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Target cell membrane sialic acid modulates both binding and fusion activity of influenza virus

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Abstract

Influenza virus binds to cell surface sialic acid receptors, and following endocytosis fuses with the endosome membrane at low pH. Whether sialic acid plays a role in the virus–cell membrane fusion step is not known. We investigated the effect of the removal of cell membrane sialic acid on the fusion activity of influenza virus (A/PR/8/34 strain) toward human T lymphocytic leukemia (CEM) cells at low pH. Fusion was monitored by fluorescence dequenching of octadecylrhodamine incorporated in the virus membrane. Removal of sialic acid by neuraminidase resulted in a drastic reduction in both viral binding and fusion. The association of the virus with neuraminidase-treated cells was enhanced at pH 5, compared to that at neutral pH, probably due to the unfolding of the hemagglutinin and the resulting increase in viral surface hydrophobicity, but the fusion capacity of the virus was reduced significantly. The results were analysed with a mass-action kinetic model which could explain and predict the kinetics of fusion. Our results indicate that binding of influenza virus to sialic acid residues on the cell surface leads to rapid and extensive fusion and partially inhibits the low pH-induced viral inactivation.

Keywords: Influenza virus; Sialic acid; Membrane fusion; Membrane binding; CEM cell; Neuraminidase; Fluorescence

1. Introduction

Cell surface sialic acid residues are considered to be the primary receptors for influenza virus [1–4]. Initial binding of the virus is followed by endocytosis, and fusion of the viral membrane with the endosomal membrane upon acidification of the endosome lumen [5–7]. Structural studies of the influenza hemagglutinin have shown sialic acid as the main component of the cellular receptor for the virus [8]. An indirect role for sialic acid in helping sialoglycoproteins attain a critical structure has also been proposed [9]. Whether the cell membrane sialic acid is also involved in the membrane fusion reaction is not known. Previous studies on the fusion of influenza virus with liposomes have indicated that the presence of ganglioside G_{D1a} increases the rate constant of adhesion, but not that of fusion [10], while studies utilizing liposomes containing varying amounts of gangliosides have shown an optimal surface concentration for virus–liposome fusion [11]. It has been suggested that the interaction of gangliosides G_{T1b} or G_{D1b} with influenza virus hemagglutinin causes an increase in the fluidity of the viral envelope, thereby promoting fusion with target membranes [12]. We previously examined the low pH-induced fusion of influenza virus with the plasma membranes of living CEM cells as a model to study the fusion of the virus with endosome membranes [13]. Here we have utilized this model system to investigate the role

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of cell membrane sialic acid in the fusion reaction of influenza virus with cellular membranes.

2. Materials and methods

2.1. Materials

Influenza virus, A/PR/8/34 (H1N1) strain was obtained from SPAFAS (Preston, CT). The virus was grown for 48 h at 37° C in the allantoic cavity of 11-day-old specific pathogen-free embryonated eggs, purified by discontinuous sucrose density gradient centrifugation and stored at −70° C in phosphate buffered saline. Trypsin, trypsin inhibitor, neuraminidase (from *Vibrio cholerae*, 1 unit/ml specific activity), endoglycosidase H (1 unit/ml specific activity) and octaethyleneglycol dodecyl ether ($C_{12}E_8$) were purchased from Calbiochem (San Diego, CA). Tes buffer and NaCl were from Sigma (St. Louis, MO). Proteinase K was obtained from Boehringer-Mannheim, endoglycosidase F (200 units/ml specific activity) from Oxford GlycoSystems (Rosedale, NY), and octadecylrhodamine B chloride (R18) from Molecular Probes (Eugene, OR).

2.2. Cells

CEM cells (obtained from the UCSF Cell Culture Facility) were maintained in RPMI 1640 medium containing 25 mM Hepes buffer, supplemented with 10% foetal bovine serum (FBS) and antibiotics. The cells were grown in T-75 flasks under a 5% CO_2 atmosphere at 37° C up to a cell density of $1 \cdot 10^6$–$1.5 \cdot 10^6$/ml. The cells were harvested by centrifugation at $180 \times g$ for 8 min at room temperature, washed twice in Dulbecco's PBS, and once in phenol red-free RPMI 1640 containing 25 mM Hepes buffer (pH 7.4) (Medium A). The cells were resuspended in the latter buffer at a stock cell density of 10^8/ml and kept on ice in polypropylene centrifuge tubes until use the same day. Cell viability was determined by Trypan blue exclusion and was routinely above 95%.

