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Influenza virus infection of desialylated cells

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Sialic acid has long been considered to be the sole receptor for influenza virus. The viral hemagglutinin (HA) is known to bind cell surface sialic acid, and sialic acids on viral glycoproteins are cleaved by the viral neuraminidase (NA) to promote efficient release of progeny virus particles. However, NWS-Mvi, a mutant virus completely lacking NA, grows well in MDCK cells continuously treated with exogenous neuraminidase (sialidase). Exogenous sialidase quantitatively releases all sialic acids from purified glycoproteins and glycolipids of MDCK cells and efficiently removes surface sialic acid from intact cells. Binding of NWS-Mvi and parent influenza viruses to MDCK cells is indistinguishable, and is only partially reduced by sialidase treatment of the cells. Both mutant and wild-type viruses enter enzymatically desialylated cells and initiate transcription. The ability of influenza A reassortant viruses to infect desialylated cells is shared by recent H3N2 clinical isolates, suggesting that this may be a general property of influenza A viruses. We propose that influenza virus infection can result from sialic acid–independent receptors, either directly or in a multistage process. When sialic acid is present, it may act to enhance virus binding to the cell surface to increase interaction with secondary receptors to mediate entry. Understanding virus entry will be critical to further efforts in infection control and prevention.

Key words: influenza/virus receptor/virus binding/virus entry/sialic acid

Introduction

Influenza virus particles carry both a sialic acid–specific lectin (hemagglutinin, HA) and a sialidase (neuraminidase, NA). Influenza HA mediates both binding to the cell surface and fusion of viral and host membranes. The activities of HA and NA were initially demonstrated by the ability of the virus to agglutinate and elute from red blood cells (Hirst, 1941). The moiety removed by the viral enzyme activity was shown to be sialic acid (Gottschalk, 1957), and treatment of red blood cells with bacterial sialidases prevented hemagglutination. NA is critical in virus dissemination; mutant viruses lacking NA activity form large aggregates upon budding (Palese *et al.*, 1974; Liu *et al.*, 1995), presumably by interaction of HA with sialic acid attached to HA and NA glycoproteins of neighboring virus particles. The infectivity of the aggregated progeny of NA-minus mutant viruses is extremely low, but increases upon disaggregation by sialidase or sonication.

To study the roles of HA and NA in viral entry and budding, we previously isolated mutant viruses completely lacking sialidase activity due to massive deletions of almost all of the NA gene segment, including all residues of the enzyme active site. Propagation of these mutant viruses requires large amounts of exogenous sialidase added to the medium (Yang *et al.*, 1997). This observation raises the paradox that influenza viruses, thought to require sialic acid for binding to cells, can be efficiently propagated in the continuous presence of high levels of sialidase. Therefore, we have investigated the requirement for sialic acid for entry of mutant or wild-type viruses in Madin-Darby canine kidney (MDCK) cells, the most commonly used cell line for laboratory cultivation of influenza virus. Our results show that virus can bind to and infect cells that have no accessible surface sialic acid. Thus, sialic acid–mediated binding of virus to target cells is not required for virus entry, although the presence of sialic acid does increase the efficiency of virus infection.

We propose that multiple receptors are used by influenza virus, mediating entry either independently of sialic acid or via a multi-step entry pathway. Initial binding may be via an abundant low affinity receptor (sialic acid) which then allows virus to interact with a rarer determinant of entry. At higher multiplicity of infection, an apparent requirement for sialic acid is circumvented. Understanding the nature of the multiple interactions required for virus entry may provide further targets for antiviral therapy.

Results

As NWS-Mvi infects cells in the presence of large amounts of sialidase, we wished to determine if any sialic acid was retained at the surfaces of treated MDCK cells, whether influenza viruses infect cells in the absence of surface sialic acid, and if the NA-minus mutant NWS-Mvi and wild-type NWS-Tok viruses use the same kinds of molecules as cellular receptors. We compared binding of mutant and wild-type viruses to desialylated and untreated MDCK cells and assayed influenza-specific mRNA (coding for HA) as a measure of productive virus entry. We assessed the ability of both viruses to initiate

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infection in desialylated and untreated MDCK cells, and also examined multicycle virus growth under the same conditions.

Complete removal of susceptible sialic acids from intact MDCK cells by sialidase treatment

Treatment of MDCK cells with different sialidases released a maximum of approximately 1.6 nmol sialic acid per million cells at 37°C (Figure 1a). Release of sialic acid from MDCK cell monolayers with varying amounts of *Micromonospora viridifaciens* (*Mvi*) sialidase on ice reached a maximum of approximately the same amount (Figure 1b), indicating that all accessible sialic acids were being removed under the conditions used for virus binding (Figure 2), and that a small proportion of total sialyloglycan is translocated to the cell surface over the course of the 2 h digestion at 37°C. Even at 0°C, all available sialic acids were completely released by *Mvi* sialidase, as no further increase in released sialic acid was detected by increasing the amount of sialidase used. Given that *M.viridifaciens* sialidase was found to be ~60-fold less active on ice than at 37° (data not shown), the conditions used for desialylation at 37°C prior to binding and infection studies represent vast excess of the sialidase activity required for complete removal of susceptible sialic acids.

The sialidase of *Salmonella typhimurium* (*Sty*) cleaves α2–3 linked sialic acid approximately 1000-fold more efficiently than those sialic acids linked α2–6, while sialidases of *Arthrobacter ureafaciens* (*Art*) and *Vibrio cholerae* (*Vch*) have much broader substrate preferences. Both cleave α2–3 and α2–6 linked sialic acids (Reuter and Schauer, 1994). *M.viridifaciens* sialidase has similar specificities to those of *Art* (Uwajima, 1989; S.J.Stray and G.M.Air, unpublished observations). Based upon the activities determined for each enzyme using fetuin as a substrate, even the least amount of enzyme used in this study (*Vch*) would release the measured amount of free sialic acid within the first ten min of the 2 h incubation. We can therefore conclude that the treatments at 37°C removed all susceptible surface sialic acids and that the difference in amounts of sialic acid released by the different sialidases represents differences in the amounts of available substrates for each enzyme. As *S.typhimurium* sialidase removes only about half the sialic acid released by other sialidases, MDCK cells must have both α2–3 and α2–6 linkages to sialic acid (Figure 1a). The only limitation on the release of sialic acids by *Mvi* sialidase is thus the presence at the surface of susceptible sialylated molecules; all accessible molecules are cleaved to completion.

