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An alternative route of infection for viruses: Entry by means of the asialoglycoprotein receptor of a Sendai virus mutant lacking its attachment protein

(galactose-terminated protein/lectin/hepatoma/cell fusion/paramyxovirus)

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ABSTRACT During the first stage of infection, the paramyxovirus Sendai virus attaches to host cells by recognizing specific receptors on the cell surface. Productive virus-cell interactions result in membrane fusion between the viral envelope and the cell surface membrane. It has recently been shown that the ganglioside GD1a and its more complex homologs GT1b and GQ1b are cell surface receptors for Sendai virus. We report in this paper that the temperature-sensitive mutant *ts*271 of the Enders strain of Sendai virus lacks the viral attachment protein HN and the biological activities of hemagglutination and sialidase activity associated with it when the virus is grown at 38°C. This HN^- virus was unable to infect or agglutinate conventional host cells that contained receptor gangliosides and were readily infected by the parental wild-type virus. The HN^- virus did, however, attach to and infect Hep G2 cells, a line of hepatoma cells that retains the asialoglycoprotein receptor (ASGP-R) upon continuous culture. This receptor is a mammalian lectin that recognizes galactose- or *N*-acetylgalactosamine-terminated proteins. In accordance with the known properties of this receptor, infection by the HN^- virus was abolished by treatment of Hep G2 cells with sialidase, by the presence of Ca^{2+} chelators, and by competition with *N*-acetylgalactosamine, asialoorosomucoid, and antibody to the receptor. F, the only glycoprotein on the HN^- virus, was shown to compete with the galactose-terminated protein asialoorosomucoid for the ASGP-R. The ability of the HN^- virus to cause cell–cell fusion of Hep G2 cells indicated that attachment of this virus to the ASGP-R still permitted viral entry by its usual mode—i.e., membrane fusion at the cell surface. These results open up the possibility that enveloped viruses, which contain glycosylated proteins or lipids, may make use of naturally occurring lectins in addition to their normal receptors as a means of attachment to host cells.

The entry of enveloped viruses into cells occurs by at least two general routes, both dependent on the recognition of specific cell surface receptors and membrane fusion. The togaviruses, rhabdoviruses, and myxoviruses enter cells via adsorptive endocytosis, a process that delivers the entire virus particle to the intracellular endosomal compartments (1–4). Viral membrane fusion with the endosomal membrane is dependent on acidification (to pH 5–6) of this compartment (5) and is mediated by the viral spike glycoproteins (6, 7). On the other hand, adsorption and membrane fusion of the paramyxoviruses, such as Sendai virus, occurs at neutral pH at the cell surface (8), allowing direct release of the viral capsid into the cytoplasm.

Wild-type (wt) Sendai virus contains only two glycoproteins, HN and F, in its lipid envelope. These form the viral spikes and are known to function during the adsorption-fusion phase of infection. The HN protein of paramyxoviruses contains the receptor-recognition and receptor-destroying (sialidase) activities of the virus (9–11). Recently members of the gangliotetraose family of gangliosides have been identified as receptors for Sendai virus (12) and as substrates for the viral sialidase (13).

It has been suggested that the action of the viral sialidase on the first host cell receptor for HN creates a second cell receptor for F (14). The availability of the temperature-sensitive mutant *ts*271, a mutant devoid of HN protein when grown at the restrictive temperature (15), provides a system in which the roles of HN and F in adsorption and membrane fusion could be separately examined. This mutant does not infect conventional host cells, which are readily infected by the wt virus (16). The F protein, the only glycoprotein of the mutant, contains a terminal galactose moiety (17) and thus is a likely ligand for recognition by the hepatic asialoglycoprotein receptor (ASGP-R), a mammalian lectin that specifically recognizes proteins terminal in galactose or *N*-acetylgalactosamine (18). Therefore, the human hepatoma Hep G2 cell system, which expresses abundant ASGP-R (19), was investigated as a special host for Sendai virus, which might support infection, by having F serve as its own attachment factor.

