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THE ROLE OF TARGET MEMBRANE SIALIC ACID RESIDUES IN THE FUSION ACTIVITY OF THE INFLUENZA VIRUS: THE EFFECT OF TWO TYPES OF GANGLIOSIDE ON THE KINETICS OF MEMBRANE MERGING

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Abstract: The influenza virus enters target cells via the action of hemagglutinin proteins (HA) inserted into the viral envelope. HA promotes membrane fusion between the viral envelope and endosomal membrane at low pH, following viral binding to sialic acid-containing receptors on target cells, and internalization by endocytosis. The effect of target membrane sialic acid residues on the fusion activity of the influenza virus towards model membranes was evaluated by both reduction, (i.e. treating somatic cells with neuraminidase- (NA-) prior to virus-cell interactions), and by supplementing liposomes with the gangliosides GD1a and GT1b. The harshness of the neuraminidase pretreatment of target cells required to affect virus-induced membrane merging was found to greatly depend on the assay conditions, i.e. whether a virus-cell prebinding step at neutral pH was included prior to acidification. Minor concentrations of neuraminidase were found to greatly reduce virus fusion, but only in the absence of a prebinding step; they had no effect if this step was included. Although membrane merging was greatly reduced following cell neuraminidase pretreatment, virus-cell association at low pH was not disturbed proportionately. This probably reflects unspecific virus-cell binding under these conditions, probably of inactivated or aggregated virus particles, which does not translate into membrane merging. This seems to suggest both that target membrane sialic acid can protect the virus from losing its activity before triggering membrane merging, and that the importance of this interaction is not merely to ensure virus-target proximity. With liposomes, we found that both types of ganglioside supported efficient

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fusion, with GD1a promoting a slightly faster initial rate. However, in this case, virus-target proximity closely mirrored fusion activity, thus pointing to differential specificity between targets routinely used to assay influenza virus fusion activity.

Key Words: Influenza Virus, Membrane Fusion, Sialic Acid, Gangliosides, Liposomes

INTRODUCTION

The influenza virus enters its target cells by first binding to sialic acid residues on the cell surface, and subsequently being internalized by receptor-mediated endocytosis, and then delivered to endosomes. Viral access to the cytosol occurs following fusion between the viral envelope and the endosomal membrane, an event that is triggered by conformational changes in the envelope hemagglutinin (HA) at the acidic pH (±5.0) normally found in the endosomal lumen (for reviews see [1-3]). HA is a homotrimeric glycoprotein synthesized as an inactive precursor (designated HA0) in infected cells; it only becomes functionally active following proteolytic cleavage, which results into two distinct subunits (HA1 and HA2) linked by disulfide bonds. The low pH-induced conformational change includes dramatic changes in the membrane-bound HA2 subunits, the result of which is the exposure of a short N-terminal hydrophobic peptide that is projected into the medium and inserted into the target membrane. The insertion of the "fusion peptide" is thought to mediate physical changes in the apposing lipid-bilayers, thus bridging them and facilitating membrane merging (for reviews see [2-4]). Interestingly, if the conformational change cannot be translated into membrane fusion (i.e., if it takes place in the absence of any target membrane, for example) the viral HA quickly and irreversibly loses its membrane-perturbing qualities, a process dubbed "viral inactivation" [5], although some studies suggest both that the HA can retain some fusogenic ability following inactivation [6], and that at least some aspects of the low pH-induced conformational change may be reversible [7, 8]. It is also worth noting that most studies have been performed in "bulk" systems (i.e. looking at and averaging out the activity of many HA trimers), and that only a few molecules may actually participate in membrane merging, and thus undergo physiologically relevant changes [1, 9].