All MDCK cell sialic acids are inherently cleavable by sialidase

To determine if all sialic acids in MDCK cells are cleavable by *Mvi* sialidase, we fractionated whole cells and analyzed the susceptibility of isolated glycolipid (organic extract) and glycopeptide (protease-treated, post-extract residue) fractions to both sialidase and mild acid release of sialic acid. Chemical analyses of conjugated sialic acids with or without sialidase treatment of MDCK cells were performed. To enhance sensitivity, cells were metabolically radiolabeled with [^{3}H]-glucosamine, which is taken up by cells and incorporated almost exclusively into N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc) and sialic acid (SA) in glycoconjugates (Varki, 1994). Total glycopeptide and glycolipid

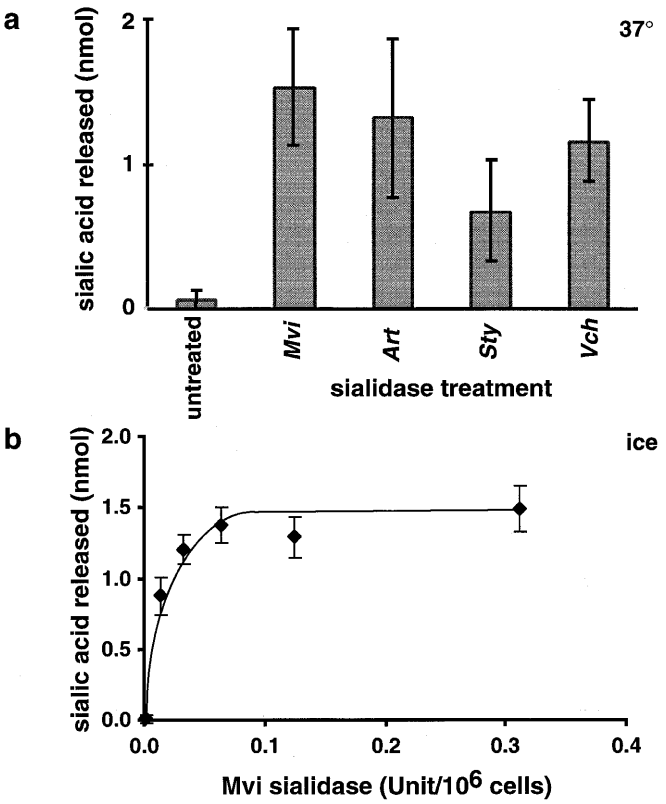

Fig. 1. Surface sialic acids released from MDCK cells *in situ.* (**a**) Sialic acid liberated from MDCK cells by sialidase treatment: cells (35 mm well, approximately 1×10^6 cells) were treated for 2h at 37°C with sialidases from *Arthrobacter ureafaciens* (*Art*, 6.5 mU), *Micromonospora viridifaciens* (*Mvi*, 31 mU), *Salmonella typhimurium* (*Sty*, 4.5 mU) or *Vibrio cholerae* (*Vch*, 0.12 mU). Note that activities were determined using fetuin as a substrate and thus do not necessarily reflect the optimal substrate cleavage of each enzyme (1 mU of enzyme releases 1 nmol of sialic acid per minute). These concentrations of enzyme were empirically determined to be in excess of that required to release all susceptible sialic acids (see *Materials and methods*). Data reported in both panels are mean ± SD for six wells. Sialic acid release determinations were performed on the supernatants from the same cells used for binding studies (Figure 2). (**b**) Sialidase treatments release all accessible sialic acid. Surface sialic acid released by increasing amounts of *Mvi* sialidase was determined in supernatants of cells treated with sialidase for 2 h on ice (to prevent *de novo* transport of sialyloglycans to the cell surface). Sialic acid release determinations were performed on the supernatants from the same cells used for infectivity studies (Figure 4).

fractions were prepared from MDCK cells, and sialic acids were quantitatively released by mild acid hydrolysis. The released radioactive sialic acid was compared to release by *in vitro* sialidase digestion (Tables I and II). Mild acid hydrolysis releases all sialic acid from glycoconjugates, regardless of linkage or modification, whereas many sialidases demonstrate distinct preferences for cleaving sialic acids in particular linkages or are inhibited by certain modifications (e.g., 4-O-acetylation) or when sialic acid is present as an internal branch (e.g., in gangliosides GM2 and GM1) (Reuter and Schauer, 1994).

Comparison of acid hydrolyzed and enzymatically digested glycolipid and glycopeptide fractions showed that *M. viridifaciens* sialidase quantitatively released sialic acid compared to mild acid treatment (Table I). Sialic acid released from glyco-

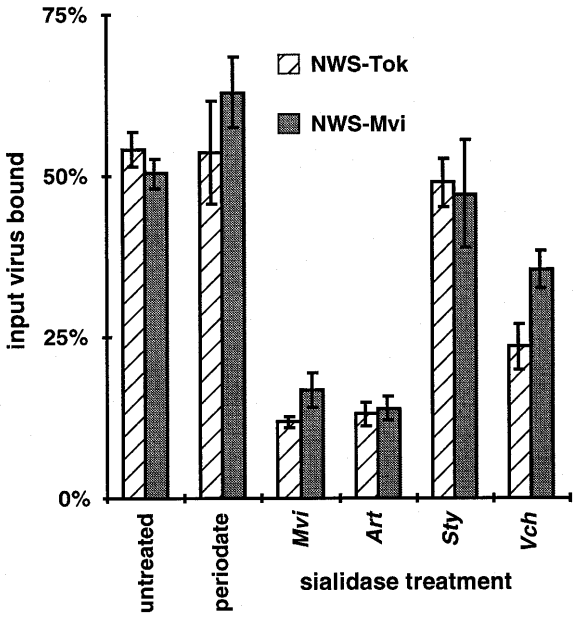

Fig. 2. Reduction of surface sialic acid reduces but does not abolish virus binding. (**a**) Metabolically radiolabeled virus was incubated with treated MDCK cell monolayers (35 mm well, approximately 1×10^6 cells) on ice, 60 min. Bound (cell associated) and free (supernatant) virus was determined. Cells had been pretreated with periodate (10 mM, on ice) or excess sialidase as in Figure 1.

