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Swine Testis Cells Contain Functional Heparan Sulfate but Are Defective in Entry of Herpes Simplex Virus

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Herpes simplex virus (HSV) enters and infects most cultured cells. We have found that swine testis cells (ST) produce yields of infectious HSV-1 up to four orders of magnitude lower than those of human embryonic lung (HEL) and HEp-2 cells because of a defect in virus entry. For ST cells, virus binding is reduced, DNA from input virus cannot be detected, and virus proteins are not synthesized. Polyethylene glycol treatment of ST cells after exposure to HSV allows viral entry, protein synthesis, and productive infection. Transfection of viral genomic DNA that bypasses the normal entry process produces similar yields of infectious virus from ST, HEL, and HEp-2 cells. Therefore, all three cell lines can support the HSV replicative cycle. Biochemical analyses and inhibition of sulfation by sodium chlorate treatment show that ST cells contain amounts and types of heparan sulfate (HS) similar to those of highly susceptible cells. HSV infection of sodium chlorate-treated HEL and ST cells indicates the presence of a second, non-HS receptor(s) on susceptible HEp-2 and HEL cells that is missing, or not functional, on poorly susceptible ST cells. We conclude that ST cells are defective in HSV entry, contain functional HS, but lack a functional non-HS receptor(s) required for efficient HSV-1 entry. Further, ST cells provide a novel resource that can be used to identify, isolate, and characterize an HSV non-HS receptor(s) and its role in the entry and tropism of this important human pathogen.

Herpes simplex virus (HSV) is a common human pathogen that causes oral and genital lesions and widespread morbidity and mortality, especially in immunocompromised hosts. In its natural human host, HSV undergoes both lytic and latent infections that may involve multiple tissue types (35). The virus can infect and replicate in a wide range of cultured cells. Virus entry into cultured cells by fusion with the plasma membrane occurs through a cascade of virus-cell interactions and involves at least two types of binding to trigger efficient penetration at neutral pH (7, 10, 27, 35, 41). Initial attachment of HSV-1 is to heparan sulfate (HS), a ubiquitous cell surface proteoglycan (24, 34, 42). A subsequent stable attachment involves binding that is resistant to heparin washing and involves a saturable cell surface receptor(s) that is not HS (7, 17, 18, 21, 26, 27). Glycoprotein C (gC) and gB bind to HS (13, 33). At least gD, and perhaps gH, is required for binding to the saturable stable attachment receptor(s) (6, 18, 21). The molecular interactions of the many viral surface components and cell components during HSV attachment and entry, or the nature of the stable attachment receptor(s), have not been elucidated.

For viruses for which cell receptors have been identified, an important consideration is availability of a cell line that is poorly susceptible to infection. The most elegant studies done to prove a virus receptor and roles of receptors in infection, pathogenesis, and tropism take advantage of a limited range of cell susceptibility and availability of cells that lack a required receptor (2, 3, 11, 25, 29, 31, 36, 39, 43, 44). For HSV-1, one obstacle to studies of receptors for entry has been lack of a cell line that does not replicate HSV-1 because of a defect in entry. The few characterized cells that are poorly susceptible to this virus, for example, *Xenopus* oocytes, erythrocytes, and Chinese hamster ovary (CHO) cells, appear to be defective in steps other than entry. More recently, GroC mutant mouse L cells (12) or CHO cells (34), used to study the role of HS in HSV infection, have been shown to be defective in or produce altered HS. These cells seem to contain components which allow entry and production of virus (12) or immediate-early virus products (34).

In this report, we establish that swine testis (ST) cells are minimally susceptible to HSV-1 infection because of a defect in virus entry. Moreover, these cells are the first cells characterized to contain functional amounts and types of HS used in attachment but lack a functional, stable non-HS receptor(s) required for the stable attachment(s), which leads to efficient HSV-1 entry. These results define one determinant of HSV-1 cell tropism. Further, they provide a novel and valuable resource for studying binding to multiple receptors during entry of HSV and identifying the nature and function of the stable-attachment non-HS receptor(s) for HSV-1.

