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Mismatched hemagglutinin and neuraminidase specificities in recent human H3N2 influenza viruses

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Abstract

The hemagglutinin (HA) of influenza viruses initiates infection by binding to sialic acid on the cell surface via α2,6 (human) or α2,3 (avian) linkage. The influenza neuraminidase (NA) can cleave both α2,3- and α2,6-linked sialic acids, but all influenza NAs have a marked preference for the non-human α2,3 linkage. Recent H3N2 influenza viruses have lost the ability to agglutinate chicken red blood cells. To determine if changes in HA specificity or affinity correlate with NA specificity or activity, we examined red cell binding and elution of a series of H3N2 viruses. We found that the NA activity of many influenza viruses does not release binding by their HA. In some egg-adapted strains, lack of elution correlates with low levels of viral NA activity, and these elute rapidly when bacterial NA is added. However, a Fujian-like virus, A/Oklahoma/323/03, does not elute by its own NA or with *Vibrio cholerae* sialidase, and it binds to red cells pre-treated with *V. cholerae* sialidase. It elutes after addition of the broad specificity *Micromonospora viridifaciens* sialidase. Human glycophorin inhibits A/Oklahoma/323/03 hemagglutination 6-fold better than fetuin. We conclude that specific forms of sialic acid are used as receptor by recent human H3N2 influenza viruses, perhaps involving branched α2,6 sialic acid or α2,8 sialic acid structures on O-linked carbohydrates. The virus itself has no O-linked glycans, so even though the NA is not able to cleave receptors on cells, the viruses will not self-aggregate. It will be important to monitor efficacy of neuraminidase inhibitors in case there are NA-resistant receptors in the human respiratory tract that allow the viruses to be less dependent on NA activity.

Keywords: Human influenza virus; Specificities; Elution; Sialidase; Glycophorin; α2,6 sialic acid; α2,8 sialic acid; Hemagglutinin; Neuraminidase activity

Introduction

Extending from the plasma membrane of mammalian cells is an array of glycoproteins, glycolipids, and proteoglycans known as the glycocalyx. Many of the glycan chains terminate with sialic acid, in α2,3, α2,6, or α2,8 linkages to galactose, *N*-acetylgalactosamine, or *N*-acetylglucosamine in linear or branched structures. Sialic acids in specific linkages are recognized by sialic acid binding proteins of many pathogens. Most pathogens bind to α2,3-linked sialic acid, with some preferring α2,8 linkages (Angata and Varki, 2000; Gagneux et al., 2003; Karlsson, 1995). Among the few pathogens that show a preference for α2,6-linked sialic acids are human influenza viruses. Avian viruses bind to α2,3-linked sialic acid (Connor et al., 1994; Rogers et al., 1983), so adapting an avian virus to mammalian cells is accompanied by a specificity change from α2,3 to α2,6. There have been reports that new pandemic viruses show altered specificity over the first few

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years (Carroll and Paulson, 1985; Connor et al., 1994; Matrosovich et al., 2000). Receptor binding is mediated by the viral hemagglutinin (HA) and the binding site has been delineated by X-ray crystallography of human and avian HAs with bound sialylated oligosaccharides (Eisen et al., 1997; Skehel and Wiley, 2000). The NA cleaves sialic acid from glycoconjugates and therefore is considered a receptor-destroying enzyme.

The activities of HA and NA were initially demonstrated by the ability of the virus to agglutinate and elute from red blood cells. Absence of neuraminidase activity leads to aggregation of viruses due to the HA binding to sialic acid on adjacent viral particles. Although it is often said that NA activity is required to prevent viruses binding to receptors on the cell surface, electron micrographs in two different studies show virus aggregates far outnumber virus-cell binding and it was concluded that virus–virus aggregation is the reason viruses do not spread in the absence of NA activity (Liu et al., 1995; Palese et al., 1974). If destruction of receptors on the cell surface was the only function of NA, then it would follow that viruses must have matched HA and NA specificities. There is some evidence that changes in HA receptor binding activity are accompanied by concomitant changes in the viral NA to maintain a functional balance between receptor binding activity and receptor destroying activity (Baum and Paulson, 1991; Kaverin et al., 1998; Mitnaul et al., 2000; Wagner et al., 2000). However, there are instances of mismatched specificity such as in early H2N2 viruses (Baum and Paulson, 1991; Choppin and Tamm, 1959). There are also reports that NA is required for entry, perhaps destroying decoy receptors such as 2,3-linked sialic acid on mucins (Matrosovich et al., 2004) or removal of sialic acid from the vicinity of the HA binding site (Ohuchi et al., 1995).