Table I. Sialic acid released from purified glycopeptide and glycolipid fractions by *in vitro* treatment with sialidase or mild acid hydrolysis[h]

Fraction	SA radiolabel released (% total radiolabel)[a]		SA released (nmol/10^7 cells)[b]	
	Mild acid[c]	Sialidase	Mild acid[b]	Sialidase
Peptide[d]	30 ± 3%	34 ± 2%	17 ± 3[e]	14 ± 2[e]
Lipid[f]	45 ± 7%	55 ± 8%	10 ± 1[g]	12 ± 3[g]

[a]Identification of sialic acid was verified by high pH anion exchange chromatography (HPAEC) in which sialic acid radioactivity coeluted with authentic sialic acid standards. Residual oligosaccharide radioactivity is due to GlcNAc and GalNAc.
[b]We estimate sialic acid content of MDCK cells to be 25–30 nmol/10^7 cells, in good agreement with reports that ganglioside contributes 40–65% of total macromolecular sialic acid (Markwell *et al.*, 1981). GM_3 accounts for approximately 70% of ganglioside sialic acid (Markwell *et al.*, 1984), and the GM_3 concentration is 0.8 nmol/10^6 cells (Brändli *et al.*, 1988); i.e.: total sialic acid is approximately 23 nmol/10^7 cells.
[c]Some degradation of free sialic acid (≤10% of total) occurs during acid hydrolysis.
[d]Free sialic acid was separated from glycopeptides by descending paper chromatography.
[e]Sialic acid content of glycopeptide fraction was calculated from released radioactivity using specific activity of released glycolipid-bound sialic acid from the same labeling experiments. Sialic acid content of this fraction could not be determined directly due to interference of high peptide concentration.
[f]C18 reverse-phase chromatography was used to separate released sialic acid from residual glycolipid.
[g]Sialic acid released from glycolipids was determined colorimetrically (Warren, 1959).
[h]Data represent at least five independent labeling experiments. Data are reported as mean ± SD.

Table II. Proportion of total sialic acid released from intact MDCK cell monolayers *in situ* or from purified glycolipid or glycopeptide fractions *in vitro*

Fraction	% Total sialic acid released (compared to mild acid treatment *in vitro*)[a]			
	In vitro treatment		*In situ* treatment	
	Mild acid[b]	Sialidase	Sialidase	Periodate
Peptide	100%	100%	66%[c]	62%[d]
Lipid	100%	100%	70%[e]	48%[f]

[a]In each case, released sialic acid radioactivity was normalized to "backbone" radioactivity (i.e., radioactivity retained in GlcNAc and GalNAc moieties of oligosaccharides remaining after removal of sialic acid) which should be unaffected by the treatments to which material was subjected.
[b]Defined as 100% release.
[c]16 ± 1% of residual radioactivity in purified glycopeptides after *in situ* treatment was due to sialic acid.
[d]17 ± 1% of residual radioactivity in purified glycopeptides after *in situ* treatment was due to sialic acid.
[e]19 ± 2% of residual radioactivity in purified glycopeptides after *in situ* treatment was due to sialic acid.
[f]30 ± 0% of residual radioactivity in purified glycopeptides after *in situ* treatment was due to sialic acid.

lipid fractions was determined colorimetrically. Due to interference from the high peptide concentration in the glycopeptide fraction, the amount of sialic acid released from glycopeptides had to be calculated from released radioactivity using specific activity values determined directly from the analysis of the glycolipid fractions. Sialidase treatment of the glycolipid fraction released 12 ± 3 nmol of sialic acid from 10^7 cells, while mild acid treatment released 10 ± 1 nmol. Sialidase treatment of the glycopeptide fraction released 34 ± 2% of total radioactivity (14 ± 2 nmol/10^7 cells), while mild acid hydrolysis released 30 ± 3% (17 ± 3 nmol/10^7 cells). Some sialic acid radioactivity released by acid hydrolysis is lost due to keto-enol tautomerization of the glycerol sidechain of sialic acid, which occurs more rapidly at higher temperatures. This loss, from both glycolipid and glycopeptide fractions, is approximately 10% of total radioactivity over 20 min acid hydrolysis. Thus, within the limits of detection, all sialic acid in glycoconjugates of MDCK cells is releasable by *M.viridifaciens* sialidase.

Ganglioside digestion *in vitro* poses a special problem due to the limited solubility of glycolipid in aqueous solution. Commercial preparations of purified ganglioside GM3 were digested to completion without detergent, whereas branched gangliosides such as GM2 and GM1 were at least 75% digested in one h without detergent (data not shown). The rate of digestion of commercial or purified MDCK cell gangliosides by *M.viridifaciens* or *A.ureafaciens* sialidases was enhanced by the addition of detergent, presumably by increasing the solubility of the lipid substrates. Digestion was optimal in the presence of 0.1% sodium deoxycholate, although some enhancement of digestion was also observed in the presence of 0.1% sodium cacodylate (data not shown). However, the unbranched ganglioside GM3 accounts for 70–90% of all gangliosides in MDCK cells, depending upon culture conditions (Markwell *et al.*, 1984). Thus, the lack of detergent in our *in situ* sialidase reactions has a negligible effect on the efficiency of sialic acid release.

Some sialoglycoconjugates are inaccessible to sialidase digestion in situ

We compared the amount of sialic acid cleaved by sialidase from isolated glycopeptide and glycolipid fractions to the amount removed by digestion of intact, viable MDCK cells (Tables I and II). Under the conditions used, identical to those used in infectivity experiments, 66% of the total glycopeptide-associated sialic acid and 70% of that in glycolipids was removed. All sialic acids were removed by enzymatic cleavage *in vitro*. This demonstrates that residual sialic acid–containing glycoconjugates after *in situ* digestion are inaccessible to exogenous sialidase, and are probably intracellular.

