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Fusion Properties of Cells Persistently Infected with Human Parainfluenza Virus Type 3: Participation of Hemagglutinin-Neuraminidase in Membrane Fusion

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Cells persistently infected with human parainfluenza virus type 3 (HPF3) exhibit a novel phenotype. They are completely resistant to fusion with each other but readily fuse with uninfected cells. We demonstrate that the inability of these cells to fuse with each other is due to a lack of cell surface neuraminic acid. Neuraminic acid is the receptor for the HPF3 hemagglutinin-neuraminidase (HN) glycoprotein, the molecule responsible for binding of the virus to cell surfaces. Uninfected CV-1 cells were treated with neuraminidase and then tested for their ability to fuse with the persistently infected (pi) cells. Neuraminidase treatment totally abolished cell fusion. To extend this result, we used a cell line deficient in sialic acid and demonstrated that these cells, like the neuraminidase-treated CV-1 cells, were unable to fuse with pi cells. We then tested whether mimicking the agglutinating function of the HN molecule with lectins would result in cell fusion. We added a panel of five lectins to the neuraminic acid-deficient cells and showed that binding of these cells to the pi cells did not result in fusion; the lectins could not substitute for interaction of neuraminic acid with the HN molecule in promoting membrane fusion. These results provide compelling evidence that the HN molecule of HPF3 and its interaction with neuraminic acid participate in membrane fusion and that cell fusion is mediated by an interaction more complex than mere juxtaposition of the cell membranes.

Human parainfluenza virus type 3 (HPF3), an important agent of lower respiratory tract disease in children, is a member of the paramyxovirus family of negative-strand RNA viruses. The HPF3 proteins and molecular organization of the genome follow the general pattern observed for all paramyxoviruses (20). Infected cells contain two virus-encoded glycoproteins on the cell surface: a receptor-binding protein (HN) and a fusion protein (F). The receptor-binding protein of parainfluenza virus contains both hemagglutinating and neuraminidase activities (12). A characteristic feature of paramyxovirus infection in cell culture is the induction of cell fusion, resulting in the formation of syncytia. While several elements of the fusion process have been extensively studied, the precise role of each of the glycoproteins and the critical determinants of fusion remain unclear.

We previously reported the molecular characterization of three cell lines persistently infected (pi) with HPF3 (13). Immediate persistent infection with HPF3 was readily established in CV-1 cells by infecting at high multiplicities of infection (MOIs). In the pi cells, all six structural protein genes were transcribed into monocistronic and polycistronic RNAs. All of the viral proteins were present in the pi cells, including both the uncleaved precursor fusion protein (F_o) and the cleaved active fusion protein (F). Each of the pi cell lines contained standard HPF3 genomic RNA and a distinct subgenomic RNA species. These subgenomic RNAs were present in purified nucleocapsid cores, were stably maintained, and contained only polymerase gene sequences and the authentic 5′ ends of the genomic RNA.

The pi cells remained free of cytopathic effects while shedding infectious virus into the medium. The pi cells demonstrated a novel fusion phenomenon: they were entirely refractory to self-fusion. Although the pi cells did not exhibit fusion or syncytium formation with each other, they were able to undergo rapid and complete fusion when seeded with uninfected cells.

In this report, we identify the molecular basis for the ability of cells persistently infected with HPF3 to fuse with uninfected cells while remaining refractory to self-fusion. We use this model to derive information about the requirements for virus-mediated cell fusion. We demonstrate that HN protein function, as well as F protein activity, is necessary for fusion of cells persistently infected with HPF3.

MATERIALS AND METHODS

Virus and cell lines. CV-1 (African green monkey kidney) cells were grown in monolayers in Eagle's minimal essential medium (MEM) supplemented with 10% fetal bovine serum. The pi cell lines were cultured as previously described (13). The neuraminic acid-deficient cell line Lec 3281 (kindly provided by Pamela Stanley, Albert Einstein College of Medicine) was cultured in MEM-alpha medium with 10% fetal calf serum (21).

Neuraminidase treatments. Cells were prepared for neuraminidase treatment by trypsinization and resuspension in 0.14 M NaCl–0.7 mM Na_2HPO_4–25 mM piperazine-*N*,*N*′-bis(2-ethane sulfonic acid) (PIPES), pH 6.0. Neuraminidase (0.5 U; from *Clostridium perfringens*; Sigma Chemical Co., St. Louis, Mo.) was added, and the cells were incubated at 37°C for 30 min. The cells were then pelleted, washed with MEM, and added to confluent monolayers of pi cells. Control cells were incubated identically except that neuraminidase was not added.