2.3. Virus labeling

The virus was labeled with R18 as described previously [14]. A 4.8 μl aliquot of a 3.12 μmol/ml ethanolic fluorophore solution was injected under vortex mixing into a viral suspension containing 2 mg of viral protein/ml. The final concentration of added probe corresponded to approx. 4 mol% of total viral lipid, and that of ethanol was less than 1% (v/v). The mixture was incubated in the dark for 0.5–1 h at room temperature. R18-labeled virus was separated from uninserted fluorophore by chromatography on Sephadex G-75 (Pharmacia, Uppsala, Sweden) using 10 mM Tes, 150 mM NaCl (pH 7.4) as elution buffer. The protein concentration of the labeled virus was determined by the Lowry assay.

2.4. Fusion of R18 labeled influenza virus with CEM cells

Fusion was monitored continuously with the R18 fluorescence assay [13–16]. Fusion was initiated either by rapid injection of R18-labeled virus (4 μg viral protein) into a cuvette containing the cell suspension ($4 \cdot 10^7$ cells) at pH 5, or by lowering the pH (to 5) of the suspension of cells and virus preincubated at pH 7.4. The final incubation volume was always 2 ml Medium A and the pH was measured at the end of each experiment with a Beckman pH meter. The fluorescence scale was calibrated such that the initial fluorescence of the R18-labeled virus and cell suspension was set at 0% fluorescence. The value obtained by lysing the virus and cellular membranes after each experiment with $C_{12}E_8$ (at a final concentration of 2 mM), was set to 100% fluorescence. Fluorescence measurements were performed in a Spex Fluorolog 2 fluorometer using the front-face configuration in the emission channel, with excitation at 560 nm and emission at 590 nm, using 5 and 25 mm slits in both monochromators. A high-pass filter (50% transmission at 590 nm; Schott Glass OG590, Melles-Griot) was placed between the cuvette and the emission monochromator. The sample chamber was equipped with a magnetic stirring device and the temperature was controlled with a thermostatted circulating water bath. Some experiments were also carried out using a Perkin-Elmer LS-5B fluorometer, operated with a Softways (Moreno Valley, CA) computer program.

2.5. Binding and cell association

Fluorescently labeled influenza virus was incubated with CEM cells in identical amounts as in the fusion experiments, in a final volume of 2 ml Medium A, either for 5 min at 37° C or for 30 min at 0° C with stirring. Cell association experiments were carried out in the same way but at pH 5, with or without virus-cell prebinding at pH 7.4. Incubations were carried out either at 37° C or 0° C and for 1 min or 5 min at pH 5, as described under Results. The cells were sedimented by centrifugation at 4° C for 7 min at $180 \times g$ and the fluorescence was measured at 37° C in the supernatant and pellet following detergent lysis. In some experiments the virus was precentrifuged at $180 \times g$ for 7 min to eliminate any aggregated virions, and exclude the possibility of virus co-pelleting with the cells.

2.6. Enzymatic treatment

CEM cells ($4 \cdot 10^7$) were incubated with the various enzymes, trypsin (0.0025% (w/v), 0.05%, and 0.1%), proteinase K (0.01% (w/v) and 0.05%), neuraminidase (0.005 and 0.05 unit/ml), endoglycosidase H (0.005

unit/ml) and endoglycosidase F (1 unit/ml) in a final volume of 2 ml Medium A at 37° C for 20 min. Following enzymatic treatment, cells were placed on ice for 5 min to stop the reaction, except for trypsin where the reaction was terminated by adding a twofold molar excess of trypsin inhibitor. The cells were centrifuged at 4° C for 7 min at $180 \times g$, washed once in cold Medium A, resuspended in the same medium and kept on ice until use. Control cells were treated in parallel under the same conditions in Medium A without the enzyme. The use of either 0.005 or 0.05 unit/ml neuraminidase produced the same effect on both virus binding and fusion, and higher enzyme concentrations did not result in further inhibition. Cell viability was shown not to be affected by the enzymatic treatment at the enzyme concentrations used.

