The Use of a Quantitative Fusion Assay to Evaluate HN–Receptor Interaction for Human Parainfluenza Virus Type 3

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Sialic acid is the receptor determinant for the human parainfluenza virus type 3 (HPF3) hemagglutinin–neuraminidase (HN) glycoprotein, the molecule responsible for binding of the virus to cell surfaces. In order for the fusion protein (F) of HPF3 to promote membrane fusion, HN must interact with its receptor. In addition to its role in receptor binding and fusion promotion, the HPF3 HN molecule contains receptor-destroying (sialidase) activity. The putative active sites are in the extracellular domain of this type II integral membrane protein. However, HN is not available in crystalline form; the exact locations of these sites, and the structural requirements for binding to the cellular receptor, which has not yet been isolated, are unknown. Nor have small molecular synthetic inhibitors of attachment or fusion that would provide insight into these processes been identified. The strategy in the present study was to develop an assay system that would provide a measure of a specific step in the viral cycle—functional interaction between viral glycoproteins and the cell during attachment and fusion—and serve to screen a variety of substances for inhibitory potential. The assay is based on our previous finding that CV-1 cells persistently infected (p.i.) with HPF3 do not fuse with one another but that the addition of uninfected CV-1 cells, supplying the critical sialic acid containing receptor molecules that bind HN, results in rapid fusion. In the present assay two HeLa cell types were used: we persistently infected HeLa-LTR-βgal cells, assessed their fusion with uninfected HeLa-tat cells, and then quantitated the β-galactosidase (βgal) produced as a result of this fusion. The analog α-2-S-methyl-5-N-thioacetylneuraminic acid (α-Neu5thioAc2SMe) interfered with fusion, decreasing βgal production by 84% at 50 mM and by 24% at 25 mM. In beginning to extend our studies to different types of molecules, we tested an unsaturated derivative of sialic acid, 2,3-dehydro-2-deoxy-n-acetyl neuraminic acid (DANA), which is known to inhibit influenza neuraminidase by virtue of being a transition-state analog. We found that 10 mM DANA inhibited neuraminidase activity in HPF3 viral preparations. More significantly, this compound was active in our assay of HN–receptor interaction; 10 mM DANA completely blocked fusion and βgal production, and hemadsorption inhibition by DANA suggested that DANA blocks attachment. In plaque reduction assays performed with the compounds, the active analog α-Neu5thioAc2SMe reduced plaque formation by 50% at a 50 mM concentration; DANA caused a 90% inhibition in the plaque reduction assay at a concentration of 25 mM. Our results indicate that specific sialic acid analogs that mimic the cellular receptor determinant of HPF3 can block virus cell interaction and that an unsaturated n-acetyl-neuraminic acid derivative with affinity to the HN site responsible for neuraminidase activity also interferes with HN–receptor binding. Strategies suggested by these findings are now being pursued to obtain information regarding the relative locations of the active sites of HN and to further elucidate the relationship between the receptor-binding and receptor-destroying activities of HN during the viral life cycle. The quantitative assay that we describe is of immediate applicability to large-scale screening for potential inhibitors of HPF3 infection *in vivo*.   © 1999 Academic Press

INTRODUCTION

The envelope of HPF3 contains two viral glycoproteins designated HN (hemagglutinin–neuraminidase) and F (fusion protein). Attachment of the virus to the host cell is initiated through interaction of HN with the sialic acid containing cell surface receptors. The ensuing fusion of the viral envelope with the cellular membrane, resulting in the release of the nucleocapsid into the cytoplasm, is mediated by F. However, in the case of HPF3, and some other paramyxoviruses, both HN and F are involved in membrane fusion. Cofunction of both glycoproteins was found to be necessary for syncytium formation (Horvath *et al.,* 1992; Hu *et al.,* 1992; Lamb, 1993; Moscona and Peluso, 1991), and it has been proposed that interaction of HN with the receptor triggers the conformational change in F required for its fusion promoting activity (Lamb, 1993). By virtue of its N (neuraminidase) moiety, HN also has a receptor-destroying potential, which plays a role in the spread of infection. Comparison in this laboratory of HPF3 HN variants with mutations in N have demonstrated the dependence of viral release on the level of receptor-destroying neuraminidase activity (Huberman *et al.,* 1995).

An assay system that would quantify functional viral HN–receptor contact and allow easy screening for inhibitory compounds was suggested by the properties of our *in vitro* model for persistent HFP3 infection (Moscona

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and Galinski, 1990; Moscona and Peluso, 1991). CV-1 cells infected at high multiplicity of infection (m.o.i.) do not form syncytia because their surface is depleted of the sialic acid receptor determinant necessary to interact with HN, but have the ability to fuse with uninfected cells (Moscona and Galinski, 1990; Moscona and Peluso, 1991). In order to obtain a more sensitive and quantitative assay for fusion we now used two HeLa cell types, one possessing the gene for β-galactosidase (lacZ) under the control of the HIV LTR and one expressing HIV tat protein. Once fusion between the two cell populations occurs, tat from one transactivates lacZ expression in the other population of cells, leading to the production of β-galactosidase (βgal).