Much attention has focused on the later (i.e. HA2-dependent) steps in influenza virus-mediated membrane fusion. However, relatively little attention has been paid to the initial contact between HA1 and the target membrane [27, 29], and on the influence this interaction may ultimately have on membrane merging, possibly by accurately positioning a sufficient number of trimers relative to the endosome membrane. It has been found that target membrane components modulate the fusion activity of lipid-enveloped viruses by interfering with HA interactions with cellular membranes [10, 11] Indeed, it has been proposed that target membrane sialic acid residues, which modulate

influenza virus binding to target cells, play a role in the membrane fusion step itself [10]. We have addressed this issue by applying a dual experimental approach, and studying the binding and fusion activity of the influenza virus to two types of target membrane: on the one hand, cells from which sialic acid moieties were removed following neuraminidase treatment; and, on the other, artificial target membranes (liposomes) supplemented with different types of gangliosides. Viral fusion activity towards these two types of target membranes was determined using the octadecylrhodamine B chloride (R18) fluorescence dequenching assay [5, 6, 12].

MATERIALS & METHODS

Chemicals

All the chemicals used were obtained from the Sigma Chemical Company (St Louis, MO), unless stated otherwise.

Virus

The A/PR/8/34 (H1N1) strain of the influenza virus was grown for 48 h at 37ºC in the allantoic cavity of 11-day-old embryonated eggs, purified by discontinuous sucrose density gradient centrifugation and stored at -70ºC in phosphate buffered saline.

Cells

CEM cells were donated by Dr. Nejat Duzgunes (University of the Pacific School of Dentistry, San Francisco CA). Cells were grown in a RPMI 1640 medium containing 25 mM Hepes, supplemented with 10% foetal bovine serum and antibiotics. Cells were grown in T-75 flasks up to a cell density of $1\text{-}1.5x10^6$/ml under a 5% CO_2 atmosphere in a Forma Scientific incubator. For experimental purposes, the cells were harvested by centrifugation at 180xg for 8 min at room temperature, washed twice in PBS, once in phenol red-free RPMI 1640 supplemented with 25 mM Hepes (Medium A), and resuspended in the latter medium. Cell viability was determined by Trypan blue extrusion, and was routinely above 95%. CEM cells were used since they exhibit very low endocytic ability [10], and thus virus-cell interactions take place solely at the plasma membrane level.

Liposome preparation

Phospholipids were obtained from Avanti Polar Lipids (Alabaster, AL). Liposomes (LUVs – large unilamellar vesicles), composed of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) in a 2:1 molar ratio, and containing 5 mol % of the gangliosides GD1a or GT1b, were prepared in 85 mM NaCl, 50 mM KCl, 1mM EDTA, 10 mM Hepes, 10 mM Mes, 10 mM sodium citrate, buffered at pH 7.4 (Medium B) by the reverse-phase evaporation method [13]. The vesicles were sized through 0.1 μm polycarbonate filters, and their concentration was determined by a phosphate

assay. Multilamellar vesicles (MLVs) were prepared by gently rehydrating dried lipid films with Medium B.

Virus labeling

Influenza virus samples were labeled with the fluorescent probe octadecylrhodamine B chloride (R18; Molecular Probes Inc., Eugene, OR) at a selfquenching concentration, as described previously [5, 6, 12]. The final concentration of added probe corresponded to approximately 5 mole % of the total viral lipid, and that of ethanol was less than 1% (v/v). The mixture was incubated in the dark for 30 min at room temperature. R18-labeled virus was separated from non-inserted fluorophore by chromatography on Sephadex G-75 (Pharmacia, Uppsala, Sweden) using 150 mM NaCl, 10 mM Hepes, pH 7.4 as an elution buffer. The protein concentration of the labeled virus was determined using the Lowry assay.

Viral fusion activity

Fusion was monitored continuously at 37ºC as a function of R18 fluorescence dequenching, dependent on probe dilution into a target membrane upon virus fusion [5, 6, 12]. For experiments with CEM cells, influenza virus (2 µg of viral protein/ml) was added to 4 x 10^7 cells in a final volume of 2 ml of Medium A. Membrane merging was triggered by adjusting the medium pH to 5.0, either immediately before virus addition, or following a 5 min virus-cell incubation at pH 7.4 and 37°C, to allow viral binding (see Results). When liposomes were employed as viral target membranes, influenza virus (2 µg of viral protein) was added to 200 nmoles of LUVs in a final volume of 2 ml of Medium B at pH 7.4 and 37°C. Following a 15 min incubation, the pH was lowered to 5.0, and virus-liposome fusion followed. The binding periods chosen were those that guaranteed maximal fusion activity in each system.