MATERIALS AND METHODS

Cells and viruses. The cells used were human embryonic lung (HEL), swine testis (ST), African green monkey kidney (Vero), human larynx epidermoid carcinoma (HEp-2), and swine kidney (SK-6) cells. HEL, HEp-2, and Vero cells are highly susceptible cells commonly used to culture and study HSV-1. All of the cells except Vero cells were grown in Dulbecco's modified Eagle medium (DMEM; Gibco Laboratories) supplemented with 10% fetal bovine serum (FBS; Hyclone). Vero cells were passaged in medium 199 with Hanks salts supplemented with 5% FBS or with 5% calf serum when prepared for virus titer determination. The viruses used were HSV-1(F), HSV-2(333), pseudorabies virus (PRV) strain Rice, and vaccinia virus (WR). Stocks of all viruses, except where noted, were grown on HEp-2 cells except PRV, which was grown on swine cells. Mutant viruses that lack either the immediate-early gene for ICP4 or the gene for gC or thymidine kinase and express β-galactosidase from the virus genome were kindly provided by Neal DeLuca (5). These viruses were grown on HEp-2 or the required complementing cell line (5) and

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were used at a multiplicity of infection of 10 or 2 PFU per cell to determine the number of HSV-infected cells.

Determination of virus yields and infectious centers. Monolayers containing approximately 4×10^6 cells in 25-cm^2 dishes were infected with viruses for 90 min at 37°C. After removal of the inoculum, monolayers were overlaid with medium 199 containing 2.5% calf serum and incubated at 37°C until the indicated time for harvest. Yields of infectious virus were determined in duplicate on Vero cells by using an overlay of medium 199 containing methylcellulose. Titers included infectious virus from both cells and medium. Infectious centers of HSV-1 were determined by infecting approximately 3×10^6 cells with virus at 0.1 or 2.0 PFU per cell. After 90 min of incubation, the inoculum was removed and residual virus was inactivated with citrate buffer (pH 3.0) (27). At 3.0 h postinfection, the cells were trypsinized, diluted in medium 199 containing 2.5% calf serum and plated on Vero cells. After the cells had attached, the monolayers were overlaid for 48 h with medium 199 containing methylcellulose to determine the number of infectious centers.

Electron microscopy of infected cells. ST or HEL cells were mock infected or infected with HSV-1 or PRV at 0.01 or 3.0 PFU per cell. Cells were fixed at 10 or 30 h postinfection and processed for thin-section electron microscopy as previously described (7).

Preparation and binding of purified, radiolabeled virus to cells. Radiolabeled HSV-1 was purified by dextran gradient centrifugation as previously described (9). Aliquots of ^{3}H-labeled virus that contained 8.9×10^8 PFU/ml and 12,500 cpm/0.10 ml were stored at −80°C. A dose response of virus binding at 4°C was determined as previously described (9). For virus binding under differential washing conditions, radiolabeled, purified virus was incubated with cells for 90 min and the supernatant was removed to quantitate unbound radioactivity. Wells were washed five times with phosphate-buffered saline (PBS), PBS containing 500 μg of heparin per ml, or 1.0 M NaCl. These supernatants were monitored as the virus in wash. Monolayers with bound virus were solubilized with Triton X-100. Counts in the unbound virus, washing fluid, and bound virus were determined by liquid scintillation counting. The percentage of recovered virus was 90 to 100% of the input, and the percentage of bound virus was calculated from the average of triplicate samples as percent bound = bound counts per minute/unbound + bound + wash counts per minute.

Southern hybridization of viral DNA. Confluent monolayers of 3×10^5 HEL or ST cells were infected with HSV-1, and DNA was isolated at the times indicated in the figures. To detect input viral DNA (see Fig. 4A) prior to onset of DNA synthesis, cells were infected at 40 PFU per cell and Southern hybridization of DNA from nuclear extracts was performed. At 30 min or 2 h postinfection, the viral inoculum was removed and monolayers were washed three times with PBS to remove the unbound virus. Cells were trypsinized, resuspended in PBS, and pelleted at $200 \times g$ for 5 min before nuclei were isolated and nuclear DNA was extracted as described previously (40). This procedure eliminates any signal from viral DNA that might be sequestered in cytoplasmic vesicles because of noninfectious endocytosis (10, 35) or virus attached to cell surfaces via HS (27). Moreover, it examines DNA in the nucleus, where HSV initiates gene expression. To determine DNA from virus replication (see Fig. 4B), ST or HEL cells were infected at 0.01 PFU per cell for 90 min, the virus inoculum was removed, and the cells were washed and incubated at 37°C for 24 h. Total cellular DNA was isolated by chloroform-phenol extraction and ethanol precipitation, digested with *Bam*HI (Gibco-Bethesda Research Laboratories), separated on a 0.8% agarose gel, and transferred to a nylon filter (Schleicher & Schuell) for hybridization as described elsewhere (37). Filters were probed with an HSV-1 DNA *Sma* fragment containing the gD-1 gene that had been radiolabeled by random priming.