Lectins. The following lectins were purchased from Sigma: concanavalin A, lentil lectin, peanut lectin, wheat germ

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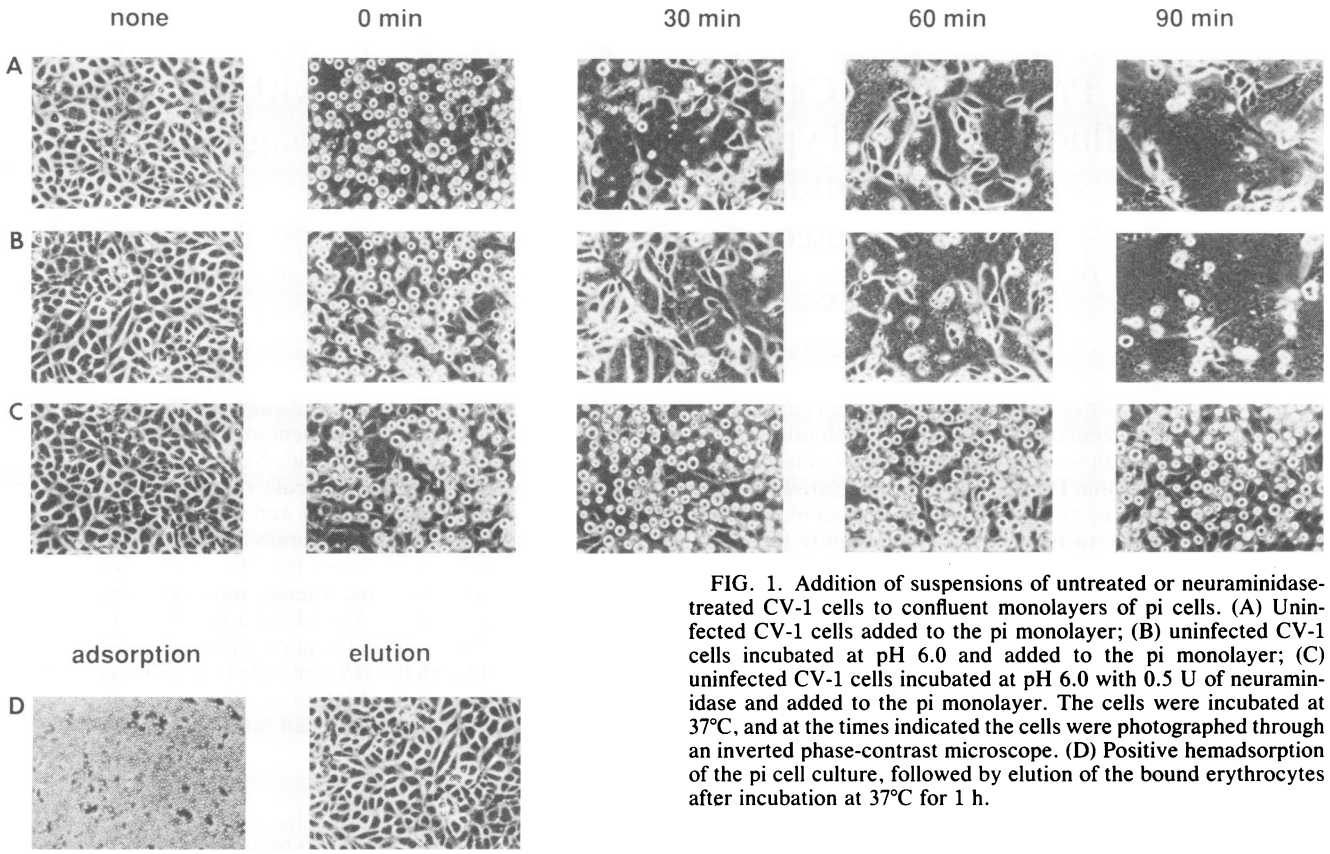

FIG. 1. Addition of suspensions of untreated or neuraminidase-treated CV-1 cells to confluent monolayers of pi cells. (A) Uninfected CV-1 cells added to the pi monolayer; (B) uninfected CV-1 cells incubated at pH 6.0 and added to the pi monolayer; (C) uninfected CV-1 cells incubated at pH 6.0 with 0.5 U of neuraminidase and added to the pi monolayer. The cells were incubated at 37°C, and at the times indicated the cells were photographed through an inverted phase-contrast microscope. (D) Positive hemadsorption of the pi cell culture, followed by elution of the bound erythrocytes after incubation at 37°C for 1 h.

agglutinin, and asparagus pea lectin. All lectins were used at a concentration of 20 μg/ml except wheat germ agglutinin, which was used at 5 μg/ml.