In the present study we show that this particular assay can be used to assess HN–receptor interaction during the binding and fusion processes and to address functional questions about HN. We began by examining the possibility that compounds that mimic the sialic acid moiety of the cellular receptor for HPF3 would block HN–receptor interaction. The relative inhibitory action of such analogs should provide information about the structure of the binding site of HN, as well as permit the design of low-molecular-weight inhibitors of HPF3 infection and pathogenesis.

Using our cell fusion assay as one indication of HN–receptor interaction during binding between HN and receptor and cell fusion, we analyzed synthetic sialic acid analogs for their ability to inhibit fusion between persistently infected (p.i.) cells and uninfected cells. The naturally occurring sialic acid, Neu5Ac, was modified at particular sites to determine which functional groups may be important in recognition and binding of HN to sialic acid-containing molecules. The analogs were also analyzed for their ability to inhibit viral infection as determined by a standard plaque assay. We found that the analog α-2-S-methyl-5-N-thioacetylneuraminic acid (α-Neu5thioAc2SMe), which blocked fusion between persistently infected and uninfected cells, also inhibited plaque formation.

Our studies also included 2,3-dehydro-2-deoxy-n-acetylneuraminic acid (DANA), an unsaturated sialic acid derivative, which is an inhibitory transition-state analog of influenza virus neuraminidase. We found that this compound inhibited the neuraminidase activity in HPF3 viral preparations. In our quantitative assay, DANA blocked the fusion of p.i. cells with uninfected cells. The ability of DANA to interfere with viral entry was indicated by its inhibitory effect in the plaque reduction assay. Blockade of attachment of erythrocytes to p.i. cells by DANA during hemadsorption supported the postulate that DANA's interference with fusion in the quantitative assay was due to interference with receptor interaction.

The results demonstrate the broad utility of the herein described quantitative fusion assay for assessing receptor interaction, for identifying potential inhibitors of para-influenza virus type 3 infection, and for uncovering new information about the nature of HPF3–receptor interaction.

RESULTS/DISCUSSION

Development of the fusion assay

To further our understanding of the interaction between the HN glycoprotein and its sialic acid-containing receptor, we developed a quantitative assay that allows us to test the ability of molecules to interfere with the interactions between the viral glycoproteins and the target cell that are required for fusion. To set up this assay, we took advantage of the fusion properties of our in vitro model of persistent infection with HPF3 (Moscona and Galinski, 1990; Moscona and Peluso, 1991). Persistent infection with HPF3 is readily established in cultured CV-1 cells by infection of cell monolayers at high multiplicity of infection (Moscona and Galinski, 1990). While the p.i. cells do not fuse with one another, they undergo rapid and complete fusion when seeded with uninfected cells. We showed that the lack of fusion between the p.i. cells was due to an insufficiency of sialic acid-containing receptors for the viral HN glycoprotein and that their fusion with uninfected cells results directly from the interaction of HN with receptors available on the uninfected cells (Moscona and Peluso, 1991).

To quantitate fusion we used two HeLa cell types, one (HeLa-CD4-LTR-βgal) containing the β-galactosidase gene under control of the HIV LTR and another (HeLa-tat) expressing the HIV tat protein. Fusion between the two different cell types leads to the production of β-galactosidase, which is then quantitated. These HeLa cell types were used previously to assess cell fusion mediated by expressed individual HPF3 proteins (Tanaka and Galinski, 1995). A somewhat different approach (using T7 RNA polymerase expressed by recombinant vaccinia virus in one cell population to drive lacZ synthesis in the other) was applied to demonstrate fusion mediated by HIV envelope proteins (Nussbaum et al., 1994) and by expressed HPF3, NDV, and SV5 proteins (Bagai and Lamb, 1995) and to assess the relationship between the surface density of expressed HPF3 HN protein and the extent of HPF3 F protein promoted fusion (Dutch et al., 1998). For our experiments, we established a persistent infection by HPF3 in HeLa-CD4-LTR-βgal cells and assessed the fusion of these cells with uninfected HeLa-tat cells as a measure of interaction between HN on p.i. cells and sialic acid receptor on uninfected cells.

In the present assay system, HeLa-CD4-LTR-βgal cells persistently infected with HPF3 were seeded in 96-well plates and allowed to adhere overnight. Uninfected HeLa-tat cells were then added and βgal activity, resulting from the fusion between the two cell types, was determined in lysates as described under Materials and Methods.
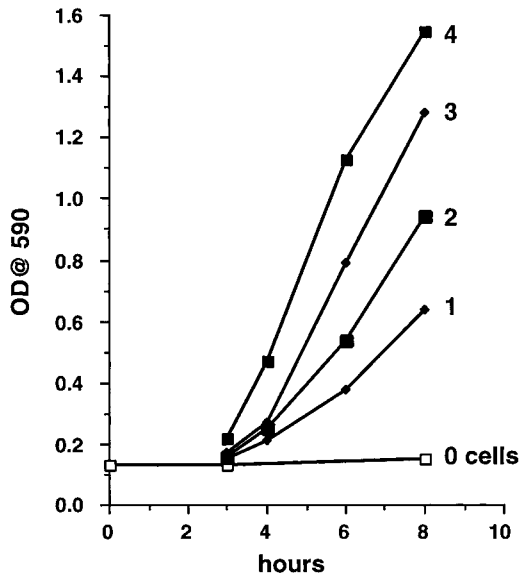
FIG. 1. Time course of βgal activation resulting from the fusion of varying numbers of uninfected HeLa-tat cells with the adherent layer of p.i. HeLa-CD4-LTR-βgal cells. βgal activity (ordinate) was determined after the indicated hours of incubation (abscissa). The numbers of uninfected cells added at time 0 were 0.38×10^4, 0.75×10^4, 1.5×10^4, and 3×10^4 for graphs 1, 2, 3, and 4, respectively. The adherent layer contained 3×10^4 cells.