Production of virus proteins. Synthesis of virus-specific proteins was determined as previously described (10), by pulse-radiolabeling of confluent monolayers of Vero, ST, and HEL cells in 24-well dishes. The cells were infected at 37°C with the amounts of virus indicated in the figures and labeled at 4 h and 10 min postinfection for 20 min with [^{35}S]methionine at 20 μCi/ml in methionine-free medium. Cells were washed with cold PBS containing 1.0% glucose and 2.5% calf serum. Cell proteins were solubilized in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) buffer and analyzed on an 8.5% polyacrylamide gel cross-linked with *N,N'*-diallyl tartardiamide. Total protein was quantitated by the Bio-Rad protein assay, and samples were equalized for SDS-PAGE. The gels were processed with Amplify (Amersham), dried, and exposed to film for fluorography. The major viral capsid proteins (4, 14) for HSV-1 and HSV-2 (large arrows) and for PRV (small arrow) are indicated in Fig. 3.

Polyethylene glycol (PEG) treatment for HSV-1 infectivity. Confluent monolayers of cells in 30-mm-diameter dishes were infected with HSV-1 at 40 PFU per cell for 1 h at 37°C. The viral inoculum was removed, and the monolayers were exposed to PEG 6000 at 50% (wt/vol). Successive washes were immediately performed with 1:3 and 1:7 dilutions of PEG (10). Cells were washed with DMEM and overlaid with DMEM containing 8% FBS for 3.5 h. Cells not exposed to PEG were treated similarly in PBS without PEG. For analysis of protein synthesis, at 4.5 h postinfection, the cells were labeled for 30 min with medium that contained [^{35}S]methionine. Cells were washed and solubilized, and proteins were analyzed by SDS-PAGE. To determine yields after PEG treatment, cells were incubated in DMEM and harvested at 10 h postinfection. Cells were detached at 3.0 h postinfection and diluted, and infectious-center titers were determined.

Transfection of viral DNA. HSV-1 or PRV genomic DNA was isolated from infected cells as previously described (20). Cells were plated in DMEM containing 10% FBS for 16 to 20 h prior to transfection of 10^6 cells per 60-mm-diameter dish. Fine precipitants were obtained by adding 31 μl of 2 M $CaCl_2$ to 250 μl of 2× *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES)-buffered saline (pH 7.05) containing various amounts of viral DNA. A 500-μl volume of the precipitant was added to cells at room temperature for 10 min, and the mixture was incubated for 30 min at 37°C. DMEM containing 5% FBS was added, and the cells were incubated for 4 to 6 h. The medium was removed, and the cells were washed with warm DMEM and overlaid with DMEM containing 5% FBS. To determine the specific activity of viral DNA at 12 to 14 h posttransfection, the cells were overlaid with medium and methylcellulose to isolate plaques. HSV-1 and PRV plaques were quantitated on Vero cells. To determine the time for one round of growth by transfection of DNA, HEL cells were transfected with HSV DNA and harvested at the indicated time points and virus titers were determined on Vero cells to obtain yields from a single round of growth by transfection. Efficiency of DNA transfection was monitored in all transfections by spectrophotometric analysis (determination of optical density at 420 nm) of β-galactosidase activity from a plasmid containing the *lacZ* gene under control of the cytomegalovirus promoter. HEL and ST cells consistently produced similar levels of β-galactosidase activity from the transfected plasmid. The first burst of virus yields from transfection occurred at approximately 20 h for HSV on HEL cells

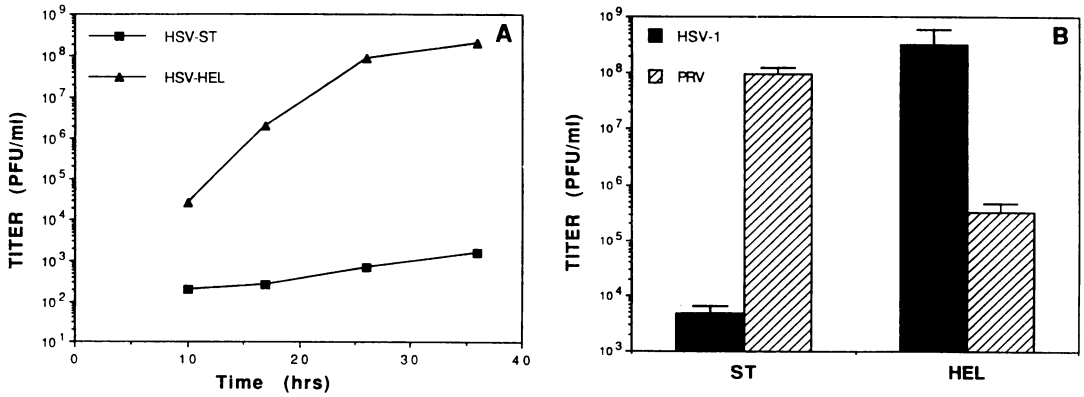

FIG. 1. Infectious yields of HSV-1. Confluent monolayers of ST and HEL cells were infected with HSV-1 or PRV at 0.01 PFU per cell for 90 min at 37°C. Cells were harvested at the indicated times postinfection (A) or at 24 h postinfection (B). Infectious virus titers were determined in duplicate on Vero cells. The graphs represent average yields from triplicate dishes.