Hemadsorption assays. Monolayer cultures of pi cells were washed twice with cold medium lacking serum and then incubated with human erythrocytes at 4°C for 75 min. Nonadherent erythrocytes were removed by repeated washing with cold medium lacking serum.

Cell fusion assays. CV-1 or Lec 3281 cells (2×10^6 cells) were added to confluent monolayer cultures of pi cells in 60-mm dishes. Fusion of the cells with the pi cells was assessed at various times after incubation at 37°C as indicated in the text. Photographs of the cells were taken through a phase-contrast microscope.

RESULTS

Fusion characteristics of the pi cells. Infection of CV-1 cell monolayers with plaque-purified HPF3 at an MOI of 10 reliably produced persistent infection (13, 25). While the pi cells did not exhibit syncytium formation with each other, they underwent rapid and complete fusion when seeded with uninfected cells. Figure 1A shows the extent of cell fusion at 0, 30, 60, and 90 min after the addition of uninfected CV-1 cells to one of the pi cell lines; Fig. 1B shows the same experiment performed after incubation of the uninfected cells at pH 6 and demonstrates that the ability of the uninfected cells to fuse with the pi cells was not affected by this treatment. The viral fusion protein in the plasma membrane of the pi cells clearly is functional and capable of mediating fusion once a critical component, missing from the pi cells, is provided by uninfected cells.

Effects on the fusion process of neuraminidase treatment of uninfected cells. Experiments were performed to determine whether the presence of neuraminic acid residues on the surface of the uninfected cells was necessary for fusion to occur when these cells were added to the pi cells. Uninfected CV-1 cells were treated with neuraminidase at pH 6 to remove neuraminic acid residues from the cell surface. Figure 1C shows the extent of cell fusion at 0, 30, 60, and 90 min after the addition of neuraminidase-treated uninfected CV-1 cells to the pi cell monolayer. The ability of these cells to fuse with the pi cells was totally abolished. Even after several hours, there was no fusion. This result demonstrates that neuraminic acid residues provided by the uninfected cells are an essential factor in promoting fusion. The presence of functional HN on the surface of pi cells was demonstrated by the binding of erythrocytes to all the cells in the pi monolayer (Fig. 1D) followed by elution of the erythrocytes at 37°C as a result of the activity of the viral neuraminidase. Presumably, the presence of HN on the surface of the pi cells results in the pi cell surfaces becoming denuded of neuraminic acid residues. These cells then cannot fuse until provided with neuraminic acid-containing macromolecules on adjacent cell membranes.

Fusion experiments with Lec 3281 cells. To confirm the need for sialic acid residues on the surface of the uninfected cells, experiments were performed using cells that lack cell surface sialic acid. These cells were derived by selection for resistance to limulin, a lectin with binding specificity for neuraminic acid (21). Figure 2 shows the extent of cell fusion at 0, 30, 60, and 90 min after the addition of Lec 3281 cells to the pi cell monolayer. These cells did not fuse with the pi cells. CHO cells (from which the neuraminic acid-deficient cells were derived) were able to fuse with the pi cells

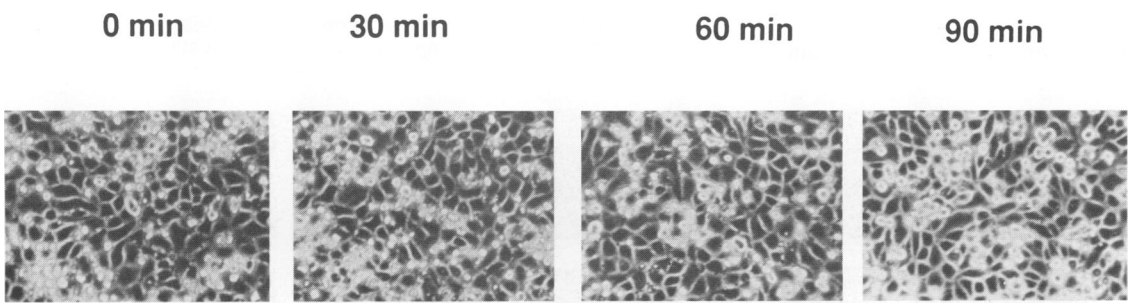

FIG. 2. Addition of suspensions of cells lacking surface neuraminic acid to confluent monolayers of pi cells. Lec 3281 cells lacking neuraminic acid were added to cultures of pi cells and incubated at 37°C for the times indicated. Cells were photographed as described in the legend to Fig. 1.

similarly to the CV-1 cells. These findings provide further evidence that the critical molecule supplied by the normal uninfected cell membrane is indeed sialic acid, the receptor for HN.