In all our experiments, the fluorescence scale was calibrated such that the initial fluorescence of R18 labeled virus and cells was set at 0% fluorescence, and the value obtained by detergent lysis after each experiment (maximal probe dilution) was set at 100% fluorescence. In experiments with CEM cells, the detergent octaethyleneglycol dodecyl ether ($C_{12}E_8$, CalBiochem, San Diego, CA) was used for calibration purposes, at a final concentration of 2 mM. For experiments with liposomes, Triton X-100 was employed (1% v/v final concentration). In fusion experiments, unbound virions were not separated from bound virions contrary to what takes place for binding and cell association experiments (see below).

In some cases, both the extent of dequenching and the initial rate (% dequenching/min during the first few seconds following pH reduction) were quantified. Fluorescence measurements were performed in a SPEX Fluorolog or a Perkin-Elmer LS-50B spectrofluorometer, with excitation at 560 nm and emission at 590 nm. The sample chamber was equipped with a magnetic

stirring device, and the temperature was maintained at 37ºC with a thermostated circulating water bath.

Binding and cell association

To quantify virus-cell binding, R18-labeled influenza virus (2 µg of viral protein) was added to 4 x 10^7 CEM cells in a final volume of 2 ml of Medium A. The virus-cell suspension was then incubated at 37ºC and pH 7.4 for 5 min. For virus-liposome binding, influenza virus (2 µg) was added to 400 nmoles of MLVs, in a final volume of 2 ml of Medium B and incubated for 15 min at pH 7.4 and 37ºC. Following these incubations, cells and MLVs were centrifuged, and fluorescence was measured in the pellet and supernatant after the addition of either $C_{12}E_8$ (2 mM, for CEM cells) or Triton X-100 (1% v/v, for MLVs). Binding was calculated as the percentage of fluorescence in the pellet relative to total fluorescence (pellet + supernatant).

Cell association was also measured for influenza virus interactions with CEM cells. This monitors not only bound virions at neutral pH (that may, or may not, have fused with the target), but also virus that may have associated non-specifically with the cells following acidification. Therefore, the virus-cell incubation at pH 7.4 detailed above was followed by a further 5 min incubation at pH 5.0 (37ºC). The cells were then centrifuged, and cell association was calculated as described above [10]. In these experiments, total fluorescence (Supernatant + Pellet) was equivalent to the 100% calibration done in the fusion experiments (not shown).

Enzymatic treatment

To assess the importance of target membrane sialic acid on influenza virus fusion activity towards target membr**a**nes, CEM cells were pretreated with neuraminidase (NA from *Vibrio cholerae*, 1 unit/ml specific activity) obtained from CalBiochem. Three different concentrations of the enzyme were used (0.5, 5 and 50 mu/ml). In all cases, incubation of CEM cells (4 x 10^7) with NA took place in 2 ml Medium A for 20 min at room temperature. Following enzymatic treatment, the cells were centrifuged (180 x g, 8 min), washed once with Medium A, and ressuspended in 2 ml of the same medium.

Statistical analysis

Statistical analysis of all the data generated was carried out using the Student-Newman-Keuls or the Tukey-Kramer multiple comparison tests.

RESULTS

Effect of neuraminidase pretreatment on influenza virus interaction with CEM cells