An independent measure of accessibility of sialic acid is provided by periodate oxidation of MDCK monolayers. Periodate destroys the glycerol side chain (C7–C9) of sialic acids which are solvent-accessible at the apical surface of the cell, but does not permeate the membrane bilayer. Periodate is toxic to metabolically active cells, so cannot be used to treat cells throughout infection as we did with sialidase. Periodate treatment destroyed 62% of glycopeptide sialic acid, but only 48% of glycolipid sialic acid compared to untreated control (Table II). Because there is no detectable population of sialic acids which is susceptible to periodate but resistant to sialidase, and because we demonstrated that removal of susceptible sialic acid at both 0°C and 37°C is complete (Figure 1), we can conclude that all surface sialic acids are cleaved by sialidase. This is consistent with the observation that all sialyloglycans in isolated glycolipid and glycopeptide fractions from MDCK cells are sensitive to sialidase digestion. Our observation that more ganglioside sialic acid is removed by sialidase treatment at 37°C (2 h) than is destroyed by periodate oxidation (20 min) at 0°C (Table II) is consistent with both glycoproteins and glycolipids moving to the cell surface during the course of the sialidase treatment (Palestini *et al.*, 1998; Reichner *et al.*, 1988). Indeed, sialidase treatment on ice (2 h) removed slightly less sialic acid than at 37°C (Figure 1). Periodate and sialidase susceptibility of sialic acids of chilled cells on ice probably represents a "snapshot" of the membrane localization of oligosaccharides. Over the course of sialidase digestion of cell monolayers (2 h pretreatment or continuously throughout 2–4 days infection), sialyloglycans may be translocated to the apical surface of the polarized MDCK cell monolayer where they become accessible to digestion by exogenous sialidase.

Desialylation reduces but does not abrogate virus binding to cells

Periodate treatment of MDCK cells had little if any effect on binding by NWS-Tok and NWS-Mvi (Figure 2); cells treated with periodate on ice, after quenching with glycerol and washing to remove the toxic periodate, were permissive for virus growth to levels similar to untreated cells (results not shown). This contrasts with the observation that hemagglutination (virus-mediated red blood cell crosslinking) by NWS-Mvi is abrogated by periodate treatment of red blood cells (Yang *et al.*, 1997). Our results indicate that virus receptors on red blood cells and permissive tissue culture cells are distinct.

We used a variety of sialidases which cleave substrates with different efficiencies due to sialic acid linkage (SAα2–3Gal, SAα2–6Gal, or SAα2–8SA) or modification (e.g., acetylation at positions 4, 7, 8, or 9; hydroxylation of the 5-N-acetyl group of Neu5Ac). Treatment with the sialidases having the broadest substrate specificity (*A.ureafaciens* and *M.viridifaciens*) reduced virus binding by 70–80%, while treatment with sialidases with more restricted specificity (*S.typhimurium*) had very little effect upon binding although some sialic acid was released from the cells, suggesting that terminal α2–3 linked sialic acids make little contribution to virus binding and infection (see Figure 3). All sialidase treatments released sialic acids from intact MDCK cells (Figure 1). All sialidases tested failed to abrogate virus adsorption to cells. As desialylated cells retain the ability to be infected by virus, this residual binding must be physiologically relevant to virus entry (see below). Due to the vast difference in size between the sialidase (49 Å diameter from x-ray crystal structure (Gaskell *et al.*, 1995)) and the virus (diameter approximately 1000 Å, diameter of HA trimer from crystal structure 68 Å; Knossow *et al.*, 1986), it is physically impossible that sialic acids which are inaccessible to sialidase cleavage could be bound by virus particles.

Cells depleted of sialic acid are permissive for virus infection

To test for productive virus entry, a ribonuclease protection assay (RPA) was used to measure mRNA transcribed from the viral HA gene segment. Entry of NWS-Tok and NWS-Mvi into cells treated with broad-specificity sialidases (*A.ureafaciens* or *M.viridifaciens*) was reduced but not abolished (Figure 3). Results for NWS-G70c were similar (data not shown). *S.typhimurium* sialidase had a negligible effect on entry in accord with its minor effect on virus binding, while digestion with *V.cholerae* sialidase had an intermediate effect on entry as upon binding (Figures 2,3). PhosphorImager quantitation of mRNA radioactive signal indicated that the RPA mRNA signal was linear with the amount of virus used to infect untreated cells up to three particles per cell. Treatment with *M.viridifaciens* sialidase reduced entry, as measured by transcription, of NWS-Tok and NWS-Mvi to 4–11% of control (Table III). Combined treatment with both *M.viridifaciens* and *V.cholerae* sialidases reduced entry approximately the same amount as treatment with *M.viridifaciens* sialidase alone. The amount of *Mvi* sialidase used in these treatments was in excess of that

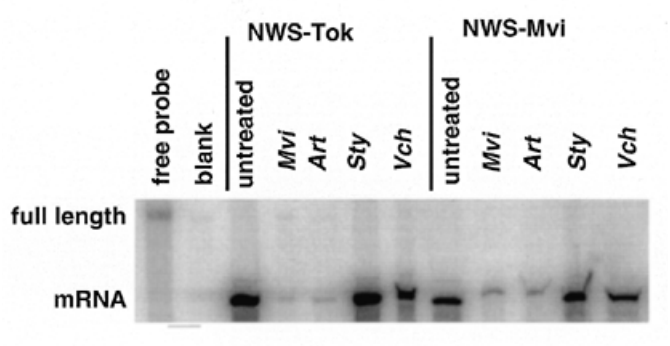

Fig. 3. Virus infection of sialidase-treated MDCK cells. (**a**) Productive entry of virus was determined by measuring mRNA transcribed from the viral HA gene by ribonuclease protection assay (RPA). MDCK cells (16 mm well, approximately 2.5×10^5 cells) were pretreated with the indicated sialidase for two h prior to infection (as in Figure 1) with 1.6 HAU virus (5 particles per cell). Sialidase was present throughout adsorption and infection. RNA was harvested from cells at 7 h postinfection.