and also for PRV on ST cells. The highest efficiency of virus DNA transfection was obtained by using 5 μg of HSV DNA, which had a specific activity of 350 PFU on Vero cells. Virus yields from $CaCl_2$ transfection into ST and HEL cells were calculated as described for specific activity except that the overlay did not contain methylcellulose. To equate yields from transfection and infection, 350 PFU (total) of HSV-1 DNA or stock virus was inoculated for one round of replication in ST and HEL cells.

Purification of HSPG. HS proteoglycans (HSPG) were solubilized and characterized as described by McClain and Fuller (27). To label cellular glucosaminoglycans, approximately 3×10^6 cells were plated in 25-cm^2 flasks and radiolabeled with [^{35}S]sulfate and [^{3}H]glucosamine at 37°C for 24 h. To purify radiolabeled HSPG, guanidine HCl extracts of cell monolayers and media were analyzed by Sephadex G-50 column chromatography. Radiolabeled macromolecules were isolated and quantified by scintillation spectroscopy and analyzed by Q-Sepharose anion-exchange chromatography. After elution with a 40-ml linear gradient of sodium chloride (0.3 to 1.2 M) in 8 M urea–0.5% 3-[(cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS)–50 mM sodium acetate (pH 6.0), 1-ml fractions were collected and monitored for radioactivity and conductivity. Proteoglycan fractions from Q-Sepharose chromatography were identified by their [^{35}S]radioactivity profiles. Pooled samples of proteoglycans were digested with chondroitin sulfate ABC lyase (100 mIU/ml) in 0.1 M Tris–0.1 M acetate (pH 7.3) for 60 min at 37°C, and digested proteoglycans were analyzed by Q-Sepharose anion-exchange chromatography. Fractions containing ^{35}S radioactivity were pooled, dialyzed against H_2O, and lyophilized.

Characterization of HSPG. HSPG pools for HEL or ST cell fractions were reconstituted in H_2O and vacuum dried. Glucosaminoglycans were prepared by treating labeled HSPG at 45°C for 24 h with alkaline borohydride in 1-ml volumes of 0.05 M sodium hydroxide–1.0 M sodium borohydride. Excess borohydride was destroyed by neutralizing the solution with 5.0 M acetic acid. Glucosaminoglycans were differentially cleaved by either heparitinase digestion or nitrous acid treatment. Heparitinase digests were performed identically to chondroitin sulfate ABC lyase digests, except that 5 mIU of enzyme per ml was used. Nitrous acid treatment was performed at low pH. Following treatment, the volumes of the samples were reduced to 250 μl. An equal volume of 8 M guanidine HCl was added to bring the final concentration of the treated samples to 4 M guanidine HCl. Alkaline borohydride-treated samples were analyzed by chromatography on Superose 6 columns, and both heparitinase- and nitrous acid-treated samples were analyzed by chromatography on Superdex 75 columns. Columns were equilibrated and eluted in 4 M guanidine HCl–50 mM sodium acetate–0.5% Triton X-100 (pH 6.0) at a flow rate of 0.4 ml/min.

Inhibition of sulfation by sodium chlorate. All chlorate experiments were performed in sulfate-free DMEM (Gibco-Bethesda Research Laboratories). Medium was supplemented with 8% FBS that had been dialyzed for 72 h against buffer containing 150 mM NaCl and 50 mM HEPES (pH 7.4) in distilled, deionized water. Sulfate-free DMEM and serum contained approximately 0.013 mM of free sulfate per ml. HEL or ST cells were replated 16 h prior to infection into 60-mm^2 dishes in sulfate-free DMEM that contained the concentrations of sodium chlorate or sodium sulfate indicated in Fig. 7. Monolayers were infected with HSV-1(F) at 2.0 PFU per cell in sulfate-free PBS containing 1% calf serum and 1% glucose for 90 min, and extracellular virus was inactivated with citrate buffer (pH 3.0) for 2 min (27). Monolayers were washed twice with PBS and overlaid with 5 ml of sulfate-free DMEM until 10 h postinfection, when cells and virus were harvested. The concentrations of chlorate or sulfate indicated in the figures were maintained throughout infection and overlaying. Virus was released from infected cells by three freeze-thaw cycles, and its titer was determined in duplicate on Vero cells.

