158* (GS131- and GS158-deprived, respectively). The effect of losing glycosylation sites on virus affin-
Influenza virus A/USSR/90/77 isolate affinity for naturally occurring and synthetic sialosides (in $K_{ass}$ units)

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>USSR/90</th>
<th>131*</th>
<th>158*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dextran sulfate**</td>
<td>4</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Monomers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neu5Ac</td>
<td>0.005</td>
<td>0.003</td>
<td>0.002</td>
</tr>
<tr>
<td>3'-sialyllactose (3'SL)</td>
<td>0.003</td>
<td>0.002</td>
<td>0.001</td>
</tr>
<tr>
<td>Synthetic polymers:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3'SLN-PAA</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>6'SLN-PAA</td>
<td>1</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>YDS-PAA</td>
<td>0.3</td>
<td>0.7</td>
<td>2</td>
</tr>
<tr>
<td>Naturally occurring sialosides:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human glycophorin</td>
<td>7</td>
<td>15</td>
<td>20</td>
</tr>
<tr>
<td>Equine macroglobulin</td>
<td>5</td>
<td>6</td>
<td>15</td>
</tr>
<tr>
<td>Bovine macroglobulin</td>
<td>5</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Chicken mucin</td>
<td>1</td>
<td>0.3</td>
<td>0.1</td>
</tr>
<tr>
<td>Allantoic fluid</td>
<td>0.1</td>
<td>0.03</td>
<td>0.02</td>
</tr>
<tr>
<td>Mouse trachea mucin</td>
<td>0.1</td>
<td>0.5</td>
<td>0.15</td>
</tr>
<tr>
<td>Mouse serum***</td>
<td>100</td>
<td>20</td>
<td>200</td>
</tr>
</tbody>
</table>

Note: 131* and 158* are Flu mutants deprived of glycosylation sites 131 and 158.

** Affinity for dextran sulfate given in (mg/ml)-1.

*** Affinity for mouse serum given in 50%-inhibitory serum titers.

Association constants for other sialosides are given in mM-1 confidence interval of average values.

Inhibitors and viruses were used in a competitive inhibition assay [16]. The table gives association rate constants for the three isolates in reactions with Neu5Ac, 3'-sialyllactose (3'SL), and synthetic polyacrylamide (PAA) polymers containing the following groups: 3'SL, 6'-sialyllactosamine (6'SLN), and (Neu5Ac2-6Galβ1-4GlCNacβ1-2Manα1-3,6Manβ1-4GlcNAcβ1-4GlcNAc, a bifurcating chain bearing two 6'SLN residues (YDS). It also gives estimates of virus affinity ($K_{ass}$ values) for a number of naturally occurring sialosides: human glycophorin, equine and bovine macroglobulins, chicken esophagus mucin, and liquids used in selecting the viral isolates—alantoic fluid, mouse serum, and mouse respiratory tract mucins. As evident from the table, the mutants have lower affinity for monomeric sialosides. In contrast with the mother isolate, their affinity for polymer sialylglycoconjugates depends on the type of bond between sialic acid and galactose. Virus binding to 3'SL-containing polymers decreases and, alternatively, increases to 6'SLN-PAA. The same behavior is observed with respect to naturally occurring sialosides. Human glycophorin and equine macroglobulin, which both bear 6'SLN groups, are bound by the 158* isolate with maximal efficiency, while bovine macroglobulin and chicken mucin exhibit the lowest affinity for the virus. Presumably, Neu5Ac2-3Gal represents a dominant surface group in these glycoproteins.