2.7. Analysis of fusion kinetics

The analysis of prebinding experiments was based on a slight modification of the procedure in Nir et al. [17]. The fraction of the virus prebound to the cells at neutral pH is denoted by B_1, whereas B_2 denotes the fraction of virus associated with the cells at the end of the incubation period at low pH. An expression for the fusion rate constant, f (s^{-1}), that accounts for inactivation is given by

$$f(t) = f(0)[\exp(-\gamma t) + \gamma_2(1 - \exp(-\gamma t))/\gamma] \quad (1)$$

in which t is the time and $\gamma = \gamma_1 + \gamma_2$. In Eq. (1) γ_1 and γ_2 represent forward and reverse rate constants of inactivation. In our analysis we set $\gamma_2 = 0$. If B is constant and $\gamma_2 = 0$, the fraction of fused virus, $F(t)$, is given by

$$F(t) = [1 - \exp[f(0)(\exp(-\gamma t) - 1)/\gamma]B \quad (2)$$

In the analysis of virus fusion with neuraminidase-treated cells, where B_2 was significantly larger than B_1, we divided the time t into small segments and assumed a linear increase in B with time in Eq. (2).

3. Results

3.1. Dependence of influenza virus fusion activity on neuraminidase pretreatment of CEM cells

The fluorescence dequenching of R18 has been used as a reliable indicator of virus–cell fusion in numerous systems [13,16,18–23]. We investigated the role of cell surface sialic acid residues in influenza virus fusion with CEM cells using this assay. The time-course of R18 fluorescence dequenching during the incubation of influenza virus with neuraminidase-treated and untreated (control) CEM cells is shown in Fig. 1. In preliminary experiments the toxic effects of a range of neuraminidase concentrations on cells were determined by Trypan blue exclusion. A concentration of neuraminidase was chosen (0.005 unit/ml) which was well below the toxic limit. Concentrations of neuraminidase 10-fold higher than the chosen concentration, did not cause any further inhibition of binding at 37° C, indicating that the significant sialic acid residues (i.e, those relevant to virus binding) had been removed by the lower enzyme concentration utilized in most of our binding and fusion studies.

In one set of experiments, the virus was allowed to bind to the cells for 5 min at 37° C (pH 7.4), and the pH was lowered to 5 to induce membrane fusion (Fig. 1A). Neuraminidase pretreatment of the cells caused a drastic reduction in virus fusion activity (curve b). In the other set of experiments, the virus was bound to the cells for 30 min at 0° C (pH 7.4), and the unbound virus was removed from the medium before lowering the pH (Fig. 1B). Under these

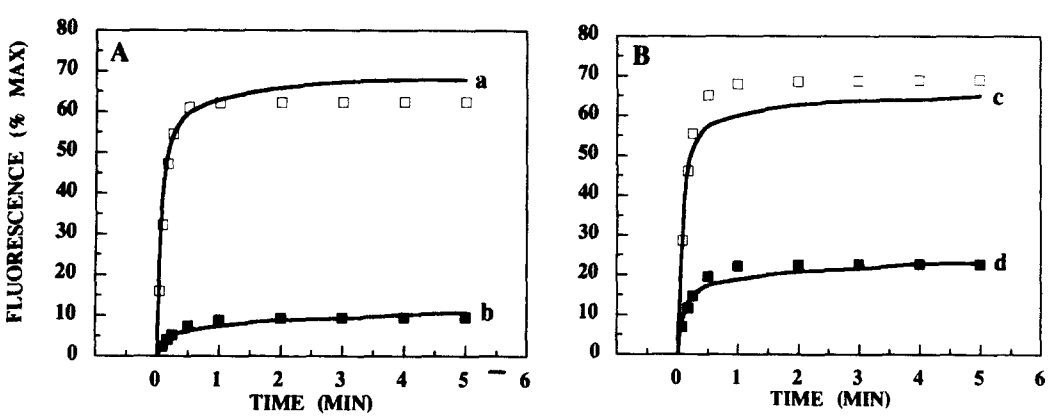

Fig. 1. Effect of neuraminidase pretreatment of CEM cells on influenza virus fusion activity. (A) Influenza virus (2 μg viral protein/ml) was added to $4 \cdot 10^7$ control (a) or neuraminidase-treated (b) CEM cells in a final volume of 2 ml, and R18 dequenching was monitored for 5 min at 37° C and pH 5, following virus–cell preincubation for 5 min at 37° C. (B) Influenza virus (2 μg viral protein/ml) was added to $4 \cdot 10^7$ control (c) or neuraminidase-treated (d) CEM cells in a final volume of 2 ml, and incubated for 30 min at 0° C. The unbound virus was removed by centrifugation in the cold, and R18 dequenching was monitored for 5 min at 37° C and pH 5. The symbols represent the values calculated by using the mass action kinetic model for control (□) and neuraminidase-treated cells (■).