Table III. Effect of desialylation on virus binding, entry, and multicycle growth

	Residual activity after sialidase treatment[a] (compared to untreated control)				
	NWS-Tok	NWS-Mvi	NWS-G70c	3003[b]	5098[b]
Amount of virus bound[c,d]	23 ± 3%[e]	32 ± 7%[e]	ND[f]	ND	ND
Viral mRNA synthesis[c]	11 ± 5%[g]	4 ± 2%[g]	5 ± 3%	ND	ND
Multicycle growth[h]	10^{-4}	10^{-1}	10^{-5}	10^{-2}	10^{-2}

[a]Cells were treated with *Mvi* sialidase (30 mU) for 2 h prior to adsorption of virus; enzyme was present and active throughout virus adsorption and growth (see *Materials and methods*).
[b]Human clinical isolates, subtype H3N2 isolated 1996–97.
[c]Compared to same virus on untreated cells.
[d]Binding experiments were as described for Figure 2.
[e]Data are mean ± SD of three to five experiments.
[f]Not determined.
[g]Determined by infecting treated or untreated cells with 0.6 HAU virus (two particles per cell, determined to be within the linear range of mRNA dose response). Infections were performed in triplicate, the error is calculated from the sum of proportional error calculated from the ratio of standard deviation to mean of mRNA signals in untreated and sialidase-treated cells.
[h]Input virus dilution at last well showing infection in untreated cells divided by input virus dilution at last well showing infection in sialidase treated cells. Virus growth endpoints were determined by hemagglutination.

required to release all surface sialic acid from MDCK cells (Figure 1b).

Treatment with increasing amounts of sialidase reduced mRNA signal compared to untreated cells, but a plateau of infectivity is reached which is not further diminished by increasing the amount of sialidase used to treat the cells prior to and during infection (Figure 4). Sialidase removes all the surface sialic acid (Tables I and II), release is complete at 0.06 mU/10^6 cells, and re-elaboration of sialic acids to the cell surface cannot explain this residual infectivity as the cells were maintained on ice throughout sialidase treatment and virus adsorption to prevent vesicular transport to the surface. The mRNA signal measured from cells desialylated and infected on ice then warmed to 37°C in the continuous presence of sialidase was well above background as measured from cells allowed to adsorb virus but maintained on ice. Thus, all susceptible sialic acids can be removed without protecting cells from virus infection, and no evidence was found of inherently uncleavable sialic acids. Entry by influenza must therefore occur by some means independent of a sialic acid receptor.

Multicycle growth in desialylated cells

The efficiency of infectious entry of NWS-Tok and NWS-Mvi into sialidase-treated cells was approximately equal (Figure 3). However, multicycle growth of NWS-Mvi infection of sialidase-treated cells was only reduced 10-fold with respect to nondesialylated cells (Table III), in contrast to NWS-Tok in cells treated with and maintained in *M. viridifaciens* sialidase which was reduced to 10^{-4} that seen in untreated cells. Thus, phenotypic differences in events subsequent to virus entry permit more efficient growth of NWS-Mvi than NWS-Tok in the presence of sialidase.

Two 1996 H3N2 clinical isolates, designated 3003 and 5098, also grew to high titer in the presence of sialidase. Infectivity as assayed by multicycle growth was reduced to 1% compared to untreated cells (Table III). Virus grew to the same HA titer as in untreated cells (to 640 HAU/ml) provided the input dose was at least 1000 particles per well. Virus production in MDCK cells ceases after 3–4 days, presumably due to depletion of nutrients. The ability of these viruses to grow in desialylated cells suggests that this may be a property of natural viruses which is lost on adaptation to growth in tissue culture.

Discussion

While it is clear that sialic acid enhances influenza binding and entry, the requirement for accessible surface sialic acid is not absolute. We present multiple lines of evidence for sialic acid-independent entry by influenza A viruses. Binding of permissive cells by virus is not abolished by sialidase treatment (Figure 2), as reported previously (Pedroso de Lima *et al.*, 1995). In contrast, hemagglutination (red-blood cell cross-linking) is prevented by sialidase (Burnet *et al.*, 1946; Air and Laver, 1995; Yang *et al.*, 1997). Desialylated cells retain the ability to bind virus and be infected by numerous influenza strains, even though all susceptible sialic acids have been released (Figures 1 and 4; Table III; Fazekas de St. Groth, 1948; Ghendon, 1977; Liu and Air, 1993).

We were unable to detect intrinsically sialidase-resistant sialic acids in MDCK cells (Table I, II); thus, cleavage by sialidase is regulated solely by sialic acid accessibility. Treatment with periodate confirms that MDCK cells have a population of sialic acid-containing glycoconjugates which are not accessible to small molecular probes, presumably due to their residence in internal membranes. Thus, virus binding and infection under the conditions used must occur independently of a requirement for accessible sialic acid.

The specificity of HA for different sialic acid–containing molecules has been investigated either by hemagglutination using derivatized red blood cells (Rogers and Paulson, 1983) or by virus binding to purified substrates *in vitro* (Sauter *et al.*, 1989; Suzuki *et al.*, 1992; Takemoto *et al.*, 1996; Matrosovich *et al.*, 1997). It has also been demonstrated that NA from different strains of virus exhibits different specificities for sialic acids in differing linkages and bearing differing modifications (Corfield *et al.*, 1981; Corfield *et al.*, 1982; Baum and

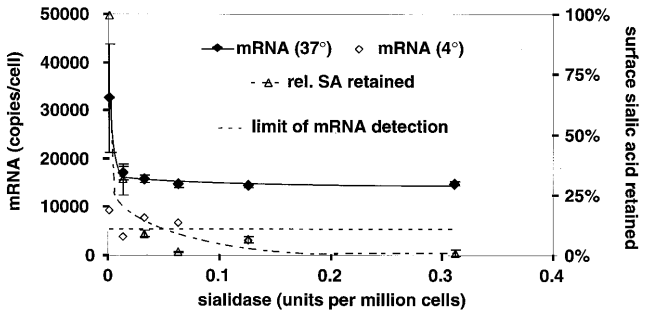

Fig. 4. Complete release of surface sialic acids fails to abrogate influenza virus infection. MDCK cells (35 mm well, approximately 1×10^6 cells) were treated on ice with increasing amounts of *Mvi* sialidase as shown; 1.6 HAU NWS-Mvi virus was then added to directly to the cells plus sialidase and allowed to adsorb on ice for 30 min. Supernatants were removed for sialic acid determination (see Figure 1b) and cells washed with ice-cold PBS prior to addition of infection medium (containing sialidase). Cells were either maintained on ice or moved to 37°C for 6.5 h for virus growth. Transcription of HA mRNA was measured by RPA. Data from cells maintained at 37°C represents mean ± SD of four determinations. The limit of detection was determined by averaging signals from cells maintained at 4°C throughout sialidase treatment, virus adsorption, and during growth period (single determination for each condition). No signal was observed in uninfected cells, and there was no cross-reaction between the mRNA-specific riboprobe and the viral RNA (negative sense). Retained sialic acid was calculated from mean ± SD of five determinations, from the same plates of cells subsequently used to determine viral infectivity under the same conditions.