Use of lectins to agglutinate the cells. We considered the possibility that the requirement for interaction between viral HN and the sialic acid on uninfected cell membranes might be overcome by the use of lectins to agglutinate the cells. If the interaction of the HN protein with sialic acid functions simply to bring the cells into close proximity so that fusion can occur, then the same effect might be accomplished by lectins. We therefore performed experiments to determine whether neuraminidase-treated CV-1 cells or Lec 3281 cells would fuse with pi cells if agglutinated by lectins. We also tested whether pi cells could be induced to fuse with each other in the presence of lectins.

Figure 3A shows the extent of fusion at 90 min after the addition of uninfected CV-1 cells to pi cell monolayers in the presence of each of five different lectins. In each case, fusion is complete, demonstrating that the lectins did not affect this process. Higher concentrations of lectins blocked the fusion of CV-1 cells with the pi cells, and lower concentrations had no effect on fusion in any of the experiments reported here. Figure 3B shows the same experiment performed after neuraminidase treatment of the uninfected cells. Although the presence of the lectins resulted in adherence of the uninfected cells to the pi monolayer, fusion did not occur. Figure 3C shows the extent of fusion at 90 min after addition of Lec 3281 cells to pi cell monolayers in the presence of the five lectins. Fusion did not occur despite adherence of the sialic acid-deficient cells to the pi monolayer. In neither experiment did agglutination of the cells by the lectins substitute for the interaction of viral HN with neuraminic acid.

Figure 3D shows the extent of fusion at 90 min after the addition of pi cells to a monolayer of pi cells in the presence of the five lectins and demonstrates that the pi cells remain refractory to self-fusion despite agglutination by the lectins. In additional experiments (data not shown), we noted that neither incubating confluent monolayers of pi cells in the presence of lectins nor adding lectins to dispersed pi cells followed by incubation at 37°C resulted in fusion between these cells. These results are consistent with the interpretation that it is the absence of sialic acid available to interact with HN on these cells' surfaces that accounts for their failure to fuse and that simple agglutination does not obviate this requirement.

DISCUSSION

Fusion of membranes at neutral pH is a hallmark of infection by members of the paramyxovirus family and may be important in the pathogenesis of diseases caused by these viruses (1, 2). The F glycoprotein, present on the surface of both viral particles and infected cells, is active in this process. The role of the second surface glycoprotein, HN, in this process has been less clear. The data reported here demonstrate that HN function is important during the process of membrane fusion. Paramyxovirus-infected cells have been found to have a decreased level of neuraminic acid residues on their surfaces (1). In the system described here, pi cells fail to fuse with each other because of their decreased level of cell surface neuraminic acid; this is demonstrated by the ability of these cells to fuse with uninfected cells which contain neuraminic acid but not with those lacking neuraminic acid. These experiments were performed with CV-1 cells; however, it is worth noting that fusion with pi cells is a general phenomenon not limited to this cell type; BHK cells (our unpublished data) as well as HEp-2, Vero, LLC-MK2, HeLa cells, and cells persistently infected with measles or respiratory syncytial virus have been shown to fuse with pi cells (25). Of note, neither of these latter viruses possesses neuraminidase activity in the attachment glycoprotein.

Erythrocytes, which contain sialic acid on their surfaces and were able to bind to the pi cells, did not appear to fuse with these cells. Had fusion of the erythrocytes with the pi cells occurred at very low efficiency, it would have escaped our detection. Perhaps either the differences in membrane composition between erythrocytes and other cells or the rigid cytoskeleton of the erythrocytes accounts for their failure to undergo fusion in this system.

The lack of neuraminic acid on uninfected cells, and hence the lack of a receptor for the HN molecule, could not be compensated for by the addition of various lectins. Although these lectins agglutinated the cells, mimicking one function of HN, fusion did not occur. This implies that the mechanism of HN participation in membrane fusion is more complex than mere juxtaposition of the two membranes.