We have previously shown [10] that treatment of CEM cells with 5 mu/ml of NA greatly reduces (but does not totally abolish) influenza virus fusion activity towards this particular target membrane. However, in the absence of

viral-cell prebinding (i.e. if R-18 labeled virus is added to CEM cells in a medium whose pH is already adjusted to 5), a ten-fold decrease in the amount of enzyme added during cell pretreatment is sufficient for the maximum inhibitory effect (Fig. 1). A decrease in influenza virus fusion activity towards NA-treated cells is already evident following 1 min of incubation, and an increase in the incubation time (to 5 min) only results in a slight (but significant) increase in the dequenching observed with the control cells. Increasing NA concentration to 5 or 50 mu had no further effect (Tab. 1). Interestingly, if the virus is prebound to CEM cells at neutral pH prior to acidification, pretreatment of cells with 0.5 mu of NA, which was sufficient for maximal inhibition using the previous experimental protocol, resulted in only a minor (not significant) decrease in fusion activity (Fig. 2). Influenza virus fusion with CEM cells was only significantly decreased if the cells had been pretreated with at least 5 mu/ml of NA, and a ten-fold increase in enzyme concentration had no further effect (Fig. 2). The extents of fluorescence dequenching were stable for 1 min following acidification, and no significant increases were detected if the incubation was prolonged to 5 min (data not shown).

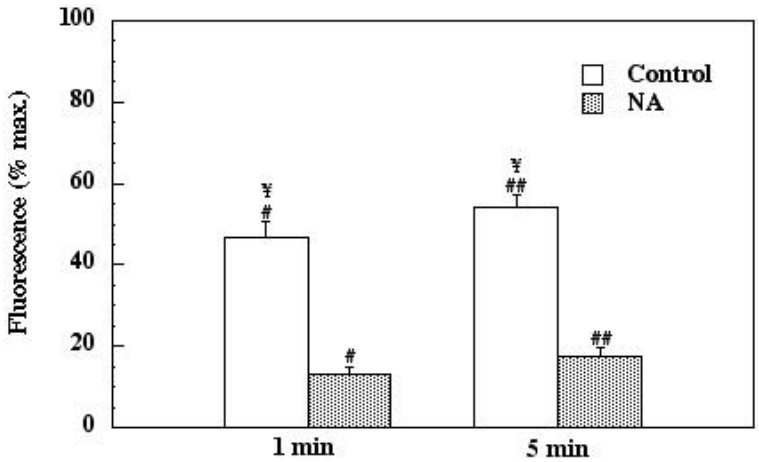

Fig. 1. Effect of neuraminidase pretreatment of CEM cells on the fusion activity of the influenza virus. R18-labeled influenza virus (2 µg viral protein/ml) was added to 4 x 10^7 Control (open bars) or NA-pretreated (shaded bars) CEM cells at 37ºC and pH 5.0. The extent of fusion, as monitored by the extent of R18 dequenching, was calculated after 1 and 5 min, as depicted. In this case, cells were pretreated with 0.5 mu of NA/ml, but similar results were obtained with 5 or 50 mu of NA/ml (data not shown). The results obtained with NA-treated CEM cells were always significantly lower than with Control cells (# and ## $p < 0.001$). Dequenching quantified 1 min after acidification was slightly enhanced by further incubation at low pH when Control cells, but not NA-treated cells, were used as target membranes for the virus (¥ $p < 0.01$). The average ± S.D. of 6 independent experiments is presented.