Recent H3N2 influenza viruses have lost the ability to bind to chicken red blood cells. This has been reported to result from decreased affinity for α2,6 sialic acid, so that although there is a considerable amount of α2,6 sialic acid on chicken red cells, the density may be too low for agglutination (Mochalova et al., 2003; Nobusawa et al., 2000; Thompson et al., 2004). Here we have studied the HA and NA specificities of H3N2 viruses by measuring their binding to and elution from red blood cells, and we found there is a mismatch in NA and HA specificities in "Fujian-like" H3N2 viruses. This study shows the possibility of an altered receptor in these recent viruses.

Results

In the early winter of 2003 there was a significant outbreak of H3N2 influenza throughout North America, and we obtained 4 Oklahoma City isolates cultured in primary rhesus monkey kidney cells (P0 and P1) from throat swabs. The four isolates have the same binding and elution properties and we have focused studies on one isolate from a young child, A/Oklahoma/323/03. This virus grows robustly in MDCK cells (10^7 TCIU or 10^{8-9} particles per 10^5 cells). The HA and NA gene sequences were determined by sequencing RT-PCR products and compared to sequences in the Influenza Sequence Database (Macken et al., 2001). The HA1 amino acid sequence is 99.4% identical to A/Fujian/411/02 and 100% identical to A/New York/50/03. There is less identity to A/Panama/2007/99 (97.9%) or A/California/7/2004 (98.2%). The NA head domain sequence is 99.7% identical to A/NewYork/50/03. NA sequences of Fujian/02 and California/04 are not in the databases, but A/OK/323/03 NA is 97.0% identical to A/Wyoming/3/2003, 97.0% to A/Wellington/1/2004, and 96.4% to Panama/2007/99.

Failure to elute from red blood cells

In common with other Fujian-like viruses, Oklahoma/323/03 proved refractory to adaptation to grow in chicken eggs and does not agglutinate chicken red blood cells. It agglutinates human red cells but does not elute from them, meaning that the NA activity does not cleave the receptor bound by the HA. We therefore carried out a series of detailed experiments to determine the red cell binding and elution properties of Oklahoma/323/03 and a panel of H3N2 viruses isolated earlier: Memphis/1/71, Udorn/307/72, Victoria/3/75, Philippines/2/82, Leningrad/360/86, Beijing/353/89, Oklahoma City isolates 3003, 5098 from 1996, and Memphis/31/98.

Red cells were agglutinated at 4 °C and elution observed at 26 °C at 2 h and overnight (Table 1). Only a few viruses (Mem/71, 5098/96 and Mem/98) eluted from red blood cells by the action of their NA, as shown by elution at 26 °C and failure to rebind when the wells were mixed and chilled again. When viruses were grown in eggs, Udorn/72 and 3003/96 also eluted and failed to rebind. MDCK-grown Victoria/75, Udorn/72, and 3003/96 completely or partially rebound to red cells, indicating that elution was the result of low affinity rather than removal of receptors by NA cleavage. Philippines/2/82, Leningrad/360/86, Beijing/353/89, Oklahoma/323/03, and OK/Tf/03 bind very tightly to red blood cells and do not elute at all either by thermal motion or with their own NA activity. We also screened the egg-grown vaccine strains Sydney/97, Panama/99, and Wyoming/03 and found they also fail to elute from human red cells. We initially studied elution at 37 °C but cell lysis at this temperature confuses the results. Elution was a little faster but not more extensive at 37 °C than at 26 °C, so we used the lower temperature for the detailed studies.