RESULTS

Susceptibility of ST and HEL cells to HSV-1 infection. The time course of HSV-1 infection (Fig. 1A) showed that at the low multiplicity of infection typically used to produce maximum virus yields, HSV-1 produced infectious-virus yields on ST cells that were 2 to 4 orders of magnitude lower than those produced on HEL cells. While HEL cells were completely rounded or detached, little cytopathic effect appeared in ST cell monolayers, even at 36 h or with a higher virus input (Fig. 2A and data not shown). In contrast, PRV, a herpesvirus native to swine, replicated to high titers in ST cells (Fig. 1B) and showed an extensive cytopathic effect. The susceptibility of a different porcine cell line, swine kidney cells (SK-6), to HSV and PRV was similar to that of ST cells (data not shown). Infectious-center assays, which measure efficiency of infection of individual cells in a monolayer, were performed to assess the

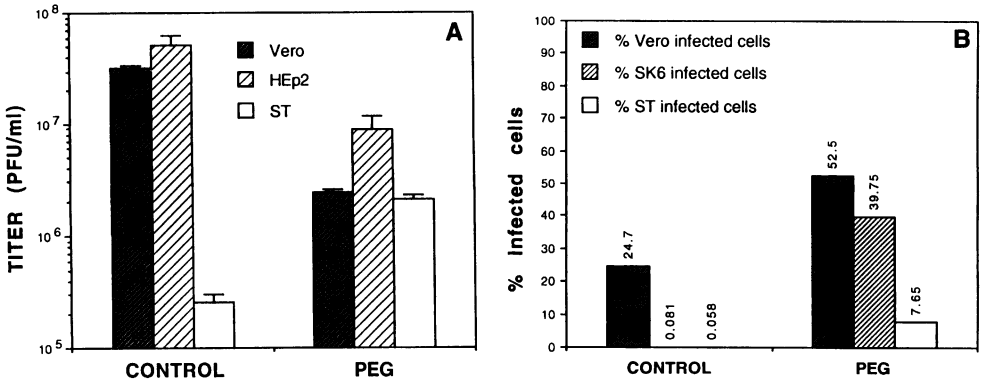

FIG. 2. Effects of PEG treatment on yields and infectious centers of HSV. Monolayers of the indicated cells at 1.0×10^6 were exposed to HSV-1 at 2.0 PFU per cell for 90 min. For one set of dishes, cells were treated with 50% PEG and washed and extracellular virions were inactivated with low-pH buffer (27). The control received the same treatment with buffers that did not contain PEG. (A) Cells were overlaid, virus was harvested at 10 h postinfection, and titers were determined on Vero cells. (B) At 3 h postinfection, the cells were detached from the dish with trypsin, resuspended, and diluted in medium containing serum. Infected cells were determined in duplicate on Vero cells. The graph represents the percentage of infectious centers compared with total cells from two experiments.

relative susceptibilities of cells to HSV-1 without amplification produced with virus yields (Fig. 2B). ST cells exposed to 2.0 PFU per cell produced a percentage of infectious centers (0.058%) over 2 orders of magnitude lower than that produced by highly susceptible Vero cells (24.7%) or HEp-2 cells (data not shown) infected in parallel. Infectious centers (Fig. 2B) and virus yields (Fig. 1 and 2A) indicated that HSV-1 did not efficiently infect two swine cell lines.

From electron microscopy of cells exposed to HSV-1 for 10 or 30 h (data not shown), the presence of virus nucleocapsids, cytoplasmic enveloped viruses, and extracellular virus at 10 h indicated HSV replication in HEL cells. However, fields of ST cells looked like mock-infected cells, with no indication of virus replication or change in cell architecture. This suggested that minimal amounts of virus entered, initiated virus gene expression, or replicated in ST cells.

In another approach, HEp-2, ST, and SK-6 cell monolayers were exposed at 10 PFU per cell to an ICP4 null mutant virus that encoded *lacZ* (5). Cells were subsequently stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) to detect production of β-galactosidase. While the entire HEp-2 monolayer stained blue from β-galactosidase activity, less than 0.5% (872 ± 22 ST cells and 568 ± 16 SK-6 cells) of approximately 3×10^6 cells were blue. We also used a gC null *lacZ* mutant virus that is capable of producing plaques because gC is not required for replication or spread of HSV-1 (13). In monolayers exposed to this mutant, the presence of individual blue cells, even at 48 h, indicated that only a few single cells in the population were susceptible to HSV infection. Results of ICP4 and gC null virus infection and counting of infectious centers (Fig. 2B) suggest that the few susceptible cells in the heterogeneous ST cell monolayer likely produce the 10^3 maximum HSV-1 yields (Fig. 1). Moreover, the electron microscopy results suggest a defect early in HSV interaction with ST cells. All of these results show that ST cells are poorly susceptible to infection by HSV-1.