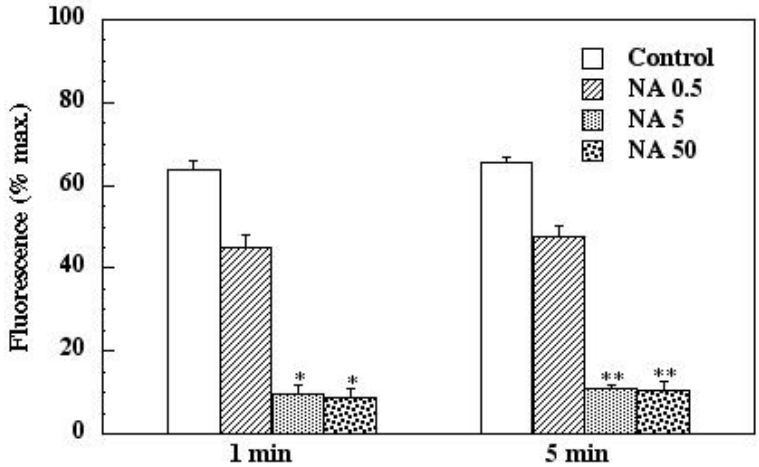

Fig. 2. Effect of neuraminidase pretreatment of CEM cells on the fusion activity of the influenza virus in the presence of virus-cell prebinding. R18-labeled influenza virus (2 µg viral protein/ml) was added to 4 x 10^7 Control (open bars) or NA-pretreated CEM cells at 37ºC and pH 7.4. In separate experiments, CEM cells were pretreated with 0.5 (stripped bars), 5 (shaded bars) or 50 (squared bars) mu of NA/ml prior to the fusion assays. Following a 5 min incubation to allow virus-cell binding, the medium pH was adjusted to 5.0, and the extent of R18 dequenching calculated after 1 min. For each experimental group, the results obtained after a 5 min incubation were not statistically different from those calculated after 1 min (not shown). The results with Control cells and cells pretreated with 0.5 mu of NA/ml were statistically indistinguishable. However, the dequenching observed following cell pretreatment with 5 or 50 mu of NA/ml was significantly reduced compared to the Control experiments (* $p < 0.001$). The average ± S.D. of at least 5 independent experiments is presented.

The results obtained with the membrane merging assays were mirrored by binding results (Fig. 3A). Thus, the binding of influenza virus to CEM cells at neutral pH was only mildly (but significantly) affected following cell pretreatment with 0.5 mu/ml of NA. A much more pronounced effect was obtained following cell pretreatment with both 5 and 50 mu/ml of NA, although (as discussed for viral fusion activity) virus-cell binding was never completely abolished (Fig. 3A). Interestingly, cell association data (which quantifies virions fused or bound after an incubation at low pH) was not as clear-cut (Fig. 3B). Thus, while cell pretreatment with 0.5 mu/ml of NA had no effect on influenza virus association with CEM cells at pH 5, the effect of higher enzyme concentrations, although statistically significant, was minor compared to the results obtained with the virus-cell binding assay at neutral pH. Indeed, although the binding data discussed here was comparable to previously published results, the effect of cell pretreatment with 5 or 50 mu/ml of NA on cell association was even slightly lower than what has been described [10].