Levels of NA activity

To determine if failure to elute is due to insufficient NA activity, we measured the level of NA activity in the viruses relative to HA titer. The NA/HA results are expressed relative to Udorn/72 (Table 1) and while the ratio itself does

Table 1
Elution of H3N2 viruses from human red blood cells[a]

Virus	MDCK-grown virus					Egg-grown virus		
	NA_{MUN}/HA (relative to Udorn/72)	Self-elution	Rebinding	Vch sialidase elution	Mvi sialidase elution	Self-elution	Rebinding	Vch sialidase elution
Memphis/71	0.6 ± 0.2	slow[b]	no	fast	fast	slow	no	fast
Udorn/72	1.0	slow	partial	fast	fast	slow	no	fast
Victoria/75	1.7 ± 0.2	slow	yes	fast	fast	–[c]	–	–
Philippines/82	0.08 ± 0.05	no	n/a[d]	fast	fast	no	n/a	fast
Leningrad/86	0.1 ± 0.07	no	n/a	slow	fast	–	–	–
Beijing/89	0.2 ± 0.06	no	n/a	fast	fast	no	n/a	fast
3003/96	1.0 ± 0.4	slow	partial	fast	fast	slow	no	fast
5098/96	3.1 ± 0.19	fast	no	fast	fast	n/a	n/a	n/a
Mem/98 (HG)	1.8 ± 0.3	slow	no	fast	fast	slow	no	fast
Memphis/98	2.2 ± 0.8	slow	no	fast	fast	nea[e]	n/a	n/a
OK/323/03	1.6 ± 0.3	no	n/a	no	slow	nea	n/a	n/a
OK/Tf/03	1.4 ± 0.5	no	n/a	no	slow	nea	n/a	n/a

[a] Results shown are from 3 or more independent experiments.
[b] Fast: eluted within 2 h; slow: eluted overnight; no: no elution overnight.
[c] – = experiment not done.
[d] n/a = not applicable.
[e] nea = did not adapt to eggs.

not have physical meaning, the comparisons between viruses show the relative levels of NA activity compared to HA titer. Table 1 shows that NA activity per HA unit is very low in vaccine strains Philippines/82, Leningrad/86, and Beijing/89 when these are grown in MDCK cells. This presumably accounts for their failure to elute, but the 2003 Oklahoma isolates have quite high levels of NA so their failure to elute is not due to insufficient NA activity. Vaccine strains Panama/99 and Wyoming/03 have similar NA/HA ratios as OK/03 and these also fail to elute (results not shown).

Elution by broader-specificity sialidases

To further investigate the specificity of binding, we asked if viruses that did not elute under their own NA activity could be eluted by broader-specificity sialidases. We tested elution by Vch sialidase which cleaves unbranched or branched α2,3 and α2,6 and some α2,8 sialic acid (Corfield et al., 1981; Schauer, 1982) and Mvi sialidase which cleaves a wider variety of sialic acids including more highly branched structures and α2,8-linked sialic acid (Air and Laver, 1995; Aisaka et al., 1991; Schauer, 1982; Stray et al., 2000). As shown in Table 1, viruses up until 1998 elute with Vch sialidase, but the 2003 isolates do not. Table 2 shows the effect of increasing sialidase concentration on elution. Beijing/89 is eluted by both Vch and Mvi sialidases but requires 100-fold more enzyme units of Vch sialidase than of Mvi. Oklahoma/03 was eluted by Mvi sialidase at 100-fold increase over the amount required to elute Beijing/89, and was not eluted by Vch sialidase even at 10-fold higher concentration than was used to elute Beijing/89.

To determine if failure to elute by Vch sialidase is due to steric inhibition such that the enzyme cannot reach the receptor after it is bound, we pre-treated red cells with sialidases and tested binding. We used red cells pre-treated with 2 mU/ml of Vch sialidase, and found that the 2003 viruses still agglutinate them, but pre-treatment of red cells with 2 mU/ml Mvi sialidase abrogated binding. These results mimic the elution results and show that the resistance of the receptor to Vch digestion is a chemical resistance rather than steric blocking by the virus.