Appearance of proteins and viral DNA in infected ST and HEL cells. One of the earliest detectable events in HSV-1 replication is production of virus-encoded proteins and shutoff of synthesis of cellular proteins by a protein carried within the virus particle. We examined protein profiles from ST and HEL cells after their exposure to HSV or PRV (Fig. 3A). At 40 PFU of input HSV per cell, virus bands were evident in susceptible HEL cells (lanes 5 to 8). However, ST cells exposed to as much as 400 PFU of HSV per cell produced no host shutoff or detectable viral proteins (lanes 1 to 4). Protein profiles in these samples were very similar to that of mock-infected ST cells. This indicated that virus did not initiate gene expression in ST cells and also suggested that the block to infection was very early, perhaps at entry. Appearance of proteins in infected cells is consistent with lack of detection of HSV DNA by Southern blot analyses (Fig. 4). When probed prior to DNA synthesis for DNA from input virus replication at 30 min or 2 h, HSV DNA could be detected in HEL cells but not in ST cells (Fig. 4A). After replication at 24 h, when abundant DNA appears in HEL cells, HSV DNA was just visible in ST cells at the higher virus input. This signal likely represents replication of HSV in the few (less than 0.5%) susceptible ST cells and demonstrates the sensitivity of the Southern analyses.

Virus binding to ST and HEL cells. Poor susceptibility of ST cells to HSV-1 infection might result from a block in any one of many steps in virus production. Electron microscopy (data not shown), protein synthesis profiles (Fig. 3A), and Southern blot analyses (Fig. 4) indicated that the block to infection was early, such as in virus binding, entry, or uncoating. Radiolabeled, purified virus was used to assess binding of HSV-1 to ST and HEL cells. Approximately 50% less input virus was required to saturate virus binding to ST (Fig. 5A). Because HSV entry involves multiple types of binding (7, 18, 21, 27, 35), the amount of radiolabeled virus that remained bound to cells after selective washing with PBS, heparin, or a high salt concentration was determined (Fig. 5B). ST cells bound approximately 50% less virus that resisted removal with PBS. For both cell lines, PBS-resistant virus could be removed by heparin while a residual amount of virus could not be removed by heparin. Although less virus attached to ST cells, the twofold reduction in virus binding seemed unlikely to account for the complete absence of DNA from the input virus, viral protein synthesis, or the 2- to 4-order-of-magnitude difference between yields (Fig. 1 and 2) or infectious centers (Fig. 2B). These results indicate possible differences in the type of, or outcome from, attachment of HSV-1 to ST and HEL cells.

Effects of PEG on virus entry. One explanation for low yields of HSV-1 from ST cells that is consistent with experimental observations (Fig. 1 to 5) was that virus could bind to but not efficiently penetrate ST cells to initiate virus gene expression.