To explore the sensitivity of the assay system, we varied the number of uninfected cells (added to a constant number of adherent p.i. cells) and followed the time course of βgal activation (Fig. 1). Fusion, assessed microscopically, was complete at 2 h; β-galactosidase activity became appreciable at 3 h, and its ensuing rise (curves 1−4, Fig. 1) was directly related to the number of uninfected cells added. This cell number-dependent gradation in the extent of fusion was most clearly reflected by βgal activity measured after the fourth hour of incu-

bation. Based on experiments like those in Fig. 1, βgal was routinely determined 6 h after the addition of 3×10^4 uninfected cells to 3×10^4 adherent p.i. cells. Wells with adherent p.i. cells to which no uninfected cells were added served as controls.

Experiments designed to confirm the specificity of the assay included pretreatment of the uninfected cells with neuraminidase to remove sialic acid prior to incubation with the p.i. cells. This (as shown previously for CV-1 cells (Moscona and Peluso, 1991)) prevented the fusion of the uninfected cells with the p.i. HeLa cells. Accordingly, βgal activity remained in the background range, whereas the control treatment, preincubation at pH 6.0 in the absence of neuraminidase, resulted in activities similar to those obtained without any pretreatment. For this experiment, prior to their addition to p.i. cells, uninfected cells were incubated at pH 6.0 without or with 0.5 units of neuraminidase (*Clostridium perfringens*) for 30 min at $37°C$ or received no pretreatment. The βgal activity values, averaged for replicate wells (10 for each experimental condition) in each experiment and given as means $\pm SD$ of results of three separate experiments, are as follows: preincubation without neuraminidase, 1.87 ± 0.15; preincubation with neuraminidase, 0.07 ± 0.03; no pretreatment, 1.70 ± 0.44.

An important confirmation of specificity was to add anti-HPF3 antiserum (which contains anti-HN antibody) or anti-HN monoclonal antibody to the assay system. These antibodies inhibited, in a dose−responsive fashion, the interaction between viral glycoprotein and cell that is required for fusion. This inhibition is demonstrated by decrease of fusion and of βgal activity (see Fig. 2). The highest levels of antibody, which completely blocked fusion of p.i. cells with uninfected cells, decreased βgal values to baseline levels. With a decreasing amount of antibody, fusion became more apparent and βgal trans-
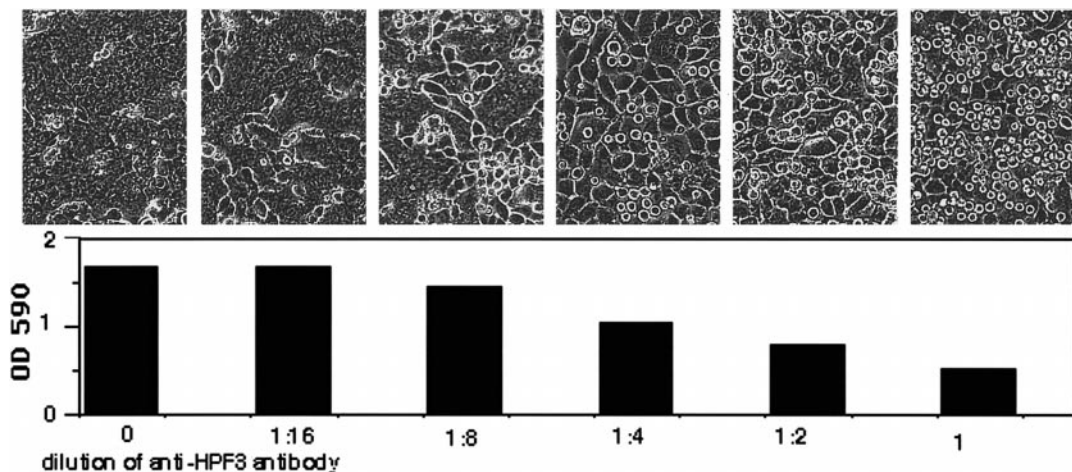