Specificities of N2 NAs

NA activity in Table 1 was measured using a low MW substrate, 4-methylumbelliferyl-*N*-acetylneuraminic acid (MUN). To see if these results are skewed by using a non-physiological substrate, we measured NA activity using the high MW glycoprotein fetuin and the trisaccharide α2,6 *N*-acetylneuraminyllactose, calculated NA activity per HA unit, and normalized this against Udorn/72 (Fig. 1). We found that the NA activities of Philippines/82 and Beijing/89 are low with all substrates, although egg-grown Philippines/82 and Beijing/89 have more NA activity relative to HA than the MDCK-grown viruses. Interestingly,

Table 2
Elution of influenza viruses from human red blood cells by bacterial sialidases

Sialidase units ($\times 10^{-5}$)	Elution by Vch sialidase			Elution by Mvi sialidase		
	H3N2		H1N1	H3N2		H1N1
	Beijing/89	OK/03	OK/01	Beijing/89	OK/03	OK/01
100	Y[a]	N	Y	Y	Y	Y
10	Y	N	N	Y	Y	Y
1	N	N	N	Y	N	N
0.1	N	N	N	Y	N	N
0	N	N	N	N	N	N

[a] Y = elutes, N = no elution.

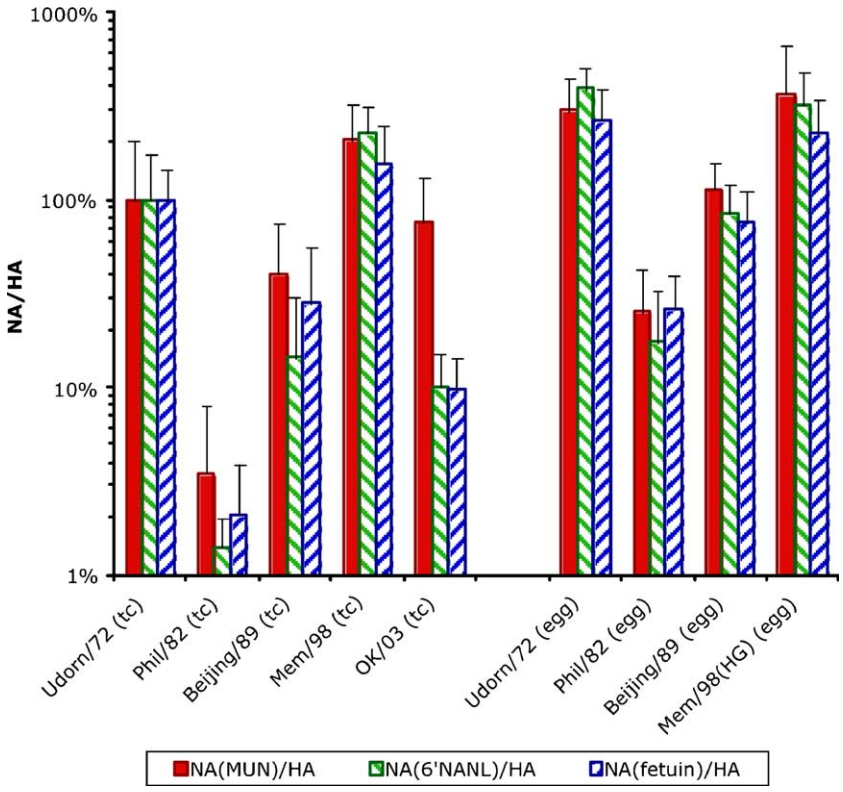

Fig. 1. NA activity of H3N2 viruses relative to HA activity. NA activity was measured by the thiobarbituric acid method using a large substrate (fetuin), a trisaccharide (α2,6 sialyllactose), and by fluorescence using a small artificial substrate (4-methylumbelliferyl-*N*-acetylneuraminic acid, MUN). The activities, as NA/HA ratios, are normalized to those of Udorn/72. The results shown are an average of 5 experiments ± standard deviation.

the "Fujian-like" virus A/Oklahoma/323/03 has a high level of NA when measured with a small substrate (MUN) (~80% of Udorn/72) but activity with fetuin is only 10% that of Udorn/72. However, the low activity with a high molecular weight substrate does not explain the inability of OK/03 to elute from red blood cells, since it is also not eluted by Vch sialidase.

A switch in HA specificity from the α2,3 to the α2,6 linkage of sialic acid when an influenza virus (or HA gene) "jumps" from avian to human host is expected to be accompanied by a similar change in NA specificity. There was evidence for some change in the H2N2 and early H3N2 viruses (Baum and Paulson, 1991; Kobasa et al., 1999, 2001) but the change was rather minor, from 5:1 to 3:1 preference for α2,3 sialic acid cleavage. We measured NA activities of a subset of viruses with α2,3 and α2,6 sialyllactose. Our results (Fig. 2) show that the increasing activity for the α2,6 linkage has not been continued after 1972. In recent viruses there is a 5:1 to 6:1 favoring of α2,3 sialic acid over the presumptive human receptor, α2,6 sialic acid.