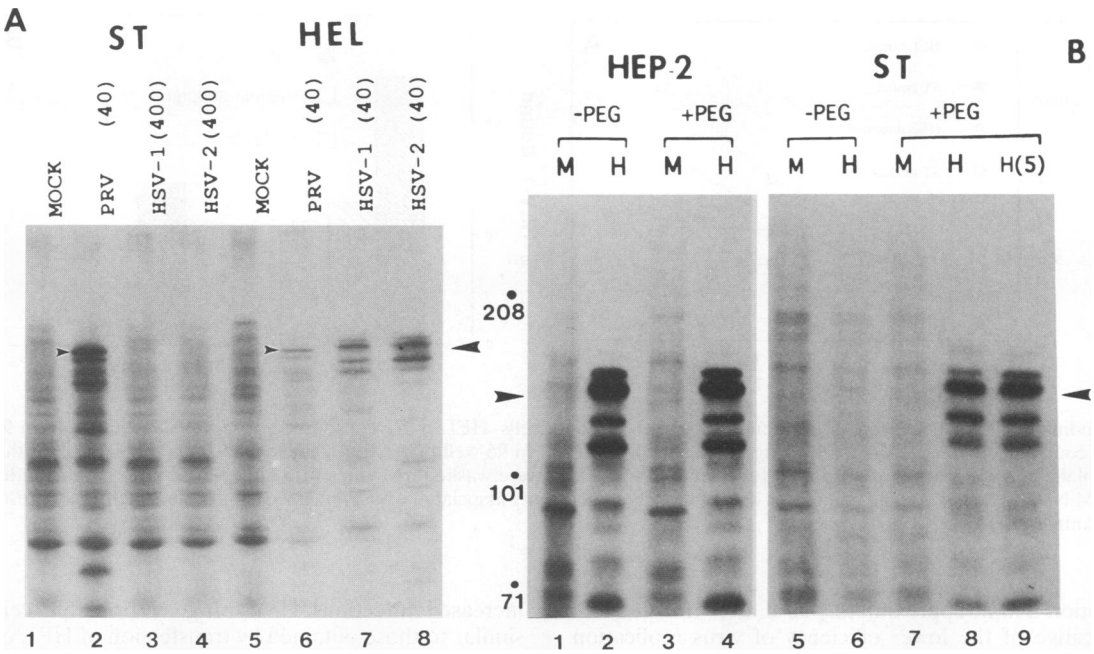

FIG. 3. Synthesis of viral proteins in ST or HEL cells exposed to virus. (A) Protein synthesized in ST or HEL cells after infection with PRV, HSV-1, or HSV-2 was determined by pulse labeling at 4 h postinfection. ST and HEL cells were grown in 24-well dishes and mock infected (lanes 1 and 5) or infected with 40 PFU of PRV per cell (lanes 2 and 6), 40 or 400 PFU of HSV-1 per cell (lanes 3 and 7), or 40 or 400 PFU of HSV-2 per cell (lanes 4 and 8). (B) Total protein synthesized in HEp-2 or ST cells after infection with HSV-1 was determined. Monolayers of HEp-2 or ST cells were treated (lanes 3, 4, 7, 8, and 9) or not treated (lanes 1, 2, 5, and 6) with PEG at 1 h postinfection. The cells were mock infected (M; lanes 1, 3, 5, and 7) or infected (H) at 40 PFU of HSV-1 per cell (lanes 2, 4, 6, 8, and 9). Cells were pulse-labeled with [^{35}S]methionine for 30 min at 4.5 and 5.5 h (lane 9) after addition of virus. Cells were washed, solubilized, and analyzed by SDS-PAGE on 8.5% *N,N'*-diallyl tartardiamide cross-linked gels. Protein profiles were visualized by autoradiography. The numbers on the left are molecular sizes in kilodaltons. The major viral capsid proteins are indicated (arrowheads).

For viruses that are blocked in infection by potent neutralizing antibodies or because of the absence of an essential envelope protein, PEG-mediated fusion of attached virions can overcome the block to infection (1, 7, 8, 10, 22). The absence of virus protein synthesis and viral DNA in ST cells (Fig. 3A and 4A) suggested that entry might be defective. We determined the effect of PEG-mediated virus entry on cells exposed to HSV by examining synthesis of viral proteins, virus yields, and numbers of infectious centers. After PEG treatment, ST cells exposed to HSV-1 (Fig. 3B) produced prominent HSV-1 protein bands and shutoff of cellular protein synthesis. The virus bands were slightly less intense in PEG-treated ST cells, but their intensity increased with a longer incubation time before pulse-labeling (Fig. 3B, lane 9). Although minor variations in sample loading might contribute to the lower intensity, the slightly lower level of virus proteins also might result from less efficient or later (treatment at 1.5 h postinfection) PEG-mediated virus entry than normal virus entry into HEp-2 cells. As also assessed by production of infectious virus (Fig. 2A), PEG treatment of ST cells exposed to HSV-1 substantially increased virus yields. Moreover, PEG-mediated cell fusion increased infectious centers on ST cells to 139-fold, compared with a twofold increase for Vero cells (Fig. 2B). SK-6 cells showed an even greater, 430-fold, increase. These results indicated that HSV-1 can initiate gene expression and replicate in ST cells. Moreover, they show that the defect in ST cells is at a cell surface event, such as stable attachment or penetration, that can be overcome by PEG-mediated virus fusion.

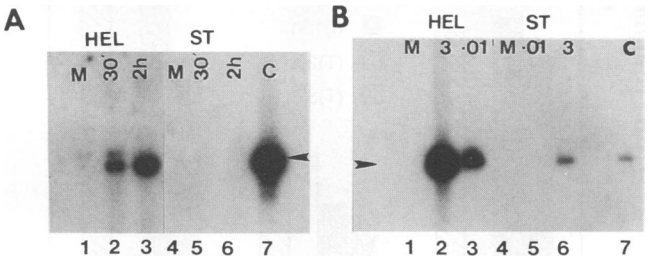

FIG. 4. Detection of HSV DNA by Southern blot analyses. Infected cells were harvested, and DNA was prepared for hybridization with an HSV-1 *Sma* fragment that contained the gD-1 sequence. Monolayers of ST or HEL cells were infected with HSV-1 at 40 PFU per cell, and nuclear DNA was isolated at 30 min or 2 h (A) and identical monolayers were infected at 3.0 or 0.01 PFU per cell (B), and total cell DNA was isolated at 24 h. One microgram of harvested DNA was subjected to Southern hybridization for each sample. Lanes M contained mock-infected cells, and lanes C contained controls that contained 25 ng of *Bam*HI-digested HSV-1 DNA. Arrowheads, HSV-1 restriction fragment.