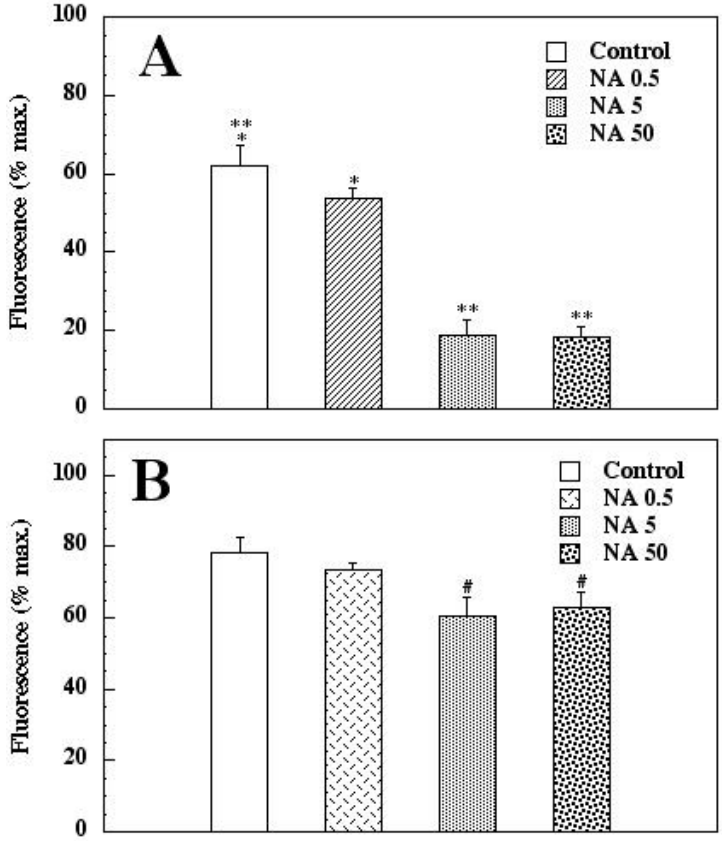

Fig. 3. Effect of neuraminidase pretreatment of CEM cells on influenza virus binding and cell association. R18-labeled influenza virus (2 µg viral protein/ml) was added to 4 x 10^7 Control (open bars) or NA-pretreated CEM cells at 37ºC and pH 7.4. In separate experiments, CEM cells were pretreated with 0.5 (stripped bars), 5 (shaded bars) or 50 (squared bars) mu of NA/ml prior to adding the virus. Virus binding (A) and Cell association (B) were calculated as described in the Methods section. Binding was found to be significantly reduced when NA-treated cells were used as viral targets, more noticeably for the pretreatment with higher enzyme concentration (* $p < 0.05$; ** $p < 0.001$). Cell association relative to control situations was only reduced when the cells had been pretreated with higher concentrations of NA (# $p < 0.01$). The results obtained following cell pretreatment with 5 and 50 mu NA/ml were always statistically indistinguishable. The average ± S.D. of at least 4 independent experiments is presented in all cases.

Our findings with this system are summarized in Tab. 1, where the percentages of inhibition found with NA-treated cells relative to the appropriate controls are listed for all our experimental designs. As discussed above, CEM treatment with the lowest concentration of NA results in either negligible (Fusion with binding, Binding, Cell Association) or significant (Fusion without binding) inhibition of virus-cell interactions. This is in contrast with what takes place

with higher concentrations of NA, where the results are always significantly lower than the controls. However, virus-cell association at low pH is always very high, and possibly reflects non-functional (probably unspecific) interactions.

Tab. 1. The effect of Neuraminidase (NA) treatment of a target membrane on interactions between the influenza virus and CEM cells[a].

	% of Inhibition Relative to the Control		
	NA 0.5 mu/ml	NA 5 mu/ml	NA 50 mu/ml
Fusion without Binding	67.5	68.9	70.2
Fusion with Binding	27.7	83.3	85.7
Binding	12.9	69.7	70.4
Cell Association	6.1	23.0	19.7

[a]Fusion, in the absence or presence of virus-cell prebinding, was monitored for 5 min at pH 5.0. Binding and Cell Association were calculated as described in the Methods section. The percentage of inhibition was calculated relative to the appropriate controls in each case. The average of at least 4 independent experiments is presented.

Effect of the gangliosides GD1a and GT1b on the fusion activity of the influenza virus towards lipid vesicles

The removal of sialic acid residues from CEM cells resulted in a clear reduction of influenza virus fusion towards these cells. Therefore, we decided to investigate to what extent introducing sialic acid residues, in the form of different gangliosides, into a target membrane would affect influenza fusion activity. As would be expected, the influenza virus bound poorly to PC/PE MLV liposomes that do not contain viral receptors (Tab. 2). The inclusion of both GD1a and GT1b in the lipid bilayer lead to a marked potentiation of virus-liposome binding, and, although values were higher for GT1b, no statistically significant differences were detected between the two gangliosides at this level (Tab. 2). However, a higher influenza virus affinity for GT1b has previously been reported, using a more sensitive methodology [14].

Tab. 2. The binding of influenza virus to MLVs[a].

	PC/PE	GD1a	GT1b
BINDING (%)	17.6 ± 3.1	45.4 ± 3.4	49.8 ± 2.9

[a]R18-labeled influenza virus (2 µg) was added to 400 nmoles of MLVs, in a final volume of 2 ml at 37ºC. Virus-cell binding was quantified following a 15 min incubation at pH 7.4. The average of at least 3 independent experiments (± S.D.) is presented.