MATERIALS AND METHODS

Virus and Cells. The origin of the *ts*271 mutant has been described (20). When grown at 30°C, this virus contains the HN protein and infects conventional host cells. When produced at 38°C, the virus is devoid of HN and noninfectious (15, 16). For this study, wt and *ts*271 viruses were propagated in chicken embryo lung cell monolayer cultures at 30 or 38°C. Virus production was quantified by hemagglutination (HA) titer using chicken erythrocytes for wt and *ts*271 (30°C) viruses and by protein determination for the purified *ts*271 (38°C) virus. For analysis of their polypeptides by PAGE, viruses were radiolabeled using [^{35}S]methionine (25 μCi/ml, 1200 Ci/mmol; 1 Ci = 37 GBq; Amersham), then purified by differential and isopycnic centrifugation as described (21). Sialidase activity of the purified viral preparations was assayed using [^{3}H]sialyllactitol as a substrate (22).

MDBK, HeLa, and MDCK cells were cultured as de-

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Abbreviations: wt, wild-type; ts, temperature-sensitive; ASGP-R, asialoglycoprotein receptor; OR, orosomucoid; ASOR, asialoorosomucoid; AGASOR, agalactoasialoorosomucoid; HA, hemagglutination(ing).

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scribed (6). N-Hepa 1 cells were grown in minimal essential medium containing 60 μM 6-thioguanine and 10% fetal bovine serum. Hep G2 cells were obtained from Barbara Knowles and were cultured in minimal essential medium containing 20 mM Hepes and 10% fetal bovine serum (hereafter called growth medium). Fresh growth medium was added to the Hep G2 cells the day before each experiment to improve their adherence to the plate.

For infection experiments, individual 35-mm plates of cells were used. The cells were washed twice with minimal essential medium containing 2 mM $CaCl_2$ and 20 mM Hepes (hereafter called binding medium). For infection with the wt or *ts*271 (30°C) viruses, 10 HA units of virus (3 plaque forming units per cell) was dispersed in 0.25 ml of binding medium containing 1% bovine serum albumin (fraction V, Miles).§ For infection with the *ts*271 (38°C) virus, an amount of viral protein equivalent to 10 HA units was used and the binding medium contained 1% cytochrome *c*. After a 15-min adsorption period, unattached virus was removed from the plates by three washes with binding medium. Two milliliters of growth medium was added to each plate. Then the plates were incubated at 30°C, the temperature at which both wt and *ts*271 produce viruses containing HN. Infection was assessed by the cytopathic effect 24 hr after infection and by virus production (HA titer) 48 hr after infection.

Treatment of the Cells with Ca^{2+} Chelators, Sialidase, and Competing Ligands. For treatment with Ca^{2+} chelators, Hep G2 cells were incubated with 1 or 10 mM EDTA or EGTA in 25 mM Tris·HCl, pH 7.2/0.7 mM sodium phosphate/140 mM NaCl (hereafter called Tris-buffered saline) for 30 min at 37°C. The cells, all of which floated off the plates during this treatment, were mixed with 0.25 ml of wt or *ts*271 virus in Tris-buffered saline. After a 15-min incubation at 37°C, the cells were pelleted through a cushion of 20% sucrose in Tris-buffered saline and replated in fresh growth medium. Treatment with sialidase was by incubation of cell monolayers for 30 or 120 min at 37°C with 50 mU of *Vibrio cholerae* sialidase (Calbiochem–Behring) in 0.5 ml of Tris-buffered saline. The sialidase was removed, the monolayers were washed once, and virus was added as described above.

For competition experiments, orosomucoid (OR), obtained from the American Red Cross, was fully desialylated to asialoorosomucoid (ASOR) (23). Agalactoasialoorosomucoid (AGASOR) was a gift of G. Ashwell. *N*-acetylglucosamine and *N*-acetylgalactosamine were purchased from J. T. Baker Chemical (Phillipsburg, NJ). Monospecific polyclonal rabbit anti-rat or anti-human ASGP-R were raised to purified receptor species and affinity purified as described (24, 25).

Hep G2 cells were washed twice with chilled incubation medium, then the various ligands (OR, ASOR, AGASOR, *N*-acetylglucosamine, *N*-acetylgalactosamine, or anti-ASGP-R antibody) were added in cold incubation medium containing cytochrome *c* at 1 mg/ml. After 1 hr at 4°C, the infecting virus was added without removal of the ligand. The incubation was continued for an additional 30 min at 4°C. The cells were washed three times with cold incubation medium to remove unadsorbed virus, then overlaid with fresh growth medium and incubated for 2 days at 30°C.