What component(s) of the red cell surface is bound by the Oklahoma/03 virus?

The Fujian-like virus does not bind to fetuin when tested in a sandwich ELISA assay, but it does bind to human red cell membranes and to human glycophorin (Gulati et al., 2005). To determine the difference in affinity we measured the ability of fetuin and glycophorin to inhibit the hemagglutinin of OK/03 and calculated the

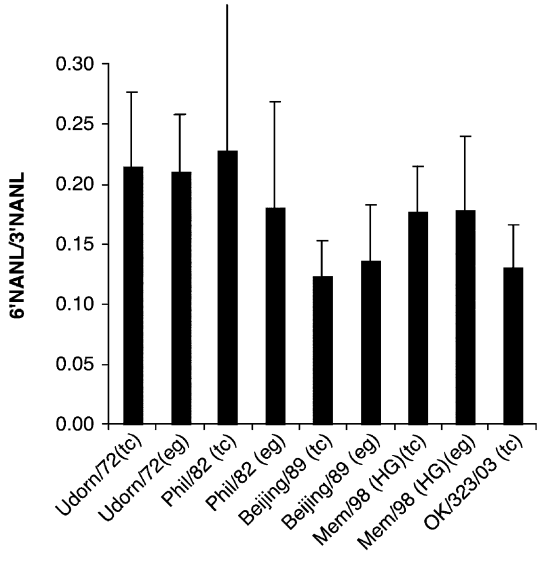

Fig. 2. The linkage preference of influenza N2 NAs was measured by the thiobarbituric acid method using α2,6 and α2,3 sialyllactoses. Viruses were grown in tissue culture (tc) or in eggs (eg). Ratios (α2,6/2,3) shown here are an average of 5 experiments ± standard deviation.

concentration, in terms of sialic acid, that blocked hemagglutination (IC_{100}). Fig. 3 shows that human glycophorin is a 6-fold more potent inhibitor of hemagglutination of the OK/323/03 virus than fetuin per sialic acid and that OK/03 has high affinity for glycophorin (IC_{100} 22 μM). The high affinity for glycophorin is shared by 3003/96, but the binding entity is different since 3003/96 is eluted partially by its own NA and completely by Vch sialidase. Affinity for fetuin and glycophorin is low and approximately equal for Udorn/72 and Memphis/98 ($IC_{100} \geq 200$ μM). We resuspended the red cells in each well and left the HI titration plate at room temperature to see if the viral NA of OK/03 could remove sialic acid from the fetuin or glycophorin and hence relieve the inhibition of hemagglutination, but it did not, confirming that the receptor is resistant to viral NA. Viruses that do not elute from red cells have high affinity for glycophorin and bind to sialic acid that is resistant to the viral NA activity (Fig. 3 and Table 1).

We compared release of sialic acid by Vch and Mvi sialidases from purified glycophorin and fetuin. For glycophorin there was no significant difference in either the kinetics of release or the total sialic acid released. For fetuin, there was a more rapid release of sialic acid by Mvi compared to Vch sialidase, but the end points were similar (results not shown), indicating that most of the O-linked sugars on glycophorin are de-sialylated by Vch sialidase and that the resistant sialic acid must be a minor species.