FIG. 2. Dose−responsive inhibition of HN−receptor interaction by antibody treatment. Standard amounts of uninfected cells plus the indicated dilutions of antibody were added to the adherent layer of p.i. cells and incubated under standard conditions for 6 h. βgal activity (see height of columns) was determined immediately after microscopic assessment of fusion (see photomicrographs).
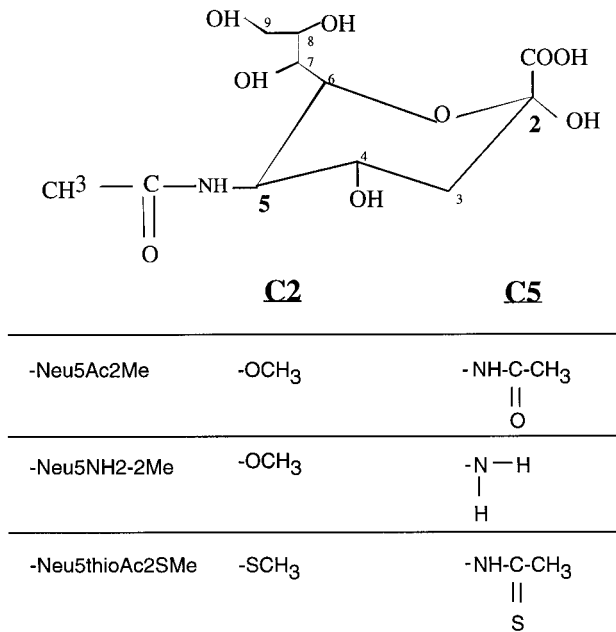
C2   **C5**

	C2	C5
-Neu5Ac2Me	-OCH$_3$	-NH-C-CH$_3$ (=O)
-Neu5NH2-2Me	-OCH$_3$	-N-H, H
-Neu5thioAc2SMe	-SCH$_3$	-NH-C-CH$_3$ (=S)

FIG. 3. Structures of the synthetic sialic acid analogs compared to the natural molecule.

Application of the quantitative fusion assay to the study of sialic acid receptor analogs

It was shown for influenza A virus that several analogs of N-acetylneuraminic acid can bind HA and inhibit virus binding to receptor at 4°C (Itoh et al., 1995; Machytka et al., 1993). If such analogs are to be useful for inhibiting infection, they must act at 37°C, and at this temperature they may be degraded by neuraminidase. In the case of influenza, replacing oxygen by sulfur at the C2 position (where natural sialic acid is linked to additional carbohydrates and cleavage by neuraminidase occurs) or removal of the acyl group on carbon 5 made the molecules resistant to neuraminidase (Itoh et al., 1995). These findings, and observations on the effect of substitutions at carbon 2 and 5 on the analogs' binding affinity to HA (Kelm et al., 1992), influenced the choice of analogs for our studies. The structures of the analogs that we describe here, compared to the natural sialic acid moiety of the receptor, are shown in Fig. 3.

Figure 4 shows the effect of the various sialic acid analogs on the level of fusion between persistently infected HeLa-CD4-LTR-βgal and uninfected HeLa-tat cells as measured by βgal activity. It may be seen that α-Neu5Ac2Me, which is identical to N-acetylneuraminic acid except for the glycosidic methyl group at C2 (Kuhn

et al., 1966), did not decrease fusion significantly. In contrast, the analog α-Neu5thioAc2SMe (α-2-S-methyl-5-N-thioacetylneuraminic acid), which differs from N-acetylneuraminic acid by containing an S-methyl group for the glycosidic group at the C2 position and N-thioacetyl at C5 (Isecke and Brossmer, 1994), decreased fusion between the two cell populations by 80% at 50 mM; the smaller, 24% inhibition at 25 mM indicates dose–responsive behavior. In this analog, the S substitution on C2 conferred neuraminidase resistance (Itoh et al., 1995). However, the S on C5 as well as on C2 may have also enhanced binding to HN; this same analog was found to be most effective in inhibiting influenza virus binding to fetuin at 4°C (Itoh et al., 1995).

The third analog tested, α-Neu5NH2-2Me, contains an O-glycoside at C2 and is lacking the acyl group at carbon 5. While this lack conferred resistance to influenza neuraminidase (Itoh et al., 1995), the C5-acyl group was found to be essential for binding to HA (Kelm et al., 1992). As seen in Fig. 4, this analog did not inhibit HN–receptor interaction in our fusion assay. It is possible that in the case of HPF3, too, removal of the C5 acyl group is abolishing not only neuraminidase susceptibility but also binding ability.

Use of the quantitative fusion assay to assess the effect of DANA

We next began to use the cell fusion assay to study the relationship between the receptor-binding and the receptor-cleaving functions of HN. In addition to the roles of the HPF3 HN in receptor binding and fusion promo-
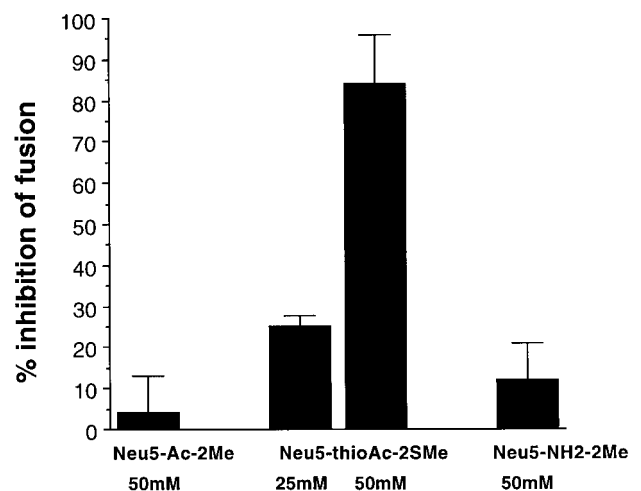
activation increased correspondingly. While this study cannot show definitively which components of the interaction are inhibited by the antibodies, the results support the notion that fusion and βgal activity in the assay relate quantitatively to viral protein interactions with the target cell.