Competition of ^{125}I-Labeled ASOR by Whole Viruses or Solubilized Viral Glycoproteins. The viral membrane envelope was solubilized from purified virus by the addition of Nonidet P-40. Then the insoluble nucleocapsid fraction was removed by centrifugation, and the protein matrix fraction of the envelope was removed by dialysis as described (26). Detergent was removed from the resulting preparations of solubilized glycoprotein by treating the dialyzed samples three times with Amberlite XAD-2 beads.

Aliquots containing 50 μg of protein of whole virus or solubilized viral glycoprotein were placed in wells containing 4 $\times 10^5$ cells in 250 μl of binding medium for 1 hr at 4°C. Then ^{125}I-labeled ASOR (6800 cpm/ng of protein) was added to a final concentration of 60 ng/ml ($0.086 \times K_d$) in the presence of the competing ligands for 2 hr at 4°C. The cells were washed and the specific binding of ^{125}I-labeled ASOR was quantified as described (19).

RESULTS

Characterization of the HN$^-$ Virus. The *ts*271 (38°C) virus had no detectable HA activity (Table 1), and the HN polypeptide was absent in this virus although it was readily detectable in the wt virus sample containing the same amount of viral protein (Fig. 1). In addition to the HA activity, the HN protein also contains the sialidase activity of the virus. When a sensitive radioactive assay (22) was used to quantify this biological activity, the wt virus hydrolyzed 650 nmol of [^{3}H]sialyllactitol/hr per mg of protein. The *ts*271 (38°C) virus at the same protein concentration had no demonstrable sialidase activity (Table 1).

The HN$^-$ mutant also lacked the ability to productively interact with conventional host cells (Table 1). It would neither agglutinate nor infect MDBK, HeLa, or MDCK cells,

Table 1. Comparison of the biological activities of the HN$^-$ and wt viruses

Biological activity	HN$^-$ virus	wt virus
Hemagglutination, units/ml per mg of protein	<1.5	4400
Sialidase activity, nmol/hr per mg of protein	<0.48	650
Infectivity, HA units/ml		
MDBK cells	<2	512
HeLa cells	<2	1024
MDCK cells	<2	512

The ability of the HN$^-$ and wt viruses to cause hemagglutination was tested using chicken erythrocytes. Sialidase activities of the two viruses were quantified using [^{3}H]sialyllactitol as the substrate in a radioactivity assay (22). Infection of host cells was measured by the production of progeny virus (hemagglutination units/ml). All assays were done in quadruplicate.

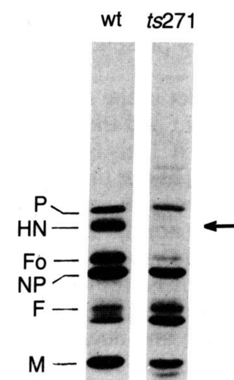

FIG. 1. NaDodSO$_4$/PAGE analysis of the [^{35}S]methionine-labeled polypeptides from wt and *ts*271 (38°C) viruses. Radiolabeled viral proteins were separated by PAGE (27) and visualized by autoradiography. Their positions are indicated by the letters on the left. The position of the attachment protein HN is indicated by the arrow to the right.

§Contaminants in preparations of bovine serum albumin interfere with the binding of ligands to the ASGP-R (23) but do not interfere with the binding of Sendai virus to its normal ganglioside receptors (12). It was therefore included during infection with wt or *ts*271 (30°C) to block the alternative route of entry via the ASGP-R.

Table 2. Interaction of the HN^- and wt viruses with hepatoma cells containing or lacking the ASGP-R

Test	Activity, %	
	Hep G2 cells	N-Hepa 1 cells
For the ASGP-R		
4°C saturation surface binding of ASOR	100	1
37°C uptake and degradation of ASOR	100	4
For infection		
By the wt virus	100	100
By the HN^- virus	100	<2

Saturable surface binding (19) of ASOR by Hep G2 cells was achieved at 2.83 ± 0.10 ng of ^{125}I-labeled ASOR per 3 × 10^5 cells. The cell-associated rate of uptake (19) at 37°C by Hep G2 cells was 152.0 ± 9.0 ng of ^{125}I-labeled ASOR/16 hr per 3 × 10^5 cells. Cells were infected with 10 HA units of wt virus or the equivalent amount of viral protein for the HN^- virus. Virus production at 30°C was quantified by HA titer of the medium 48 hr after inoculation. The value of 100% for virus production is equivalent to 128 HA units/ml for both N-Hepa 1 and Hep G2 cells. All determinations were done in quadruplicate.