conditions, the fusion activity of the bound virus was also inhibited significantly in enzyme pre-treated cells. In control cells, these two experimental approaches yielded similar values for the initial rates of fusion, as well as for the extents of fusion (Fig. 1, curves a and c). In treated cells, however, the decrease in the initial rate and extent of fusion was not as dramatic when the unbound virus was removed (curve d).

We examined the time-course of virus-cell fusion by a mass-action kinetic analysis [17]. The fusion (f) and inactivation (γ) rate constants given in Table 1 resulted in fits to the curves in Fig. 1 shown by the squares. In the case of neuraminidase pretreatment of cells, the data were fit best by invoking a decrease in f and an increase in γ, compared to the control cells (Table 1). When the virus was preincubated with the cells at 37° C, removal of sialic acid resulted in a 5-fold reduction in f, and a similar increase in γ. Preincubation in the cold resulted in a similar pattern.

We also investigated how the prebinding of the virus to the cells at neutral pH and 37° C, before initiation of the fusion reaction, affects the extent of fusion, both for neuraminidase-treated and control cells (Fig. 2). When the virions were prebound to the normal cells, the extent of fusion was higher compared to the case of no prebinding (Fig. 2, open bars). In contrast, for neuraminidase-treated cells, a slight decrease in the extent of fusion was observed with prebinding (striped bars).

Enzymes other than neuraminidase that were tested did not affect the kinetics of virus–cell fusion, at the concentrations of enzyme and incubation conditions that were necessary to retain cell viability (data not shown), and were therefore not examined further.

3.2. Virus binding and association to untreated and neuraminidase-treated cells

To further characterize the role of sialic acid in the fusion activity of influenza virus, experiments on virus binding and association to untreated (control) and neuraminidase-treated cells were performed under several experimental conditions. Fig. 3 shows that pretreatment of cells with neuraminidase at the same concentration as the fusion experiments resulted in a significant decrease in virus binding at pH 7.4. When binding experiments were

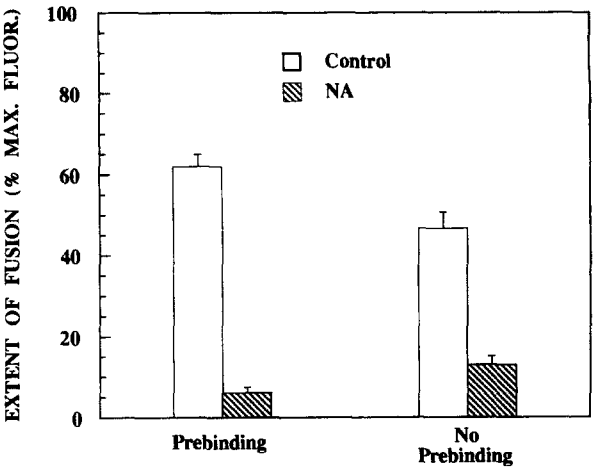

Fig. 2. Effect of neuraminidase pretreatment of CEM cells on the extent of fusion of influenza virus, with or without viral prebinding to the cells. Influenza virus (2 μg viral protein/ml) was added to $4 \cdot 10^7$ control (open bars) or neuraminidase-treated (striped bars) CEM cells in a final volume of 2 ml at 37° C, and the extent of R18 fluorescence dequenching was measured after 5 min incubation at pH 5, with or without prebinding of the virus to the cells. In the case of viral prebinding, the virus was incubated with cells for 5 min at neutral pH before lowering the pH to 5. In the absence of viral prebinding, the pH of the cell suspension was adjusted to 5 just prior to addition of virus. Values represent means of at least 5 experiments ± standard deviation.

carried out at 0° C for 30 min, the decrease in virus binding due to enzymatic treatment (24% of control) was not as pronounced as that observed when the virus was allowed to bind to pretreated cells at 37° C for 5 min (70% of control). In contrast, when the cells were pretreated with a higher concentration of neuraminidase, a similar reduction in binding was observed at both temperatures (Table 2).