Several lines of evidence from other paramyxoviruses have suggested that both the HN and F proteins participate in the fusion process. Plaque variants of bovine parainfluenza virus type 3 which exhibit altered fusion properties have been isolated (18, 19). Their alterations in fusion activity were correlated with an alteration in neuraminidase activity. Those mutants with the least active neuraminidase

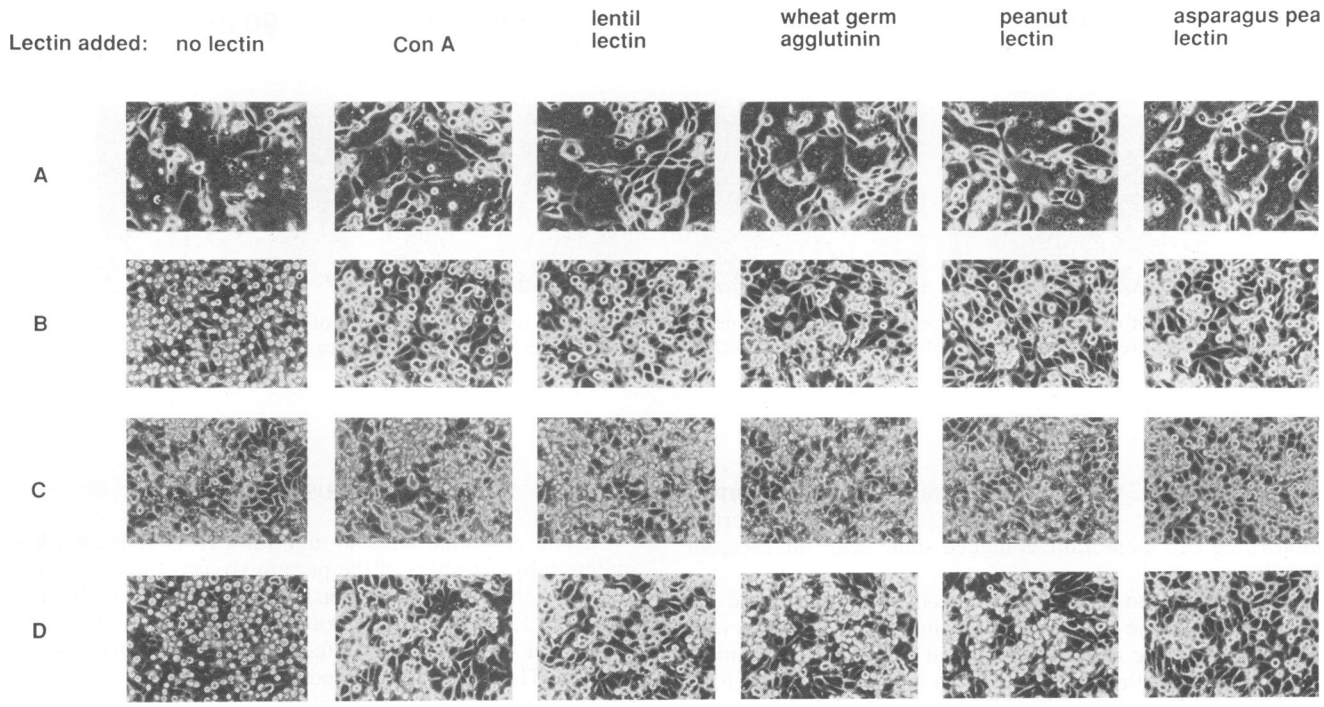

FIG. 3. Evidence that agglutination by lectins does not alter the inability of cells lacking neuraminic acid to fuse with confluent monolayers of pi cells. (A) Uninfected CV-1 cells added to the pi monolayer directly or in the presence of the five lectins listed. The lectins did not alter the normal fusion process. (B) Uninfected CV-1 cells incubated at pH 6.0 with 0.5 U of neuraminidase and added to the pi monolayer in the presence of the five lectins. (C) Lec 3281 cells lacking neuraminic acid, added to cultures of pi cells in the presence of the five lectins. (D) Pi cells added to the pi monolayer in the presence of the five lectins. The cells were incubated at 37°C and after 90 min were photographed through an inverted phase-contrast microscope.

were the most active in promoting fusion, and they could be converted to the nonfusogenic phenotype by exogenously added neuraminidase. More recently, the HN and F proteins of these viruses have been expressed from cloned copies of the genes, and the results confirmed that F alone was not sufficient for membrane fusion (17). Combinations of the F and HN proteins coexpressed in cells demonstrated not only that both F and HN were required, but that the degree of cell fusion was dependent on the type of HN protein coexpressed with F protein; those with high neuraminidase activity exhibited less syncytium formation. Syncytium formation induced by the recombinant viruses was completely blocked by an anti-HN monoclonal antibody (17).