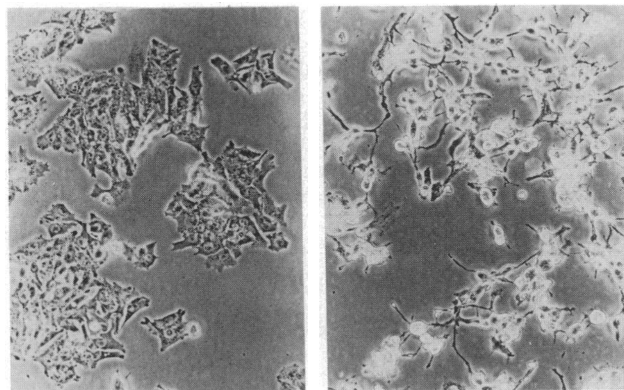

FIG. 2. Infection of Hep G2 cells by the HN^- virus. Hep G2 cells growing in monolayer (*Left*) were overlaid with the *ts*271 (38°C) virus. The cytopathic effect of infection became evident within 24 hr after inoculation (*Right*).

all of which contain receptor gangliosides (12) and are readily infected by the wt virus (512–1024 HA units/ml by 48 hr after inoculation). These cells were also readily infected by the *ts*271 (30°C) virus, which contains HN (data not shown). Thus the presence of the HN protein is required for the virus to recognize the receptors present on these bovine, human, and canine cell lines.

The only apparent defect in the *ts*271 (38°C) virus was the absence of the HN protein and the adsorption and sialidase activities associated with it. This is consistent with previous observations of this virus at the time of its original isolation (15). The second viral glycoprotein, F, a galactose-terminated protein, was present in normal amounts in the HN^- virus.

A Special Host, Hep G2 Cells. A line of liver-derived cells, hepatoma Hep G2, which is known to retain the ASGP-R upon continuous culture (19), exhibited saturable surface binding as well as uptake and degradation of the galactose-terminated ligand ^{125}I-labeled ASOR (Table 2). The HN^- mutant infected Hep G2 cells as well as did the wt virus (128 HA units/ml). Using the cytopathic effect as a criterion of infection, we observed that >99% of the cells of the Hep G2 monolayers were susceptible to the HN^- virus (Fig. 2). In contrast, N-Hepa 1 cells like most hepatoma lines lose the ASGP-R upon continuous culture. This line did not bind nor endocytose the galactose-terminated ligand ASOR (Table 2).

Table 3. Comparison of infection of Hep G2 cells by the HN^- and wt viruses in the presence of glycoproteins, carbohydrates, or competing antibody or after treatment with Ca^{2+} chelators or sialidase

Addition or pretreatment	Titer, HA units/ml	
	wt virus	HN^- virus
20 μg of OR/ml	128	128
20 μg of ASOR/ml	128	<2
20 μg of AGASOR/ml	128	128
100 mM *N*-acetylgalactosamine	128	<2
100 mM *N*-acetylglucosamine	128	128
Anti-ASGP-R antibody (0.2 mg/ml)	128–256	<2
1–10 mM EDTA for 30 min	128	<2
1–10 mM EGTA for 30 min	128	<2
Sialidase for 30 min	64	<2
Sialidase for 120 min	8–16	<2

Monolayers of Hep G2 cells were overlaid with binding medium containing various glycoproteins, carbohydrates, or competing antibody and incubated for 60 min at 4°C. Infecting virus was added without removing these and incubation was continued for an additional 30 min at 4°C. Some monolayers were pretreated with Ca^{2+} chelators or with 50 mU of *V. cholerae* sialidase at 37°C before addition of virus for 15 min at 37°C. All determinations were done in quadruplicate.

N-Hepa 1 cells were readily infected by the wt virus (128 HA units/ml) and *ts*271 (30°C) virus (data not shown) but resistant to the HN^- mutant (<2 HA units/ml).