FIG. 4. Effect of the sialic acid analogs on the degree of fusion between p.i. HeLa-CD4-LTR-βgal and uninfected HeLa-tat cells. The indicated analogs were added to p.i. cells immediately before the addition of the uninfected cells. βgal activity was determined after 6 h of incubation. The results, at 50 or 25 mM analog concentrations, are given as the percentage inhibition, i.e., relative to analog-free controls run simultaneously on the same plates. Each column is the mean (bar denotes 1 SD) of results of three or four experiments.
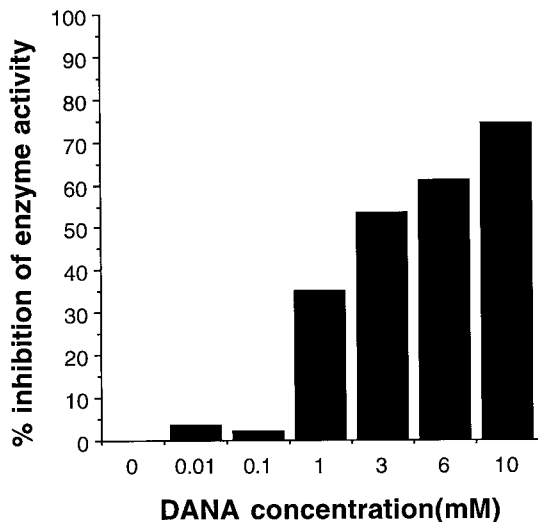
FIG. 5. Effect of DANA on activity of HPF3 neuraminidase. The results are given as percentage inhibition of enzyme activity at the indicated concentrations of DANA. Neuraminidase activities were determined using HPF3 virions at pH 4.7, using a mixture of 2,3- and 2,6-neuraminyllactose as substrate, for 30 min under conditions of substrate excess.

tion, the HN molecule has neuraminidase (or receptor-cleaving) activity. Our previous work has shown that this neuraminidase activity of HN is important for release of HPF3 virus particles from the surface of the infected cell. This neuraminidase function, release of virus particles from the surface of the infected cell, allows the virus to begin a new round of infection.

In contrast to the role of HN's neuraminidase in virus release, there is no evidence to date that neuraminidase plays a role in entry. While the binding function of HN and the neuraminidase function of HN are on the same molecule in this virus, the crystal structure has not been fully determined despite efforts to crystallize the molecule (Laver *et al.,* 1989; Murti *et al.,* 1993; Takimoto *et al.,* 1992) and it is not certain whether these two functions reside in adjacent or distant sites on the molecule (Gorman *et al.,* 1990, 1991; Iorio *et al.,* 1989; Lyn *et al.,* 1991; McGinnes *et al.,* 1993; Portner, 1981; Portner *et al.,* 1987; Sergel *et al.,* 1993; Sheehan and Iorio, 1992; Thompson and Portner, 1987).

To address the separate functions of binding and enzyme activity of the HN molecule, we studied the effects on binding and enzyme activity of an unsaturated sialic acid derivative of *N*-acetylneuraminic acid, DANA. DANA is known to inhibit influenza neuraminidase by virtue of being a transition-state analog and has been shown to inhibit the activity of the neuraminidases of HPF2 (Holzer *et al.,* 1993), Sendai virus (Portner, 1981), NDV (Portner, 1981), and mumps virus (Waxham and Wolinsky, 1986). In addition, Iorio *et al.* (1989) found that the action of monoclonal antibodies whose binding to NDV HN inhibits neuraminidase activity was blocked by DANA. For HPF2

and mumps, DANA was found to have a K_i of 10^{-5} (Holzer *et al.,* 1993) and of 4×10^{-4} (Waxham and Wolinsky, 1986), respectively. For Sendai virus and NDV, 0.1 mM was the lowest DANA concentration at which complete inhibition of enzyme activity was attained (Portner, 1981). Figure 5 shows that DANA inhibits the activity of HPF3 neuraminidase; at a 10 mM concentration, the inhibition is about 75% and at a concentration of 1 mM, the activity is reduced by only about 35%. Thus, HPF3 neuraminidase is less effectively inhibited by DANA than the paramyxovirus neuraminidases studied previously.

Figure 6 shows the effect of DANA on the extent of fusion between persistently infected HeLa LTR-βgal cells and uninfected HeLa-tat cells. Surprisingly, we found that DANA works extremely well, and in fact more effectively than the sialic acid analog compounds we tested, at interfering with the HN–receptor interaction required for fusion. DANA decreased fusion between the two cell populations by 98% at 25 mM, 80% at 5 mM, and 38% at 1.5 mM.