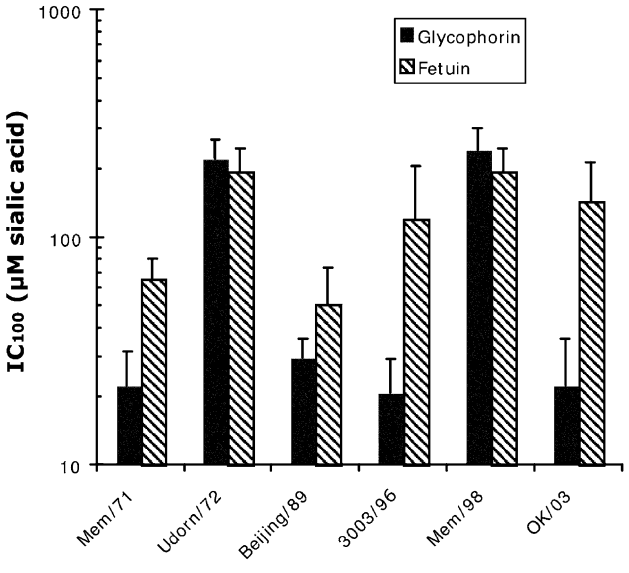

Fig. 3. Inhibition of H3N2 virus hemagglutination by glycophorin or fetuin. Glycophorin or fetuin was added to the first wells and serially diluted (1:3), then 4 HAU of virus was added to each well and incubated for 10 min at ice before adding red blood cells. The plates were placed at 4 °C for 90 min and the last well showing no agglutination was recorded. The IC_{100} is the lowest concentration of glycophorin or fetuin that inhibited hemagglutination completely. The results shown are an average of 4–6 experiments ± standard deviation.

Discussion

We have shown that the Fujian-like virus is mismatched in HA and NA specificity. The HA binds with high affinity to some moiety on the surface of human red blood cells that is highly resistant to the viral NA activity, and indeed to Vch sialidase. It is, however, sensitive to Mvi sialidase, suggesting the receptor is some form of sialic acid, a situation reminiscent of the HB binding site on N9 NA (Air and Laver, 1995). The NA activity of the Fujian-like OK/03 virus can cleave α2,6-linked sialic acid from a trisaccharide but does not release the virus from red cells. A similar lack of release from red cells in older H3N2 egg-adapted vaccine strains appears to be due to low NA activity since these viruses readily eluted with Vch sialidase. In contrast, the OK/03 virus has been passaged only in mammalian cells, has adequate levels of NA activity, and does not elute with Vch sialidase. Comparison of NA specificity among different H3N2 viruses for α2,3- or α2,6-linked sialic acid showed no significant differences, indicating there is no change in NA specificity accompanying or responsible for altered binding specificity. Also there are no amino acid changes near the active site pocket of NA of non-eluting influenza viruses. All influenza NAs have a marked preference for α2,3-linked sialic acid, and it has been suggested that this is to remove the α2,3 sialic acid from mucins that might otherwise trap virus before it reaches the cell surface (Burnet, 1948; Couceiro et al., 1993). However, we find no binding of the 2003 virus to α2,3 sialic acid (data not shown) using a direct binding assay (Wu and Air, 2004), in accord with other studies on binding specificity of recent H3N2 viruses (Mochalova et al., 2003).

The loss of ability to agglutinate chicken red cells occurred during the 1990s and has been correlated with lower affinity for α2,6 sialic acid (Mochalova et al., 2003; Nobusawa et al., 2000). The change to NA-resistant binding occurred between 1998 and 2002 (Table 1). Of the 21 amino sequence differences in HA1 between Mem/98 and Oklahoma/03, there are 4 that have been implicated in sialic acid specificity (Q156H, S186G, W222R, and G225D) (Eisen et al., 1997). However, not enough structural information is available to attribute specificity differences to these changes.

To further investigate the HA binding specificity, we compared binding to two different glycoproteins. The OK/03 virus binds to glycophorin in an ELISA assay but not to fetuin (Gulati et al., 2005), and fetuin is a poor inhibitor of hemagglutination compared to glycophorin (Fig. 3). Human glycophorin has 16 O-linked sugar chains and only one N-linked sugar chain in 131 amino acids (Pisano et al., 1993; Tomita et al., 1978), while bovine fetuin has 3 O-linked and 3 N-linked glycans in 341 amino acids (Edge and Spiro, 1987; Green et al., 1988). The numbers of sialic acids are about 30 per mole of glycophorin and about 16 per mole of fetuin. Thus, the density of sialic acids on glycophorin is considerably higher than on fetuin. The proportion of α2,6 sialic acid is 43% of total sialic acid in glycophorin, 42% in

fetuin. The chemical structures of the N-linked oligosaccharides of glycophorin and fetuin are the same; α2,6 sialic acid occurs in terminal and branched triantennary sugars, as on the viral glycoproteins.