Paulson, 1991). X-ray crystal structure determination has unequivocally demonstrated that sialic acid substrates can bind in a surface pocket at the membrane-distal region of the HA "spike" (Weis *et al.*, 1988; Sauter *et al.*, 1992b; Eisen *et al.*, 1997). Mutations altering the preference of HA for different sialic acid species map to this sialic acid binding pocket (Rogers *et al.*, 1983; Martín *et al.*, 1998), although it is not always clear how the molecular interactions have changed. Such elegant studies have led to the general conclusion that sialic acid is absolutely (and solely) required for influenza virus infection, although the vast majority of this research has been performed in systems not permissive for influenza (red blood cells) and *in vitro* studies have concentrated on the receptor function of sialylated molecules without testing the possibility of the involvement of other species. Evidence exists that other glycoconjugates such as sulfatide are also bound by the virus (Suzuki *et al.*, 1996), and that there is a second binding site on the HA molecule (Sauter *et al.*, 1992a). This apparent second binding site is not well conserved among HA sequences, so the requirements for binding are not known.

The discrepancy between MDCK cell binding and hemagglutination suggests that receptors used by influenza virus for infection of permissive cells may be distinct from those mediating agglutination into erythrocytes. The behavior of two virus mutants, HA Y98F (influenza A subtype H3) and strain NWS-Mvi, further highlights the likelihood that receptors for red cell agglutination and entry into permissive cells are not identical. A transfectant virus with a mutation of the conserved tyrosine to phenylalanine (Y98F) in the sialic acid binding pocket is infectious in MDCK cells, although cells expressing recombinant HA Y98F do not bind red cells (Martín *et al.*, 1998). The mutant virus NWS-Mvi has no endogenous NA (sialidase) activity due to a massive deletion of the NA-encoding gene segment and must be grown in the presence of high levels of exogenous sialidase to prevent self-agglutination of virus particles leading to loss of infectivity (Liu *et al.*, 1995). We have demonstrated that the binding and entry of the mutant virus NWS-Mvi is not significantly different from that of wild-type viruses in either desialylated or untreated cells.

Because treatment of cells with a panel of sialidases reduced virus binding no more than 80% and certain sialidases had more effect on virus binding than others, we originally assumed that binding would be mediated by a subclass of surface sialyloligosaccharides inherently resistant to sialidase digestion. Therefore, we wished to determine the identity and amount of sialic acids which were not sialidase-releasable. However, sialidase digestion *in vitro* of purified glycopeptides and glycolipids extracted from MDCK cells released all detectable sialic acids. Treatment of intact viable monolayer MDCK cells with *M.viridifaciens* sialidase removed only 66% of glycoprotein sialic acid and 70% of glycolipid sialic acid. As no intrinsically sialidase-resistant sialic acids are detectable in MDCK cells, those sialic acids not released by sialidase digestion of viable cell monolayers must be sequestered from the apical cell surface. Periodate oxidation confirmed that a considerable amount of sialic acid is inaccessible to small molecules. We found that approximately 40% of glycoprotein-linked sialic acid and 50% of glycolipid-linked sialic acid was not oxidized by a brief treatment with periodate of metabolically radiolabeled cells. Periodate-inaccessible sialic acid is presumably sequestered in membranes inside the cell. Accessibility of gangliosides to sialidase digestion has been previously shown to vary with the physiological state of the cell (Palestini *et al.*, 1998), and numerous sialylated glycoproteins are known to be specifically internalized or stored within the cell (Wardzala and Jeanrenaud, 1983; Duncan and Kornfeld, 1988; Reichner *et al.*, 1988). Thus, we have shown that the only restriction on sialidase digestion is when sialic acids are sequestered from the cell surface, inaccessible to periodate, sialidase (49 Å diameter), or a single HA trimer (68 Å diameter).

Our demonstration that influenza viruses infect cells without surface-accessible sialic acid leads us to propose two models for entry. Infection of desialylated cells may be via a completely sialic acid–independent receptor pathway, or by a multistep pathway which is normally initiated by virus binding to abundant surface sialic acid leading to subsequent interaction with nonsialylated receptors, but which can be bypassed at higher virus concentrations. Our results do not allow us to distinguish between the two models described.

In tissue culture systems, a proposed sialic acid–independent pathway must coexist with a sialic acid–dependent pathway, as removal of cell surface sialic acid reduces both binding and infectivity. A completely sialic acid–independent pathway appears to make a lesser contribution to virus entry than a pathway where sialic acid is required.

In a multistage model, initial interaction with the cell surface via sialic acid, occurring by multiple low affinity interactions between sialic acid and HA, may allow the virus to be retained at or roll across the surface, akin to the "browsing" model (Burnet, 1960). This facilitates interaction with secondary receptor(s) required for virus entry. Sialic acid may be important as a cell surface marker to recruit the virus to cells which

actively synthesize and secrete glycoproteins, and thus are likely to support efficient production of the viral glycoproteins required to form successful viral progeny. NA activity may be required during entry by some influenza viruses (Huang *et al.*, 1980, 1985) to aid in the process of browsing across the cell surface by sequential binding and release of individual sialic acid molecules, while in other strains the sialic acid–mediated binding of the virion to the cell surface may be weak enough for this to occur spontaneously. The secondary receptor, although possibly quite rare relative to surface sialyloglycan, would be expected to bind the virus with high affinity and to target it for rapid internalization.

Although specific sialylated molecules are readily (even constitutively) internalized, the overall rate of turnover of surface sialic acid in some cell lines is quite slow ($t_{1/2} > 8$ h; Reichner *et al.*, 1988). If the virus binds to all sialic acids on the cell surface, some virions will be bound to sialylated molecules that have long cell surface retention times and virus internalization will not be efficient. Indeed, the ability to discriminate between receptors and "pseudoreceptors," both containing sialic acid, is important in determining virulence and spread of polyomavirus (Bauer *et al.*, 1999).