Inhibition of receptor binding by DANA in a hemadsorption assay

The effect of DANA on receptor binding was assessed by a hemadsorption assay to test the ability of DANA to interfere with the adherence of erythrocytes to persistently infected cells. This experiment was designed to determine whether DANA's ability to interfere with cell fusion could be due to its effect on neuraminidase enzymatic function or due to interference with receptor interaction. To test this, we assessed binding to erythrocytes at 4°C, under conditions in which the neuraminidase enzyme is inactive, but the binding function is intact. This
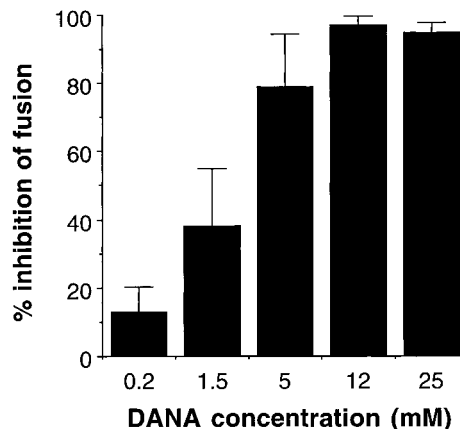

FIG. 6. Effect of DANA on the degree of fusion between p.i. HeLa-CD4-LTR-βgal and uninfected HeLa-tat cells. DANA, at concentrations ranging from 0.2 to 25 mM, was added to p.i. cells immediately before the addition of the uninfected cells. βgal activity was determined after 6 h of incubation. The results are given as percentage inhibition, i.e., relative to DANA-free controls run simultaneously on the same plates. Each column is the mean (bar denotes 1 SD) of results of four experiments.

TABLE 1

Effect of DANA on Erythrocyte Binding (HAD)
to Persistently Infected Cells

| DANA concentration (mM)	HAD (erythrocyte binding)
25	0
12.5	0
6.2	0
3.1	0
1.6	0
0.8	+
0.4	++
0.2	+++
0.1	+++
0	+++

Note. DANA, in concentrations ranging from 0.2 to 25 mM, was added to a confluent monolayer of p.i. cells immediately before the addition of a 1% solution of human erythrocytes. Hemadsorption activity was determined after 2 h of incubation at 4°C. The results are given as a score of 0 to +++ erythrocyte binding, with +++ being complete coverage of the p.i. monolayer by erythrocytes as seen in the absence of DANA. Each data point derives from the identical results of four experiments.

assay also effectively separates the step of attachment from the subsequent steps in the fusion process. Monolayers of p.i. cells were incubated with human erythrocytes at 4°C for 120 min in the presence of various concentrations of DANA. Table 1 shows that DANA effectively blocks attachment at 4°C as assessed by the adherence of erythrocytes to the p.i. monolayer. The DANA effect on blocking hemadsorption is concentration dependent, with complete inhibition of erythrocyte binding at a concentration of 1.6 mM. The range of DANA concentrations that inhibit hemadsorption is similar to the concentrations that inhibit fusion in the quantitative fusion assay. Erythrocytes may not be representative of host cells and may not contain the same complement of receptors or membrane composition as authentic target cells. Nevertheless, these results suggest that DANA's ability to interfere with cell fusion was due to its interference with receptor interaction rather than to its neuraminidase inhibition and support the notion that DANA blocks attachment.

Inhibition of viral entry and replication by sialic acid receptor analogs and by DANA

The effects of the sialic acid analogs on viral entry and replication were assessed by a plaque reduction test. α-Neu-5thio-Ac2SMe inhibited plaque formation by 60 and 10% at concentrations of 50 and 25 mM, respectively. The analogs that were ineffective in the fusion assay (Neu5Ac2Me and Neu5NH2Me) did not inhibit plaque formation.

We tested whether the ability of DANA to interfere with
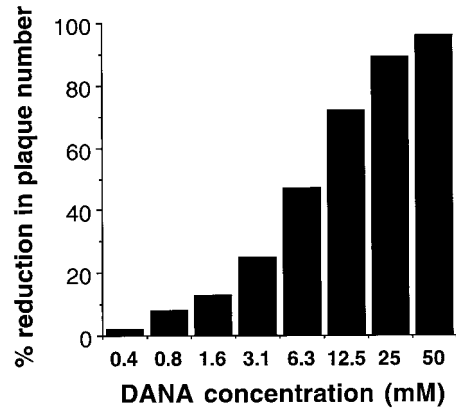
FIG. 7. Effect of DANA on viral entry and replication. Cells were infected with HPF3, with the simultaneous addition of DANA in concentrations ranging from 0.4 to 50 mM. Plaque formation proceeded under an agarose overlay for 48 h, and plaques were visualized and counted. The results are given as percentage reduction in plaque number, i.e., relative to DANA-free controls run simultaneously. Each column is the mean of results of four experiments.

HN–receptor interaction in the fusion assay would be confirmed by an ability to block viral entry as measured by the plaque reduction test. Figure 7 shows that DANA reduced the number of plaques formed in a dose-dependent manner. This inhibition, 90% at a concentration of 25 mM, was more effective than that of the sialic acid analogs.