In addition to determining virus binding to cells at neutral pH, we measured the amount of cell-associated virus after an initial 5 min prebinding at neutral pH and subsequent reduction of the pH to 5. These experiments measure the total amount of virus bound to or fused with the cell membrane, 1 min or 5 min after the reduction of the pH. Following a 5 min incubation at low pH, 81.8% of the virions became associated with the control cells, and 61.7% were associated with neuraminidase-treated cells (Fig. 4). It is interesting to note that virus association with

Table 1
Effect of neuraminidase pretreatment of CEM cells on fusion and inactivation rate constant of influenza virus

Preincubation condition [a]	Cells [b]	Fusion rate constant, f (s^{-1})	Inactivation rate constant, γ (s^{-1})	Lag time (s)
37° C for 5 min	Control	0.15	0.01	0
	NA-treated	0.03	0.05	0
0° C for 30 min, unbound virus removed	Control	0.13	0.02	0.7
	NA-treated	0.033	0.058	0

[a] In each case, influenza virus was preincubated with the indicated cells as described, and the pH was subsequently lowered to 5, at 37° C, to induce fusion.

[b] CEM cells were either treated with 0.005 unit/ml neuraminidase (NA), as described in Section 2, or mock-treated with buffer.

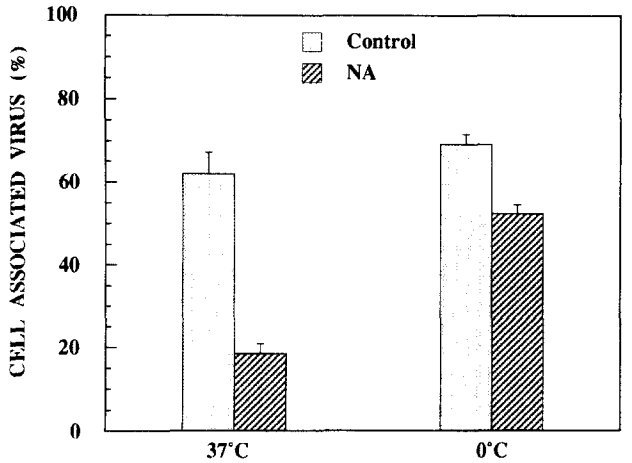

Fig. 3. Effect of neuraminidase pretreatment of CEM cells on influenza virus–cell binding. Influenza virus (2 μg viral protein/ml) was added to $4 \cdot 10^7$ control (shaded bars) or neuraminidase-treated CEM cells (striped bars) in a final volume of 2 ml at pH 7.4, and incubated at 37° C (5 min) or at 0° C (30 min). Virus–cell binding was determined from fluorescence values in the pellet and supernatant, after addition of $C_{12}E_8$ to dequench the R18 (see Section 2). Values represent means of at least 5 experiments ± standard deviation.

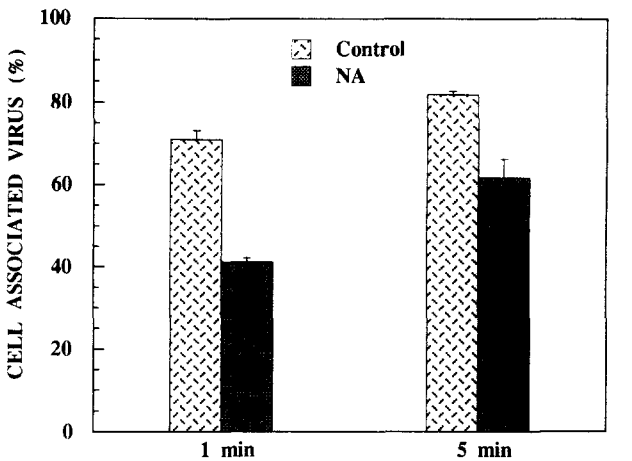

Fig. 4. Effect of neuraminidase pretreatment of CEM cells on influenza virus–cell association at low pH. Influenza virus (2 μg viral protein/ml) was added to $4 \cdot 10^7$ control (threaded bar) or neuraminidase treated CEM cells (shaded bars) in a final volume of 2 ml at 37° C. After 5 min incubation at pH 7.4, the virus–cell suspension was acidified to pH 5 and further incubated for 1 or 5 min. The fraction of cell-associated virus was determined by measuring the fluorescence in the pellet and the supernatant, after addition of $C_{12}E_8$ to dequench the R18 (see Section 2). Values represent means of at least 5 experiments ± standard deviation.