Evidence for Involvement of the ASGP-R in Infection by the HN^- Mutant. The interaction of the wt virus with Hep G2 cells was not altered by the presence of glycoproteins, carbohydrates, or antibody to the ASGP-R or by pretreatment of the cells with Ca^{2+} chelators (Table 3). Extensive treatment of these host cells with sialidase reduced their susceptibility to infection by hydrolysis of their endogenous sialic acid-containing receptors. All of these observations are consistent with the wt virus entering by attachment to its normal receptor gangliosides (12). The same results were obtained with the *ts*271 (30°C) virus (data not shown).

In contrast, the interaction between the HN^- virus and Hep G2 cells was totally disrupted by the presence of the galactose-terminated ligand ASOR, by *N*-acetylgalactosamine, by antibody against the ASGP-R, or by pretreatment of the cells with Ca^{2+} chelators (1 or 10 mM EDTA or EGTA) (Table 3). Ligands lacking a terminal galactose,¶ such as OR, AGASOR, and *N*-acetylglucosamine did not compete with the HN^- virus for the ASGP-R.

Mild treatment of the cell surface at 37°C with *V. cholerae* sialidase blocked productive binding of the HN^- virus to Hep G2 cells (Table 3). This is consistent with previous observations as to the nature of the ASGP-R (28). It is itself a sialoglycoprotein. Removal of the terminal sialic acid groups of the receptor by the action of sialidase generates a galactose-terminated protein on the cell surface that effectively competes with exogenous ligands for its own ligand-binding site. Thus, even though there is an effect of the sialidase on both wt and HN^- virus-induced infections, the time differential (120 vs. 30 min of exposure to sialidase) is consistent with their entry by different sets of receptors.

Attachment Factor on the Virus. The results described above are consistent with the HN^- virus using the ASGP-R to effect its attachment to and entry into Hep G2 cells. The assumption that F, the only glycoprotein on the HN^- virus, was the means by which the virus was attaching to the ASGP-R was further investigated in competition binding experiments. Both the whole wt virus, which contains two spe-

¶In OR the galactose moiety is covered by the terminal sialic acid; in AGASOR it has been hydrolytically removed.

Table 4. Competition of ^{125}I-labeled ASOR binding by whole Sendai virus or solubilized viral glycoproteins

Addition	^{125}I-labeled ASOR bound
None	100%
ASOR (32 ng of unlabeled)	40
wt virus (50 μg of protein)	33
Solubilized glycoproteins from wt virus (50 μg)	9
HN$^-$ virus (50 μg of protein)	51
Solubilized glycoprotein from HN$^-$ virus (50 μg)	21

The ability of whole [wt or *ts*271 (38°C)] virus or solubilized glycoprotein to compete with ASOR for the ASGP-R was tested by using radiolabeled ligand at a concentration of 60 ng/ml. Results are expressed as percent of control (no addition); 100% is 3.0 ng of ASOR/mg of cell protein.

cies of galactose-terminated proteins (HN and F), and the solubilized proteins themselves were able to effectively compete with ^{125}I-labeled ASOR for the ASGP-R (Table 4), as did also the whole HN$^-$ virus and the F protein solubilized from it.[‖]

Mode of Infectious Entry: Membrane Fusion or Adsorptive Endocytosis. Enveloped viruses enter their host cells by at least two routes. For Sendai virus, the normal route of infection is via a membrane fusion event at the cell surface (29) with concomitant release of the virus nucleocapsid directly into the cytoplasm (Fig. 3). This is in contrast to many other viruses, such as the togaviruses, rhabdoviruses, and myxoviruses, which use adsorptive endocytosis coupled with intracellular membrane fusion at acidic pH for their infectious route of entry (4). Hep G2 cells were infected by a mutant of Sendai virus lacking its normal attachment protein and by a route that bypassed its normal ganglioside receptors. It therefore became of interest to further investigate how this virus was effecting its entry to produce infection of Hep G2 cells.

‖Controls indicated that the decrease in ^{125}I-ASOR binding was not the result of the nonspecific binding of ASOR to virus or viral protein which would make it unavailable for receptor binding (data not shown).

Cell–cell fusion is a known result of envelope–cell fusion when Sendai virus is added at a high multiplicity of viral particles per cell (a process termed fusion from without). Under these conditions, Hep G2 cells are fused by the HN$^-$ virus (Fig. 4). This demonstrates the ability of the virus to cause membrane fusion in the absence of HN protein.