Mvi sialidase cleaves sialic acids in branched linkages, but Vch sialidase only cleaves sialic acid linked to internal Gal or GalNAc under certain conditions (Barton and Rosenberg, 1973; Schauer, 1982). We digested fetuin and glycophorin with Vch and Mvi sialidases to determine if the Mvi enzyme cleaves the branched O-linked structures of glycophorin more efficiently than Vch sialidase, but it did not, suggesting that the binding is not predominantly to NeuNAcα2,3Galβ1,3(NeuNAcα2,6)GalNAc-, which makes up 80% of the O-linked structures in glycophorin (Bendiak et al., 1989; Cumming et al., 1989; Fukuda et al., 1987). This experiment also showed that the increased binding of Fujian-like virus to glycophorin as compared to fetuin is not just because of increased density of sialic acid. Another candidate is the α2,8 sialylated modification of O-linked glycans of glycophorin (Fukuda et al., 1987), that is cleaved more efficiently by the Mvi sialidase than the Vch enzyme (Aisaka et al., 1991; Schauer, 1982) and binds variants of NWS HA (Wu and Air, 2004). There may also be contributions from modifications of "downstream" sugars, such as fucosylation or sulfation that might lead to resistance to cleavage by viral and Vch NAs (Mian et al., 1979). H5N1 chicken influenza viruses display a high affinity for NeuAcα2,3Galβ1,4(6−HSO_3)GlcNAc- (Gambaryan et al., 2004), but the authors did not report if human viruses bind sulfated structures or if the viral NA can cleave sialic acid from sulfated structures. In any case, the major contribution to increased binding of the OK/03 virus to glycophorin over fetuin (Fig. 3) seems to be from O-linked sugar chains. The virus itself has no O-linked glycans, so viruses that bind sialic acids specific to O-linked carbohydrates will not self-aggregate, even though the NA is not able to cleave the receptors on cells.

The glycoconjugates on the surface of human respiratory tract cells do not, of course, include glycophorin, but it is known that they do include many glycoproteins with mucin-like O-linked carbohydrates as well as the secreted true mucins. The mucins secreted by goblet cells contain only α2,3-linked sialic acid (Baum and Paulson, 1990) but the structures of O-linked glycans on other cell types are largely unknown although they are rich in α2,6 sialic acid (Baum and Paulson, 1990; Couceiro et al., 1993). It was recently reported that influenza virus A/Udorn/307/72 requires host cell N-linked glycoprotein for internalization into cells, but the virus still bound to cells lacking N-linked glycoprotein (Chu and Whittaker, 2004), so it is possible the essential N-linked glycoprotein is the co-receptor that mediates the virus entry after initial sialic acid binding (Stray et al., 2000). One difference in recent H3N2 viruses is increased binding to synthetic oligosaccharides containing 6′sialyllactosamine and loss of binding to 6′sialyllactose (Mochalova et al., 2003), but the significance of this observation for binding to cell surfaces is unclear since 6′sialyllactose does not occur in N-, O-, or lipid-linked structures.

The OK/03 virus grows efficiently in MDCK cells, indicating either that the non-cleavable receptor is not present, or that the virus is not dependent on receptor-destroying activity. We tested this by growing OK/03 in MDCK cells in the presence of oseltamivir, and found little evidence of inhibition by this potent inhibitor of NA activity as reported in other studies, where it has been shown that the NA of recent viruses is sensitive to oseltamivir in the enzymatic assay but virus replication is resistant to the drug when tested by plaque reduction assay in MDCK cells (Gubareva, 2004; Kiso et al., 2004; Thompson et al., 2004). When viruses become resistant to NA inhibitors under selection in MDCK cells, the mutation is often in the HA rather than in the NA, lowering the affinity of HA for sialic acid to overcome the requirement for NA (Air et al., 1999; Bantia et al., 1998; McKimm-Breschkin et al., 1996). It has been found that some of the viruses that are resistant in tissue culture to NA inhibitors due to mutations in the HA are fully sensitive in animal models, indicating a role of NA that is distinct from its receptor-destroying activity (McKimm-Breschkin, 2000). In accord with this, inhibitor-resistant HA mutants have not yet seen in clinical studies (Gubareva, 2004). A recent report reiterates older observations that NA activity may function in entry of influenza viruses into cells (Matrosovich et al., 2004). NA activity also exposes terminal galactose residues that are receptors for secondary bacterial infection (McCullers and Bartmess, 2003). Further understanding of the basis of this mismatch of HA and NA activities will give us insights into the functional receptors used in the human respiratory tract and the role of NA in recent human H3N2 influenza viruses.