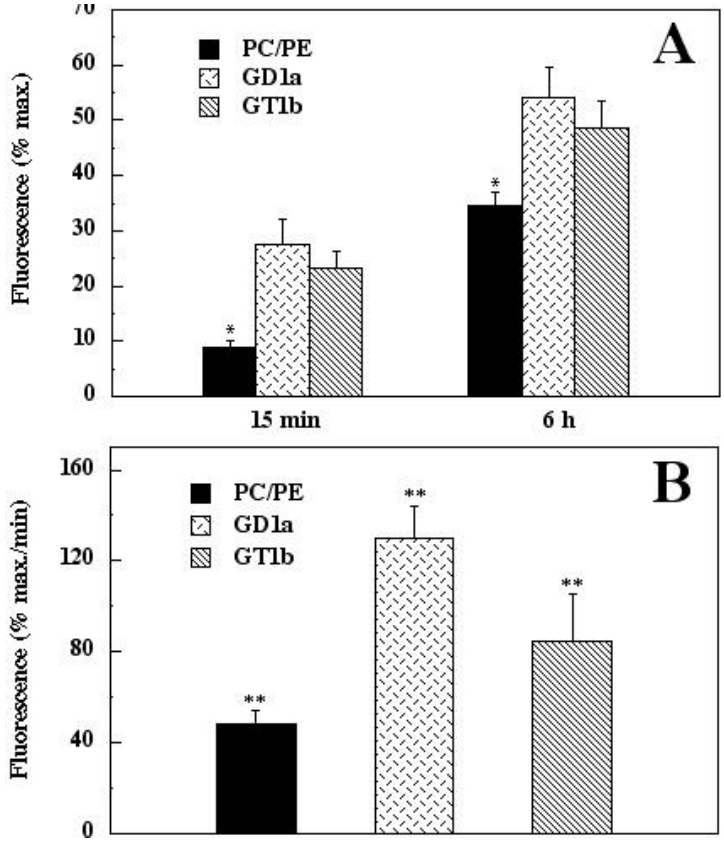

Fig. 4. Effect of gangliosides GD1a and GT1b on the fusion activity of influenza towards PC/PE liposomes. R18-labeled influenza virus (2 µg viral protein) was added to 200 nmoles of PC/PE LUVs (filled bars), and PC/PE vesicles containing 5 mol % of GD1a (etched bars) or GT1b (stripped bars) at 37ºC. Following a 15 min incubation at pH 7.4, the pH was lowered to 5.0, and virus-liposome fusion followed, and was quantified as described in the Materials section. A. The extent of fusion was quantified after 15 min at pH 5.0. The result with PC/PE liposomes was significantly different from those obtained with ganglioside-containing LUVs (* $p < 0.001$). No significant differences were observed when GD1a or GT1b were used in LUV preparation. B. The initial fusion rate was calculated by measuring the rate of dequenching in the initial stages of membrane merging (15-40 s after acidification). The results obtained with all liposome populations were significantly distinct from each other (** $p < 0.001$). The averages ± S.D. of at least 7 independent experiments are presented in all cases.

Similarly, virus fusion with liposomes was also enhanced by the inclusion of gangliosides in the target membrane (Fig. 4A). In fact, the extent of influenza virus fusion with liposomes lacking viral receptors was quite low, as reported previously [6], and only increased significantly following long incubation

periods (not shown), i.e. in an experimental situation where unspecific exchange of R18 (i.e. transfer of fluorescent probe from labeled virions to the target membrane in the absence of true membrane merging) must be taken into account [15]. As noted with binding results, the increase in the extent of dequenching was significant when either ganglioside was introduced in the target membrane, and the results obtained with GD1a and GT1b were also statistically identical (Fig. 4A). However, the effect of the two gangliosides could be readily differentiated if, instead of the final extent of fusion, the initial rate of fluorescence dequenching (i.e. the rate of fusion in the first few seconds following low pH-induced membrane merging) was quantified. Indeed, although the initial rate of fusion between the influenza virus and PC/PE liposomes was again predictably low, it could be potentiated by the inclusion of sialic acid-containing gangliosides, in this case with GD1a showing a much more pronounced effect than GT1b (Fig. 4B).