DISCUSSION

The availability of a ts mutant of Sendai virus devoid of HN protein and of a special host system for this virus has allowed us to investigate the roles of HN and F in adsorption and fusion. The fact that the HN$^-$ virus productively infects Hep G2 cells via membrane fusion indicates that F alone is sufficient for the fusion event and limits the essential role of HN to adsorption. Neither an interaction between HN and F nor the activity of a viral sialidase is apparently needed during the entry phase of the infectious process, as shown in this host system.

The suggestion that HN is essential not only for viral attachment but also for envelope–cell fusion was based on past studies using model systems either for the virus (such as viral glycoproteins incorporated into liposomes) or for the host (such as erythrocytes). In these studies the absence of the HN subunit as a result of its destruction on the intact virus by treatment with dithiothreitol (30) or trypsin (31) or of its noninclusion into the viral liposome abrogated fusion activity of the virus or of the viral liposome. It did not, however, affect their cell agglutinating activities, provided another attachment factor such as concanavalin A (30), antiglycophorin (31), or the HA glycoprotein of influenza virus (15, 30) was included in the system. However, Hsu *et al.* (32) did observe both hemagglutination and hemolysis in the absence of HN in their liposome–erythrocyte system when wheat germ agglutinin was used as means of attachment.

The results of the present study on Sendai virus are consistent with and expand those of previous studies, which used paramyxo- or myxoviruses in actual host systems. Smith and Hightower (33) produced a ts mutant of Newcastle disease virus that contained only 3% of the sialidase activity of the wt virus, but this sialidase deficiency did not

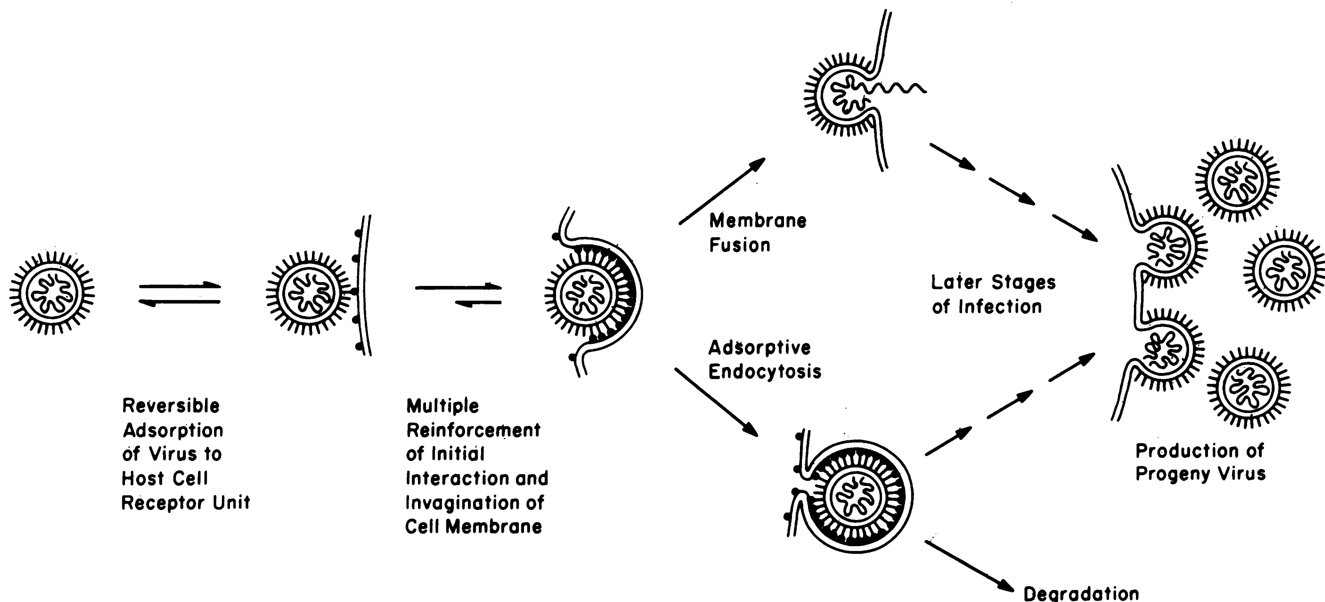

FIG. 3. Mode of entry of Sendai virus. For wt Sendai virus, the route of entry that produces infection involves fusion of the viral membrane with the cell surface and a direct release of the viral nucleocapsid into the cytoplasm. Virus particles that lack the ability to fuse at neutral pH enter by adsorptive endocytosis and are subsequently degraded. Many other types of viruses, however, use adsorptive endocytosis to achieve infection.