the neuraminidase-treated cells was much larger than that expected from fusion and binding results (compare with Figs. 1–3). The results in Fig. 4 also indicate that virus–cell association after a 1 min incubation at pH 5 was 87% of that obtained after a 5 min incubation for control cells, and 67% for neuraminidase-treated cells. The time course of cell association closely reflected the kinetics of virus fusion with control cells, where most of the fusion process was completed after 1 min. For neuraminidase-treated cells, however, the amount of virus that was cell-associated at pH 5 exhibited a time dependence that did not correlate with the viral fusion activity under the same conditions (see Figs. 1 and 2).

The observation that the percentage of cell associated virus at low pH (Fig. 4) closely reflected the percentage of fusion at 1 or 5 min (Fig. 1), indicates that most of the virus that had bound to the control cells underwent fusion. This result also indicates that virions did not merely co-sediment non-specifically with the cells during centrifugation following a low pH treatment. If the virus had aggregated and pelleted with the cells, the percentage of cell-associated virus would have been much larger than the percentage of fused virus. Previous light scattering and absorbance measurements had shown that virions did not aggregate at neutral or low pH under the experimental conditions employed [10,24]. The observations that (i) neuraminidase treatment of cells caused a large reduction of virus binding at 37° C (Fig. 3), and that (ii) essentially the same binding results were obtained when the virus preparation was pre-centrifuged before being incubated with the cells (Table 2 and data not shown), indicate that the virus did not aggregate or sediment non-specifically at neutral pH.

The higher fraction of the virus associated with neuraminidase-treated cells at pH 5, compared to neutral pH, could be the result of the increased hydrophobicity of the viral HA at low pH [25,26]. The increased hydrophobicity could arise from the dissociation of the globular heads of the HA trimers, or from exposure of the hydrophobic HA2 N-terminal fusion peptide [25,27,28]. To determine whether the enhanced binding of the virus to treated cells at low pH could be due to the unfolding of the globular heads, cell association experiments were carried out under conditions

Table 2
Influenza virus binding to CEM cells: effects of temperature and neuraminidase concentration

Preincubation conditions [a]	Incubation temperature (° C)	Binding [b] (% fluorescence)
Control	0	84.5 [3.1]
	37	77.8 [0.5]
NA (0.05 unit/ml)	0	37.7 [2.0]
	37	36.6 [1.0]
NA (0.005 unit/ml)	0	62.9 [0.9]
	37	39.4 [1.8]

[a] CEM cells were treated with either 0.05 or 0.005 unit (U)/ml neuraminidase (NA), as described in Section 2, or mock-treated with buffer (control).

[b] Influenza virus (pre-centrifuged at 180× g) was added to $4 \cdot 10^7$ control or neuraminidase-treated CEM cells, at a concentration of 2 μg viral protein/ml in a final volume of 2 ml at pH 7.4, and incubated at 37° C (5 min) or at 0° C (30 min). Virus–cell binding was determined from fluorescence values in the pellet and supernatant, after addition of $C_{12}E_8$ to dequench the R18 (see Section 2). The numbers represent the average value of duplicate determinations. The numbers in square brackets show the spread of the data in % of virions bound.

where the kinetics of unfolding is very slow, i.e., at 0° C [27]. Cell association measured in the cold after a 5 min incubation at neutral pH, was about 3-fold higher for control cells than for treated cells (data not shown). However, upon further incubation for 5 min at 0° C at pH 5, the fraction of virus bound was not significantly increased, both for treated and control cells (data not shown), in contrast to the results of virus-cell association at 37° C (Fig. 4). Thus, the lack of an increase in virus association with cells in the cold upon lowering the pH, particularly in the case of neuraminidase-treated cells, is consistent with the lack of (or very limited) unfolding of HA at low temperature. Although the fusion peptide is thought to be at least partially exposed at low temperatures [27,28], it is apparently not sufficient to enhance the binding of the virus to the cell membrane at low pH. The binding of the A/PR/8/34 strain of influenza virus to liposomes at pH 5 was also inhibited at 4° C, compared to the binding at 37° C, despite the partial exposure of the fusion peptide [28].