We determined whether DANA exerts any effect after the adsorption period or whether it blocks binding only during the adsorption period. Either the addition of DANA to the plaque reduction test was delayed until 90 min, just prior to the addition of the agarose overlay, or DANA was added at the time of infection but removed by washing at 90 min. Table 2 shows that the reduction of plaque number by DANA addition at the time of infection was not altered significantly by its removal at 90 min and that addition of DANA at 90 min caused no significant reduc-

TABLE 2

Effect of Timing of DANA Addition on Inhibition
of Viral Entry and Replication

Time of DANA addition	% reduction of plaque number
0 min	76.0 ± 1.3
0 min, removed at 90 min	67.3 ± 5.8
90 min	1.7 ± 5.7

Note. Cells were infected with HPF3. DANA (25 mM) was added immediately and remained present (line 1) or was removed at the end of the 90-min adsorption period (line 2) or was added at 90 min only (line 3), just before application of the agarose overlay. Plaque formation proceeded for 48 h, and plaques were visualized and counted. Results are presented as percentage reduction in number of plaques, i.e., relative to DANA-free controls run simultaneously. The values are means (±SD) of results of four to seven experiments.

tion in plaque number. This experiment, showing that incubation with DANA during the adsorption period alone suffices for the inhibitory effect, provides support for the notion that DANA acts at the stages of viral binding and/or entry.

Future implications

The fusion assay described here for the purpose of evaluating interaction of viral glycoproteins with the cell is carried out with a stable, standard source of material; p.i. cell lines provide the HN and F proteins, and uninfected cell lines are the receptor donors. These materials are completely reproducible from experiment to experiment and in different laboratories. Overlay cells as a source of receptor, and inhibitory compounds to be tested, can be added using any concentration or timing desired. This assay, rendered quantitative by use of a published β-galactosidase reporter system, is thus uniquely suited to large-scale screening for synthetic compounds that may interfere with viral entry. While previous routine methods of screening for antiviral agents (for example, plaque-reduction assays) reflect the end result of several steps that fall between entry and release, the present assay permits quantitative study of the early, crucial steps of functional viral glycoprotein−cell surface interaction. The hemadsorption assay can test for interference with receptor binding, but lacks quantitative resolving power and, furthermore, erythrocytes differ from authentic target cells in terms of the complement of receptors and membrane composition. The present fusion assay, which is applicable to any cell line of choice and permits sensitive quantitation, thus has greater utility as a method of screening for antiviral agents.

Our results in this first study of the effect of synthetic sialic acid analogs on HPF3 indicate that compounds that mimic the receptor determinant for HPF3 HN can block virus−cell interaction and may have the potential to counteract infection. For the influenza virus, X-ray crystallography studies yielded an atomic model for HA−sialic acid interaction, which provides a rational basis for designing receptor analogs. No such structural model is available for HPF3. However, the quantitative fusion assay we describe in this paper facilitates screening a wide range of compounds for their ability to block HN−receptor interaction. Thus, one should be able to obtain information about the binding requirements (receptor determinants) of the viral HN and, at the same time, identify sialic acid analogs with the potential to inhibit HPF3 infection.

It should be noted that, for influenza virus, monomeric sialic acid compounds, even those that blocked virus binding to fetuin and were neuraminidase resistant, failed to inhibit plaque formation. Only the polymeric forms of some of those analogs were effective in reduc-

ing plaque formation (Itoh *et al.,* 1995). Among these polyacrylamide-based polymers, that of α-Neu5thio-Ac2SMe (the active monomer in the present studies) showed the most striking interference with the replication of several different strains of influenza virus; the concentration at which this polymer completely inhibited plaque formation was 1/100 of that required to inhibit HA binding to fetuin (Itoh *et al.,* 1995). We are thus planning to extend these studies on HPF3 to test polymerized forms of analogs for effectiveness in our fusion assay and examine their ability to block viral entry in the plaque reduction test.

In our assay, the fusion between p.i. and uninfected cells was also blocked by DANA, which is known to be a transition-state inhibitor of influenza virus neuraminidase. Although, as we now show, DANA can also inhibit HPF3 neuraminidase, this inhibition is unlikely to underlie the ability of this compound to block HN−receptor interaction in the fusion assay. Since the pH optimum of HPF3 neuraminidase is 4.7 (Moscona and Peluso, 1993), it would exert only 1 to 9% of its maximal activity at the physiological pH of the fusion assay. In addition, 25% of viral neuraminidase enzyme activity is still present at a 10 mM DANA concentration that completely blocks fusion (compare Figs. 5 and 6). Most importantly, DANA blocks receptor binding in a hemadsorption assay at 4°C, further indicating that DANA's ability to block fusion is due to interfering with HN−receptor interaction rather than to inhibition of neuraminidase. While erythrocytes are not entirely representative of host cells and may not contain all the same receptors or membrane components, these results provide support for the notion that DANA blocks attachment. The possibility that DANA may also exert an effect on steps in the fusion process subsequent to HN−receptor contact, such as HN−F interaction, conformational changes in the viral glycoproteins, and F protein-mediated fusion of the lipid bilayers, remains to be explored.