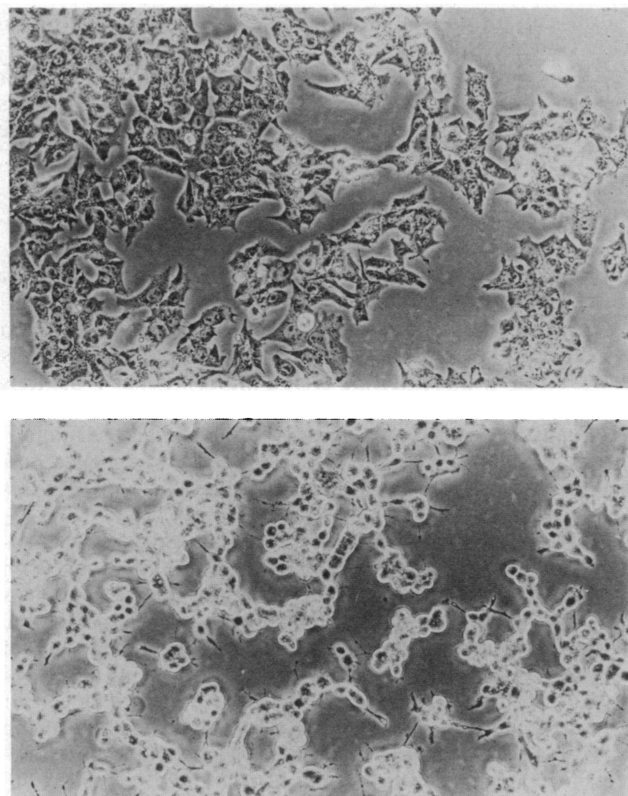

FIG. 4. Cell–cell fusion mediated by the HN$^-$ virus. Addition of a high multiplicity of HN$^-$ virus (10,000 HA units per 10^6 cells) to Hep G2 cells growing in monolayer (*Upper*) causes them to aggregate and form multinucleated structures (*Lower*).

impair its reproduction in cultured cells nor its virulence *in ovo*. Inhibition of the sialidase activity of Sendai virus by a sialic acid analog or by monoclonal antibodies did not block viral infection (34, 35). Schulze (36) has also shown that influenza virions devoid of sialidase activity, which were obtained by removing the sialidase spikes from the whole virus by trypsinization, were fully infectious. Although the viral sialidase activity does not appear to be essential for the entry phase of infection in the host system used in the present study and the others cited, it should be realized that these host systems are simplified ones. *In vivo*, the viral sialidase may play a crucial role in releasing inoculating virus from nonproductive binding to sialoglycoproteins present as mucins and in the extracellular matrix.

The results of the competition binding experiments on the infection of Hep G2 cells by the HN$^-$ virus supports the involvement of the ASGP-R. The fact that >99% of Hep G2 cells became infected when equivalent amounts of wt or HN$^-$ viruses were added indicates that both receptor systems in Hep G2 cells are apparently equally effective in virus adsorption leading to infection. The ability of the HN$^-$ virus to cause cell–cell fusion of Hep G2 cells in the neutral pH range shows that attachment of the HN$^-$ virus to the ASGP-R produces membrane fusion. This makes Sendai virus a rather unique ligand for this lectin. All of the other galactose- or *N*-acetylgalactosamine-terminated ligands recognized by the ASGP-R are internalized by adsorptive endocytosis (37). However, none of these ligands exist in the membranous type of environment that would permit fusion at the cell surface.

Infection of Hep G2 cells by the *ts*271 (38°C) virus is achieved by an alternative route that (*i*) bypasses the normal interaction of HN with its ganglioside receptors by taking advantage of the lectin capability of the ASGP-R for attachment of the virus and (*ii*) maintains the normal mode of viral entry by membrane fusion at the cell surface. This specific combination of virus and cell demonstrates that at least one type of virus that is missing its normal attachment factor can still achieve infection in special host cells. The findings reported here open up the possibility that enveloped viruses, all of which contain glycosylated proteins or lipids on their surface, may make use of naturally occurring lectins in addition to their normal receptors as a means of attachment to host cells.

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