Additional evidence for the involvement of neuraminidase in F protein-mediated membrane fusion comes from the study of mumps virus. The neuraminidase activities of several naturally occurring strains of mumps virus were compared, and it was found that the strains that exhibited high fusion activity possessed low levels of neuraminidase, while those with high neuraminidase activity were poorly fusogenic (9). Neuraminidase inhibitors were used to select sialidase-deficient variants from two of the strains that had high neuraminidase activity. These variant viruses, in contrast to their parents, produced widespread fusion in cells (23, 24). Further, enzymatic removal of the HN glycoprotein from infected cell surfaces was shown to result in an increase in syncytium formation (10). All mumps virus strains tested exhibited increased fusion after proteolytic removal of the HN glycoprotein (10). In addition, mumps-induced cell fusion was blocked by anti-HN monoclonal antibodies (22). Together, these findings support a role for HN in the fusion process mediated by mumps virus.

The evidence for the involvement of HN in cell fusion mediated by Sendai virus is conflicting. Evidence for the Sendai virus F glycoprotein being sufficient to mediate membrane fusion was provided by experiments which showed that this glycoprotein, when reconstituted into lipid vesicles, caused erythrocyte hemolysis in the presence of wheat germ agglutinin. In this experiment, HN function was replaced by the lectin (6). Perhaps in certain settings mechanisms of membrane attachment other than HN can result in membrane fusion by the F protein. In a different report, a mutant virus deficient in HN was able to infect HepG2 cells via the asialoglycoprotein receptor (7). However, this mutant was unable to infect any of the other cells types permissive for Sendai virus infection. In a separate study, this same mutant virus was able to fuse with liposomes as efficiently as wild-type virus, and radiation inactivation target size determinations suggested that F protein alone mediated the fusion reaction (4).

On the other hand, support for an essential role for HN in the Sendai virus fusion reaction comes from several lines of experimentation. A number of studies have shown that anti-HN monoclonal antibodies, while allowing agglutination to occur, interfered with F protein-mediated fusion (11, 16). Ozawa et al. (14) reported that reconstituted vesicles containing Sendai virus F glycoprotein and dithiothreitol-inactivated HN protein were not active in membrane fusion, and further that addition of concanavalin A was unable to substitute for HN function. Other data from lipid vesicle reconstitution of Sendai virus membranes are consistent with the interpretation that HN participates in the fusion process (5) and that HN and F physically interact with each

other (3). The role of the glycoproteins of Sendai virus in mediating membrane fusion warrants further investigation.

In contrast to the conflicting reports about Sendai virus, it has been demonstrated clearly that the simian virus 5 (SV5) F protein can mediate membrane fusion in the absence of HN (15). Expression of the SV5 F protein gene in CV-1 cells by using a simian virus 40 vector resulted in syncytium formation between adjacent cells. Clearly in the SV5 system, F protein activity alone was sufficient for cell fusion to occur. Additional evidence for the sufficiency of the SV5 F protein in membrane fusion has been provided by the demonstration that an anti-HN antiserum was not able to prevent cell-to-cell spread of SV5 in cell culture (8).

Our studies of cells persistently infected with HPF3 lend support to the hypothesis that the HN protein is essential for the fusion process induced by this virus, at least in cells persistently infected with HPF3, and that its role is more than simple agglutination of the two membranes. The fusion characteristics of the cells persistently infected with HPF3 that we report here have relevance to the in vivo interaction of HPF3 with its natural host, since these cells were naturally infected with a plaque-purified wild-type virus inoculum, and the results are therefore little obscured by experimental manipulations.

The findings reported here may also shed some light on the mechanism whereby persistence is easily and rapidly established by infecting at high MOI. The high levels of neuraminidase present in the high-MOI inoculum may result in rapid destruction of the cell surface sialic acid, preventing viral spread by cell-to-cell fusion and thus preventing the development of cytopathic effects. This may afford the opportunity for the development of persistent infection, accompanied by the generation and amplification of defective interfering particles. Experiments to explore both the nature of the interaction between F and HN in membrane fusion in this system and the role of the neuraminidase in establishing persistent infection are currently under way.

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