The use of multistage entry pathways is a common feature of the biology of many viruses, where a primary receptor acts to recruit virus particles to the cell surface and to enhance the likelihood of encountering a specific molecule to target them for uptake. The initial receptor recognized may be selected as a marker for cells with useful properties, such as an appropriate complement of transcription factors or efficient protein transport to the cell surface. HIV, for example, uses CD4 for initial cell recognition via its surface glycoprotein gp120; binding of CD4 causes a conformational alteration which allows gp120 to engage chemokine receptors which the virus uses as coreceptors for virus entry (Trkola *et al.*, 1996; Wu *et al.*, 1996; Kwong *et al.*, 1998). Other viruses, such as herpes viruses, express multiple surface proteins which each engage separate targets, including heparan sulfate (WuDunn and Spear, 1989; Shieh *et al.*, 1992), growth factor receptors (Montgomery *et al.*, 1996), and the polio virus receptor and related molecules (Geraghty *et al.*, 1998). It is not yet known whether these receptor-binding proteins are used independently or hierarchically as part of a multistep pathway.

Our data demonstrate that accessible surface sialic acid is dispensable for influenza virus infection, although the presence of sialic acid enhances the efficiency of influenza entry. Any requirement for initial interactions with sialic acid to enrich the virus at the cell surface may be bypassed in the presence of larger amounts of virus. Identification of additional virus receptors and a better understanding of viral entry pathways may provide insights into cellular uptake of macromolecules, and identification of other mediators of virus entry may present targets for drug intervention in the viral life cycle.

Materials and methods

Cells and viruses

MDCK cells were maintained in DMEM supplemented with 1× MEM amino acids (GIBCO BRL), 1× MEM vitamins (GIBCO BRL), sodium bicarbonate (0.225%), supplemented bovine serum (Hyclone, 5%) and gentamycin (10 μg/ml, Sigma). Virus strains were reassortants A/NWS/33_{HA}-Tokyo/67_{NA} (H1N2, designated NWS-Tok), A/NWS/33_{HA}-tern/Australia/G70c/75_{NA} (NWS-G70c, H1N9), and A/NWS/33_{HA}-G70cΔ_{NA}MviA (NWS-Mvi, H1N9^{-}) (Liu and Air, 1993). MDCK cells (ATCC) had been maintained in the laboratory for some time and thus were of a high but undetermined passage number and of uniform appearance. Human 1996 influenza A clinical isolates 3003 and 5098 (both H3N2) from Oklahoma City were passaged twice in primary monkey kidney cells and once in MDCK cells before assessment of growth in desialylated cells. Viruses were grown in MDCK cells in DMEM: Ham's F12 1:1 supplemented with 1% ITS+ (Collaborative Research) and 0.5 μg/ml trypsin as described previously (Liu and Air, 1993). Radiolabeled virus was prepared by infecting cells with 10 TCIU virus per T150 tissue culture flask, grown in the medium described above for 6–12 h prior to the addition of 10 μCi EXPRESS Protein Labeling Mix (NEN). Upon completion of cell lysis (36–72 h postinfection), virus was concentrated by centrifugation and purified by velocity sucrose gradient centrifugation (10%–40% sucrose, 35000 r.p.m., TLS55 rotor, 15 min) (Liu *et al.*, 1995). Viral titers were determined by hemagglutination, and specific radiochemical activity of each preparation was determined to allow calculation of virus titer in hemagglutination units (HAU) from radioactivity.

Binding of radiolabeled virus

MDCK cells (approx. 10^6 cells/35 mm tissue culture dish) were treated with sialidase in CMS/Ac (150 mM NaCl, 10 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM Na^+/H^+ acetate buffer pH 6.0, 2 h, 37°C), then washed once in CaMgPBS (PBS containing 10 mM $CaCl_2$ and 0.5 mM $MgCl_2$) prior to incubation with indicated amounts of metabolically radiolabeled virus on ice for 1 h in 0.5 ml total volume. Radioactivity in supernatant and attached to cell monolayer (released by treatment with 1% SDS, 55°C) was used to determine free and bound virus titer, respectively, using specific activity determined by measuring radioactivity and HA titer of stocks. NWS-Tokyo is used as wild-type control in these experiments due to the fact that NWS-G70c (H1N9), the virus from which NWS-Mvi was derived, has been shown to have hemagglutinating activity associated with its NA protein in addition to that mediated by HA (Air and Laver, 1995).

Desialylation of cells

Periodate treatment of intact, viable cells was performed with $NaIO_4$ (10 mM in PBS) for 20 minutes on ice. Digestions of MDCK cells with sialidases (E.C. 3.2.1.18) were performed using 0.5 μml sialidase stock in 0.5 ml CMS/Ac pH 6.0 per 35 mm wel of cells for 2 h at 37°C. These amounts were empirically determined to represent 10- to 100-fold excess of that releasing all susceptible cell surface sialic acid. Activities of the stocks used were determined at pH 5.0, 37°C (1 Unit = enzyme liberating 1.0 μmol sialic acid from fetuin per minute) and were found to be: *Micromonospora viridifaciens* (*Mvi*): 62 U/ml), *Arthrobacter ureafaciens* (*Art*): 13 U/ml, *Salmonella typhimurium* (*Sty*): 8.9 U/ml, and *Vibrio cholerae* (*Vch*): 0.23 U/ml. *M.viridifaciens sialidase* was the generous gift of Drs. T.Uwajima and K.Aisaka, Kyowa Hakko Kogyo Company, Tokyo, Japan. Other sialidases were from Boehringer Mannheim GmbH (*Art*, *Vch*) or Sigma (*Sty*).

Sialic acid release

MDCK monolayers were treated at 37°C with sialidases as described above, or in the same buffer incubating the culture plates on ice for 2 h with the indicated amount of *Mvi* sialidase. Sialic acid released into the supernatant was determined colorimetrically (Warren, 1959).