Since there is no crystallographic information on the structure of paramyxovirus HN, there is debate in the literature as to whether the receptor-binding and the neuraminidase activity sites of paramyxovirus HN molecules are physically adjacent, remote, or in some way associated with one another (Gorman *et al.,* 1990, 1991; Iorio *et al.,* 1989; Portner, 1981; Portner *et al.,* 1987; Sheehan and Iorio, 1992). Resolution of this question awaits determination of the crystal structure of a paramyxovirus HN. Our results on DANA are pertinent here, because they indicate that the same small molecule can inhibit HPF3 neuraminidase and specifically block HN−receptor interaction during attachment and fusion.

We propose three possible interpretations of these results with DANA. First, the receptor binding and neuraminidase enzyme sites on HN may be physically adjacent to one another, allowing one DANA molecule to interact with both sites; second, binding of DANA to the

neuraminidase active site may induce an inactivating change in a distant binding site; and third, DANA molecules may bind independently to two distinct functional sites. These three interpretations yield hypotheses testable in future experiments. Structurally distinct small molecules will be used to derive further data about the relative locations of the active sites on the HN molecule and about the relationship between receptor-binding and receptor-destroying activities of HPF3.

MATERIALS AND METHODS

Virus. Stocks of wildtype HPF3 were made in CV-1 cells from virus that was plaque-purified four times. Virus was released after freeze–thawing 36 to 48 h postinfection and stored at −80°C. Virus titer was determined by a plaque assay (see below) with CV-1 cells.

Cells. HeLa-CD4-LTR-βgal cells and HeLa-tat cells were obtained through the AIDS Research and Reference Program, Division of AIDS, NIAID, NIH. HeLa cell lines and CV-1 (African green monkey kidney) cells were maintained with Eagle's minimal essential medium supplemented with 10% fetal bovine serum and antibiotics.

Sialic acid analogs and transition-state analog. The synthesis of analogs used in this study (for structures see Fig. 3) was performed as previously described (Isecke and Brossmer, 1994). The resistance of the analogs to influenza X-31 neuraminidase was tested according to Itoh *et al.* (1995). DANA was purchased from Sigma Chemical Corp.

Cell fusion assay. HeLa-LTR-βgal cells were persistently infected with HPF3, using our methods developed for CV-1 cells (Moscona and Peluso, 1991, 1992), at a multiplicity of infection sufficient to infect all of the cells in the culture (>5 PFU per cell). Under these conditions no cell–cell fusion occurs. The presence of persistent viral infection in the cells was documented by hemadsorption positivity of the entire cell monolayer (Moscona and Peluso, 1992), release of infectious particles into the supernatant fluid (Moscona and Galinski, 1990), and viral protein synthesis in metabolically labeled cells (Moscona and Galinski, 1990). To set up the fusion assay, p.i. HeLa-LTR-βgal cells were plated in 96 well plates. After 24 h, uninfected HeLa-tat cells (3 × 10^4 per well) were added to the adherent cells, and fusion was allowed to proceed for 6 h at 37°C. β-Galactosidase activity was then measured as described (Nussbaum *et al.,* 1994). The cells were lysed with NP-40 and mixed with chlorophenol red-galactopyranoside. Absorbance was measured at an OD of 590.

Assay of viral neuraminidase. Neuraminidase activities were determined by the thiobarbituric acid assay as previously described (Merz *et al.,* 1981; Moscona and Peluso, 1993) using sucrose-gradient purified HPF3 virions. 2,3-Neuraminyllactose and 2,6-neuraminyllactose substrates were purchased from Sigma Chemical Corp.

Hemadsorption assay. Monolayers of p.i. cells were washed with cold medium lacking serum and then incubated with human erythrocytes at 4°C for 120 min in the presence of various concentrations of DANA. Nonadherent cells were removed by washing with cold medium; the extent of erythrocyte adsorption was estimated and the plates were photographed through a phase-contrast microscope.

Plaque reduction assay. CV-1 cell monolayers grown in plastic dishes of 3.5 cm in diameter were inoculated with 100 PFU of HPF3 and were incubated at 37°C in Eagle's minimum essential medium with intermittent rocking in the presence of various concentrations of sialic acid analogs or DANA. After 90 min, minimum essential medium containing 0.5% agarose was added to the dishes and incubated for 48 h. In some experiments, DANA present during the adsorption period was removed by washing three times in Eagle's minimum essential medium, and in parallel dishes DANA was absent during the adsorption period and added to the cell monolayers just prior to the addition of the agarose overlay. After the agarose overlay was removed, the cells were fixed with 10% formaldehyde in PBS and stained with a 1% crystal violet solution in methanol. Alternatively, we used an immunostaining method for plaque detection (Herold *et al.,* 1991). Briefly, after the agarose overlay was removed, the cells were fixed with methanol, washed, and blocked with PBS/3% BSA. Cells were incubated sequentially with polyclonal guinea pig anti-HPF3 antibody (BioWhittaker), biotinylated goat anti-guinea pig IgG, and streptavidin–β-galactosidase 1:150 in PBS–BSA. Each incubation was for 1–2 h at room temperature. After being washed, the cells were incubated with substrate consisting of Bluogal (BRL) in KFeCn buffer until blue plaques were visualized. The number of plaques in the control and experimental wells was counted.

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