4. Discussion

Several observations indicate that cell surface sialic acid residues modulate the fusion activity of influenza virus, as well as mediating its binding to the cell surface: (i) The fusion of virions (at 37° C) prebound to neuraminidase-treated cells (at 0° C) is inhibited compared to fusion with control cells (Fig. 1b). Under these conditions where the unbound virions were removed, the receptor binding step is bypassed, and the fluorescence dequenching provides a measure of the fusion of virions which were already bound to the cells, possibly via non-sialic acid receptors. (ii) The fusion rate constant is decreased significantly when the sialic acid receptors are removed (Table 1). (iii) Prebinding of the virus to cells at 37° C does not enhance fusion with cells treated with neuraminidase, in contrast to control cells (Fig. 2). We propose that in cells from which sialic acid residues have been removed, virus binding occurs at sites on the cell surface where fusion is unfavorable. Alternatively, the virus may be able to bind to fusion sites, but it does not exhibit the proper orientation to trigger fusion, thus becoming inactivated at low pH [13,17,29]. When the cell surface sialic acid has been removed, the viral neuraminidase would also be unable to dislocate the virion from a binding site unfavorable for fusion. The observation that trypsin or proteinase K treatment did not have a significant effect on the fusion kinetics suggests that the binding site for influenza virus on CEM cells may be gangliosides rather that glycoproteins. Although further studies are needed to ascertain this hypothesis, previous studies have also implicated gangliosides as influenza virus receptors [1]. Furthermore, gangliosides have been shown to act as cellular receptors for Sendai virus, a paramyxovirus [30].

The initial kinetics of fusion with neuraminidase-pretreated cells depends on the conditions of preincubation of the virus and the cells, being apparently slower with preincubation at 37° C (Fig. 1A, curve b) than with preincubation at 0° C followed by removal of the unbound virus (Fig. 1B, curve d). The results in Fig. 3 indicate, however, that the amount of virus bound at 0° C is considerably greater than that at 37° C, thus contributing to the more rapid kinetics observed. Indeed, the kinetic parameters describing the fusion reaction in both cases turn out to be the same, within the estimated uncertainty (Table 1). The kinetic parameters obtained for both incubation conditions were also similar in the neuraminidase-treated cells. No lag times were observed except for the case of preincubation of control cells on ice, where a lag time of 0.7 s gave the best fit to the data.

Viral fusion studies using liposomes as target membranes have shown that influenza virus fuses readily with lipid membranes of a variety of compositions in a pH-dependent manner [10,17,24,27,31,32]. It is interesting to note that although the presence of sialic acid-containing receptors in the liposomal membrane is not required for fusion of influenza virus, it enhances virus binding [10]. Although ganglioside G_{D1a} had little effect on the fusion rate constants or on the final extents of fusion, it enhanced 3-fold the rate constant of adhesion for influenza virus [10]. Our results also show that influenza virus binding is dramatically reduced upon pretreatment of target cells with neuraminidase. However, the mass action kinetic model shows that, in contrast to virus–liposome fusion, removal of sialic acid also leads to a significant decrease in the fusion rate constant (see Table 1). The results of Stegmann et al. [27] suggested that gangliosides incorporated in zwitterionic liposomes may enhance the formation of an active fusion complex, thus leading to an increase in the rate of the actual fusion process. Studies with influenza virus prebound to planar bilayers with or without gangliosides suggested that the rate of membrane fusion is enhanced by the presence of gangliosides [33]. The kinetics of fusion pore formation in planar bilayers incubated with influenza virus-infected cells was also found to be affected by gangliosides in the target membrane [34]. Recently Alford et al. [11] showed that the presence of G_{D1a} could promote the initial kinetics of lipid mixing between influenza virus and large DOPC liposomes containing 2.5 or 5 mol% G_{D1a}, whereas with liposomes containing 10 or 15 mol% of the ganglioside the initial kinetics was slower. They also demonstrated that the presence of the ganglioside in liposomes reduced significantly the low pH inactivation of the virus which was preincubated with an excess of liposomes at neutral pH. In our system we have only observed enhancement in the fusion activity of the virus when the cell surface sialic acid was intact, which suggests that the sialic acid content on the surface of control CEM cells is in the optimal range for influenza virus fusion.