The 131* and 158* mutants share the ability to bind 6'SLN better and 3'SL poorer than does the mother isolate. This is not unexpected, as GS131 and GS158 are situated close to each other (Fig. 1). The GS158 loss has a greater impact on the phenotype, presumably because Asn158 is closer to the RBS. Higher affinity for 6'SLN in GS131- and GS158-deprived isolates correlates well with the receptor group allocation in the viral RBS. X-Ray studies of hemagglutinin H3 [6] and H9 [20] complexes with a 6'SLN-containing LSTc receptor analog showed that the receptor core is tilted toward the right side of the receptor pocket and directed toward residues 158 [6] or 131 [20] (Fig. 1). It is more difficult to explain the lower affinity for Neu5Ac2-3Gal groups. X-Ray data suggest that the receptor stems bearing these groups are directed leftward [20] or up-leftward [6] of the RBS, so that glycoside residues at positions 131 and 158 should not affect the binding. However, mutant strains differ from the mother isolate not only in the absence of saccharide residues but also in occurrence of a negatively charged aspartic acid residue in place of Asn153 or Asn158 in immediate proximity to the hemagglutinin RBS.

It is likely that lower virus affinity for 3'-silosides is associated with the electrostatics of virion interaction with the negatively charged carboxyl of sialic acid. In order to estimate the possible role of these interactions in virus binding to negatively charged receptors, we studied its affinity for negatively charged dextran sulfate (see table) and also the correlation between $K_{ass}$ and the ionic strength in reactions with fetuin in the three isolates (Fig. 2). As evident from the table, mutant nonspecific affinity for dextran sulfate and for 3'SL-PAA, in contrast to the mother isolate, decreases in parallel. Figure 2 shows that the dependence between receptor binding and ionic strength in the mother and mutant isolates is inverse around 0.1 M NaCl. USSR/90 affinity for fetuin falls drastically with the increase in ionic strength, while in the case of mutant isolates it first increases and declines slightly thereafter. This suggests that in vivo electrostatic forces favor virus binding to the receptor, while in more negatively charged mutants the balance is shifted toward electrostatic repulsion. This hypothesis, rather than structural rearrangements of the RBS, might explain lower affinity of mutant isolates for sialic acid, 3'-silosides, and Neu5Ac2-3Gal-glycoconjugates.

As follows from the table, the affinity for allantoic fluid is lower in the mutant isolates than in USSR/90. Poorer binding to allantoic fluid may be a...
selective factor in the case of mutant 158* passaged in chicken eggs. Nevertheless, its affinity for mouse serum present in the culture is not lower than that in the mother isolate. A similar behavior is typical of the mouse-adapted virus, in which the affinity for mouse lung mucins also increases. Probably, the “penalty” of having a higher affinity for inhibitors is counterbalanced by a higher ability to bind sialyl-containing receptors of the host cell. To test this hypothesis, we studied the virus ability to bind plasma membrane receptor molecules of allantoic membrane and mouse lung cells (Fig. 3).

The Flu receptor binding was determined in a solid-phase enzyme-linked assay [16]. Cell membranes were sorbed on 96-well plates, incubated with the virus, and the amount of conjugate was estimated using FP. Binding to membrane receptors was compared with binding to fetuin, which effectively binds to all virus isolates. The same samples treated with Vibrio cholerae neuraminidase were used as a control of binding specificity. Such treatment completely abolished Flu binding (data not shown).

To quantitatively estimate the virus ability to bind to different membranes, it was titrated and the data were processed as Scatchard plots, with the line slope corresponding to virus affinity for substrate. The binding of USSR/90 and both its mutants to fetuin, allantoic membrane, and mouse lung cell receptors is shown in Fig. 3. For the mother isolate, the slope is similar in all cases, which suggests similar virus affinity for all substrates (Fig. 3a). The ML-adapted 131* isolate exhibits higher affinity for mouse lung cell receptors (Fig. 3b). Mutant 158* passaged in the chick embryo allantoic cavity in the presence of mouse serum has higher affinity for AM receptors (Fig. 3c). The results obtained suggest an adaptive nature of losing GS131 and GS158. Despite the similarity in mutant receptor phenotypes, they differ slightly depending on selective cultivation conditions.
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REFERENCES