If the virus binds to a receptor that is not cleaved by NA, and if this receptor is on O-linked glycans and therefore not included in the glycan structures on the virion, the need for NA activity may be reduced leading to ineffectiveness of NA inhibitors. Thus, it will be important to monitor efficacy of neuraminidase inhibitors in vivo in case these changes in binding to human red cells extend to the human respiratory tract.

Materials and methods

Viruses

The viruses used in this study include A/Oklahoma/323/03, a Fujian-like isolate; A/Memphis/31/98, a Sydney-like isolate provided by Dr. R.G. Webster (Gulati et al., 2002); two 1996 Oklahoma H3N2 isolates termed 3003, 5098; and a 2003 H3N2 isolate termed Tf; Udorn/307/72 (from Dr. Ming Luo, UAB); reassortant A/Memphis/1/71(HA)-Bel/42(NA) (Laver et al., 1979); and vaccine strains Philippines/2/82, Leningrad/360/86, and Beijing/353/89 (all high-growth egg-adapted reassortants with internal genes from

A/PR/8/34). A 2001 H1N1 Oklahoma isolate called 7485 was also used in some experiments for comparison.

The viruses were grown in Madin–Darby canine kidney (MDCK) cells (Liu and Air, 1993) or in embryonated chicken eggs and purified by sucrose gradient centrifugation (Laver, 1969).

Neuraminidase assays

NA activity was determined by the thiobarbituric acid method using fetuin, α2,3 sialyllactose (3′SL) or α2,6 sialyllactose (6′SL) as substrates (Aymard-Henry et al., 1973; Lentz et al., 1987), or by fluorescence using 4-methylumbelliferyl-*N*-acetylneuraminic acid as substrate (Potier et al., 1979).

Hemagglutination (HA) and hemagglutination-inhibition assay (HI)

Viruses were serially diluted in 50 μl of CaMg-saline (0.25 mM $CaCl_2$, 0.8 mM $MgCl_2$ in borate-buffered saline, pH 7.2) and 50 μl of washed chicken (0.5%) or human (0.8%) red blood cells added. The plates were kept at 4 °C and agglutination was read at 90 min. The plates were then left at 26 °C to monitor elution of virus from red cells (button formation). Elution was recorded at 2 h and overnight. The wells were then mixed gently and placed at 4 °C for 90 min to determine if rebinding occurred, to distinguish elution due to neuraminidase activity (no rebinding) from elution by thermal motion disrupting weak binding (re-binds on cooling). Elution by sialidase from *Micromonospora viridifaciens* (Mvi) or *Vibrio cholerae* (Vch) was done by adding the enzyme after recording the agglutination. Plates were incubated at 26 °C for 2 h and elution recorded.

Sialidase-treated red cells were obtained by incubating them in 2 mU/ml sialidase from *M. viridifaciens* (Mvi) or *V. cholerae* (Vch) at 37 °C for 1 h. Then the red cells were put on ice for 5 min. HA assays were performed at 4 °C, where the NA activity is essentially zero.

To determine the inhibitory effect of glycophorin or fetuin on hemagglutination, wells were filled with 50 μl of CaMg-saline. Glycophorin (25 μl of 2 μg/μl) or fetuin (10 μg/μl) was added to the first wells and serially diluted (1:3), then 25 μl of influenza virus containing 4 HA units was added to each well. The plates were kept on ice for 10 min and then 50 μl of 0.8% human red blood cells added. The plates were placed at 4 °C for 90 min and the last well showing no agglutination was recorded. The IC_{100} is the concentration of glycophorin or fetuin in the highest inhibiting dilution.

NA/HA ratios

To obtain a measure of NA activity of each virus preparation relative to its HA activity, the fluorescence reading generated in 20 min divided by the HA titer of 50 μl of appropriate dilution of virus was normalized to 100% for Udorn/72. For other substrates, the absorbance reading in the thiobarb method divided by the HA titer was compared to that of Udorn/72 in the same way.

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