DISCUSSION

Although the influenza virus can fuse with membranes composed solely of phospholipids [16, 17], viral fusion activity is greatly enhanced by sialic acid residues on the target membrane [10, 14, 16-22, 28]. The modulating action of sialic acid may take place at several levels, from initial virus-target binding via the HA1 subunit to the actual membrane merging step [1-3]. In this latter case, sialic acid binding may contribute to membrane proximity, affect low pH-induced conformational change, or even actively participate in the insertion of the fusion peptide into the target membrane [1, 10, 16, 19, 23]. Interestingly, it was recently proposed that not all HA proteins at the fusion site may be bound to the target membrane via sialic acid receptors, and that sialate binding can affect the contribution of an individual HA to membrane merging [24], or to dilation of the fusion pore [29]. It should also be noted that the true nature of the viral receptors (sialoglycoprotein or sialoglycolipids) is still in debate, with recent data pointing specifically to protein anchors [25], although this may vary with cell type.

However, this effect of sialic acid receptors on the target cell surface is likely to vary with the experimental approach, depending on how influenza virus fusion activity is monitored. Thus, cell pretreatment with very low concentrations of exogenous neuraminidase had little effect on virus-cell binding at neutral pH, or on virus fusion, provided that fusion was assayed by prebinding the virus to the plasma membrane at neutral pH, before triggering membrane merging by acidification. However, and contrary to what we previously described [10], this same pretreatment was sufficient to reduce fusion activity by 67%, provided the virus was directly added to the cells at pH 5.0. This suggests that the virus HA proteins undergo a pH-dependent conformational change prior to contact with the target membrane, and inactivate before being able to induce fusion [5, 6]. This inactivation may

result in the aggregation of viral particles due to an increase in surface hydrophobicity [30], although the inactivated virus still retains the ability to bind to target cells, albeit probably non-specifically [5, 10], as apparent from the large cell association of virus with CEM cells, even when binding at neutral pH is low. If the virus is allowed to contact the target membrane at neutral pH before fusion takes place, a much higher concentration of exogenous neuraminidase is needed to effectively reduce membrane merging. This indicates that target membrane sialic acid can protect the virus from inactivation/aggregation, and help convert the HA conformational change into useful membrane interactions [10]. Although we cannot discard low pH-induced changes at the cell surface, which might impair receptor availability, our results also seem to show that this effect is not solely dependent on virus-cell proximity. Indeed, although neuraminidase-treated cells are much less adequate targets for the influenza virus, the virus nevertheless binds and associates extensively with these cells, both at neutral, and, especially, at acidic pH.

Interestingly, although neuraminidase pretreatment of CEM cells negatively mirrored the effect of supplementing liposomes with gangliosides, the same conclusion could not be extended to liposome models. Virus-membrane binding closely paralleled virus-membrane fusion in this case, i.e. a very low level of binding to MLVs without gangliosides corresponded to the residual fusion activity monitored in this case. It is possible that this difference may result from neuraminidase-insensitive receptors on the surface of CEM cells. Studies with model systems have shown that although influenza virus fusion activity can be potentiated in the presence of the ganglioside GD1a, this effect depended highly on the amount introduced in the target membrane: low amounts of ganglioside stimulated fusion, while high amounts partially reversed this effect [18]. However, we have always either seen an inhibition of fusion activity when different types of target membranes are treated with neuraminidase, or seen no effect of enzymatic pretreatment. Therefore, the sialic acid content of most biological targets for the virus must be sufficient (if suboptimal) for fusion activity, but not inhibitory, although it is also possible that the viral neuraminidase may be important if such cases do occur. An additional finding deals with the fact that viral fusion kinetics are higher when GD1a is introduced into the target membrane, although the virus has been shown to bind with greater affinity to GT1b [14]. This raises two possibilities which are not mutually exclusive. On the one hand, the delay may represent the time needed for the conversion of GT1b into a more suitable receptor via the action of the viral neuraminidase [26], or this effect is due exactly to the higher binding affinity towards GT1b [14]. The fact that the virus is more tightly bound to this receptor constrains its ability to interact efficiently with the target membrane. Indeed, recent results point to the fact that tight receptor binding may delay the opening of the fusion pore [23, 29]. Taken together, these observations imply that there is an optimal interaction between the

influenza virus and the target membrane (reflected in both the number of receptors, and the affinity of viral binding to each individual receptor) that leads to efficient membrane merging.

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