The results presented in Fig. 2 indicate that the extent

of influenza virus–cell fusion is higher when the virions are prebound to cellular sialylated membrane receptors, since the virus can start fusing immediately upon acidification of the medium. Our previous studies [13,17,18] have shown that the rate constants of inactivation (γ) at pH 5 for the virus prebound to CEM cells and several other cell lines are significantly lower than the values for the unbound virus, thus contributing to the enhanced extent of fusion in the case of prebound virus. The fact that prebinding does not enhance fusion in the case of neuraminidase-treated cells suggests that the presence of sialic acid residues on control cells is essential to confer biologically relevant viral fusion activity. In neuraminidase-treated cells, fusion may exhibit some nonspecific and nonphysiological nature, and, therefore, virus binding to predominantly non-sialic acid sites will not play an important role in the fusion process. In addition, virus prebound to neuraminidase-treated cells may undergo more inactivation when the pH is lowered than virus bound to control cells.

Our experiments on virus binding to cells at neutral pH (Fig. 3) stress the importance of sialic acid residues on the cell surface as primary receptors for influenza virus. Although the amount of binding is similar at 37° C and 0° C for control cells, binding is higher at 0° C for the neuraminidase-treated cells. These results suggest that the degree of reversibility in virus binding at 37° C is more pronounced in the treated cells than in the control cells, since the rate constant of dissociation is generally very small at low temperatures [35]. However, when cells are pretreated with a higher concentration of neuraminidase, similar levels of binding are observed at 0° C and 37° C (Table 2). On the other hand, pretreatment of cells with either the high or low concentration of neuraminidase resulted in similar values for virus fusion activity (data not shown), consistent with the similar levels of virus binding at 37° C (Table 2). It is likely that following treatment with the lower concentration of neuraminidase some sialic acid residues remain on the cell surface, which can be removed by the viral neuraminidase at 37° C (but not at 0° C, since the enzyme would be expected to be inactive at this temperature). This in turn would cause the observed reduction in binding to the neuraminidase-treated cells at 37° C, compared to that at 0° C. At 0° C, there would be considerable binding to the remaining sialic acid receptors (Fig. 3). This interpretation, however, does not explain why the viral neuraminidase does not reduce binding to untreated cells. It is possible that removal of some sialic acid residues by the neuraminidase treatment exposes new sialic acid moieties susceptible to the viral neuraminidase. In an alternative or complementary scenario, clustering of the remaining sialic acid-bearing residues in the cold may provide favorable binding sites for the virus; the dispersal of these receptor sites at 37° C may reduce the affinity of the virions for the cell surface. In the case of cells treated with the higher concentration of neuraminidase, however, the virus would not be able to remove any further sialic acid residues at 37° C. Thus, the level of binding at 37° C and 0° C would be similar. In this case, even the clustering of receptors in the cold would not be sufficient to mediate the higher level of binding, since more of the sialic acid residues would have been removed.

The extensive association of influenza virus with neuraminidase-treated cells at low pH (Fig. 4), without efficient fusion (Fig. 1) suggests that the virus binds nonspecifically to treated cells under these conditions, probably due to an increase in virus surface hydrophobicity as a result of the dissociation of the globular heads of HA trimers and protonation of acidic amino acids [25,26]. The observation that the fluorescence of the virus associated with neuraminidase-treated cells is greatly quenched, while that of the virus associated with control cells is essentially dequenched (data not shown) supports this view. The results of these experiments also provide evidence that the R18 in the viral membrane does not undergo molecular exchange or transfer under low pH conditions, despite extensive binding to the plasma membrane. If molecular probe transfer had occurred, the large fraction of virus associated with treated cells at low pH should have resulted in fluorescence dequenching. This provides supporting evidence for our previous conclusion that the increase of R18 fluorescence upon incubation of influenza virus with CEM cells at low pH was mainly due to fusion [13].

In summary, our results demonstrate that the presence of sialic acid-containing receptors is required for both efficient binding and fusion of influenza virus with CEM cells. In cells pretreated with neuraminidase, binding of influenza virus at neutral pH is drastically reduced; at mildly acidic pH cell association is enhanced, probably due to an increase in virus surface hydrophobicity, but most of the virions do not engage in fusion. Thus, influenza virus is only able to fuse efficiently at low pH without becoming significantly inactivated when it is bound to sialic acid-containing receptors. Therefore, it might be suggested that in the absence of sialic acid residues in target cells, the kinetics and extent of fusion of influenza virus are low and nonspecific due to an inefficient and nonspecific binding.

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