Glycoprotein and glycolipid analyses

Cells (100 mm dish) were continuously labeled with 6-[^{3}H] glucosamine ([^{3}H]-GlcN, 5–10 μCi) for 2 days. Cells were desialylated *in situ* using *Mvi* sialidase (30 mU/0.5 ml, 2 h, 37°C), or were oxidized with 10 mM $NaIO_4$ in PBS (0°C, 20 min). To analyze sialic acids, glycolipid and glycopeptide fractions were prepared from cell monolayers as described (Wilkins et al., 1996). Monolayers were scraped from dishes and extracted with 1ml chloroform/methanol (1:1) then with 1 ml chloroform/methanol/water (10:10:3). The organic extracts were pooled and dried (glycolipid fraction). Residual cellular proteins (i.e., precipitate remaining after extraction) were digested with 10 mg/ml pronase overnight (glycopeptide fraction). Protease activity was destroyed by boiling. *In vitro* sialidase digestion of glycopeptides was performed as for *in situ* digestion. Sialic acids were released chemically by mild acid hydrolysis (20 min, 2 M CH_3COOH, 100°C); determined to be optimal for maximal sialic acid release. Glycopeptides were analyzed by descending paper chromatography using Whatman number 1 paper and ethyl acetate:acetic acid:pyridine:water (5:5:1:3) as developer. Following overnight development (total migration ~50 cm), chromatograms were cut into 1 cm sections and radioactivity determined following water elution of saccharides (Cummings, 1993).

The lipid fraction was redissolved in chloroform:methanol:water (10:10:3) and purified over Sephadex G25 (equilibrated in the same solvent) to remove hydrophilic contaminants such as nucleotide sugars (Fishman *et al.*, 1972). Flowthrough was collected, dried, and resuspended in water by sonication. Lipids were either digested with *Mvi* sialidase (62 mU/ml in NA acetate buffer 10mM pH 6.0, plus 0.1% sodium deoxycholate) or subjected to mild acid hydrolysis as above. Free sialic acid was determined directly by the method of Warren [1959]) with reaction volumes scaled down. Alternatively, assay mixes were fractionated over activated Waters SepPak™ C_{18} reverse-phase minicolumns; free sialic acid fails to bind, while residual glycolipid backbones are bound and are eluted with methanol. Due to the presence of detergent in enzyme digests, these were diluted 10-fold with water to prevent interference with lipid binding to the columns. Flowthrough and washes (10 ml) were collected and radioactivity determined by scintillation counting (free sialic acid), as was the methanol eluate (10 ml) to determine unreleased radioactivity. Errors in calculated values were derived by cumulation of the proportional error (one standard deviation) in every quantity used to generate the final value. Error in the final value was determined by multiplying the final value by the cumulative proportional error.

Infectivity in presence of sialidase

Infectivity was determined by inoculating sialidase-treated MDCK cells with 5- or 10-fold serial dilutions of virus. Infection was monitored by ribonuclease protection assay (RPA) for viral HA mRNA (single cycle), or by determining the limit dilution producing progeny virus as measured by hemagglutination at 96 h. Sialidase-treated cells were maintained in sialidase throughout virus adsorption and growth.

Ribonuclease protection assay (RPA)

Sialidase treatments were as for binding assays; sialidase was present during the entire adsorption and infection periods. MDCK cells (1.5 cm well, approximately 2.5×10^5 cells) were infected with 0.6–1.6 HAU virus (2–5 particles per cell); stocks used were 320 HAU/ml (10^8 TCIU/ml). RNA was assayed by ribonuclease protection (Zinn *et al.*, 1983) at 7 h post-infection (pi). Total RNA was harvested in 50 μl harvest/hybridization buffer (0.5 M NaCl, 50 mM PIPES pH 6.4, 1.25 mM EDTA in formamide), to which probe (approximately 5×10^5 cpm) was added directly prior to overnight hybridization at 50°C. Probe was transcribed from NWS HA cDNA (nt 1517–1746, cloned into pBluescript [Stratagene]) using T7 polymerase (Promega). After hybridization and RNase T1 digestion, products were separated by gel electrophoresis (6% polyacrylamide, 0.5 × TBE, 42% urea). Protected fragments of 215 nt (mRNA) and 230 nt (cRNA, the positive strand replication intermediate) were expected. Product size was initially verified by comparison to denatured, end-labeled DNA fragments of ϕX174 derived by *Hae*III endonuclease digestion. Quantitation was performed using Molecular Dynamics PhosphorImager SI.

High-pH anion-exchange chromatography (HPAEC)

Sialic acids were released from fractions of metabolically labeled cells, either enzymatically or by mild acid hydrolysis of fractions in 2 M acetic acid, 100°C, 20 min. Sialic acids were separated from residual glycopeptides by passage over mixed-bed resin, flow-through was collected and lyophilized. Residual glycolipids were removed from treated glycolipids by C18 reverse-phase chromatography as described above, flow-through was collected and lyophilized. Monosaccharides were analyzed by HPAEC in the presence of NaOH, using pulsed amperometric detection (PAD) on a Dionex Carbopac chromatography system (Hardy and Townsend, 1994). Digestion products were "spiked" with known monosaccharide standards (Dionex) or known Neu5Ac and Neu5Gc standards (Sigma). Analysis of neutral monosaccharide was performed using isocratic separation in 6 mM NaOH, total monosaccharides (including sialic acids) were analyzed using 30 min isocratic separation followed by a linear sodium acetate/NaOH gradient (0–100 mM NaOAc/6–100 mM NaOH over 30 min).

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Abbreviations

HA, influenza virus hemagglutinin; NA, influenza virus neuraminidase; HAU, hemagglutination unit; MDCK, Madin-Darby canine kidney cells; *Mvi*, *Micromonospora viridifaciens*; *Art*, *Arthrobacter ureafaciens*; *Sty*, *Salmonella typhimurium*; *Vch*, *Vibrio cholerae*; RPA, ribonuclease protection assay; TCIU, tissue culture infectious units; pi, post infection; Neu5Ac, N-acetylneuraminic acid; $t_{1/2}$, half-life; Neu5Gc, N-glycolylneuraminic acid; SA, sialic acid; GlcNAc, N-acetylglucosamine; GalNAc, N-acetylgalactosamine; CMP, cytidine nucleoside monophosphate; UDP, uridine nucleoside diphosphate; PBS, phosphate-buffered saline.

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