Transfection of viral DNA to overcome infection block in ST cells. Transfection of herpesvirus DNA bypasses the normal viral entry process to produce infectious progeny in cells that are able to support the virus replicative cycle. To determine if there was a block in ST cells only at virus entry, we determined yields of HSV from one round of growth by transfection into cells versus infection. To ensure harvest of virus at the appropriate times, one-step growth curves of HSV-1 obtained by infection and transfection were determined (Fig. 6A). The first burst of infectious virus occurred at approximately 8 to 10

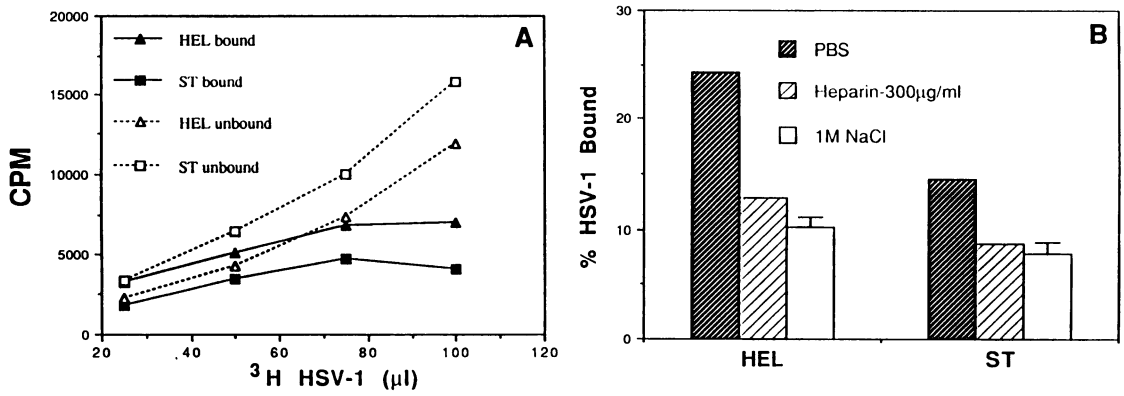

FIG. 5. Binding of radiolabeled HSV-1 to monolayers of HEL or ST cells. HEL or ST cells were grown to 95% confluency in 96-well plates. (A) The time course of radiolabeled-virus binding was determined at 4°C in 96-well plates. Each point represents the average of three wells. (B) Purified, radiolabeled virus was allowed to bind for 90 min at 4°C. Cells were washed five times with either PBS, PBS containing heparin at 500 µg/ml, or 1.0 M NaCl. The percentage bound was determined by radioactivity associated with solubilized cell lysates. Each bar represents the mean of triplicate samples.

h after infection and at approximately 20 h after transfection. This is indicative of the lower efficiency of virus replication obtained with isolated DNA than by entry of the virion particle that contains *trans*-acting virus proteins. The same number of input infectious units (a total of 350 PFU) was added to ST or HEL cells by transfection or infection. Virus was harvested, and its titer was determined on Vero cells after one round of replication by infection (8 h) and a subsequent round of infection from the progeny virus (16 h) or after one round of replication by transfection (20 h) and a subsequent time to allow infection by progeny virus from the transfection (32 h).

Comparison of infectious yields under these conditions confirmed a poor HSV-1 yield from infection of ST cells (Fig. 6B). Transfection of viral DNA produced the expected infectious yield of HSV on HEL cells and PRV on ST cells. It increased infectious HSV progeny from ST cells to levels similar to those obtained by transfection of HEL cells. Longer incubation to allow infection by progeny virus increased yields from HSV DNA into HEL cells or PRV into ST cells. However, longer incubation (32 h) did not significantly increase yields obtained with progeny virus from transfection into ST cells. This is consistent with a defect in HSV-1 entry into ST cells, since the second round of virus yield must be initiated by defective entry into neighboring cells (Fig. 6B). Transfection of virus DNA shows that bypass of entry overcomes the block to replication to confirm a defect in HSV-1 entry and indicates that ST cells are competent for HSV-1 replication if the virus genome can be introduced.

Presence of HS on ST cells. Figures 1 to 6 demonstrate that ST cells produce poor yields of HSV-1 because of a defect in

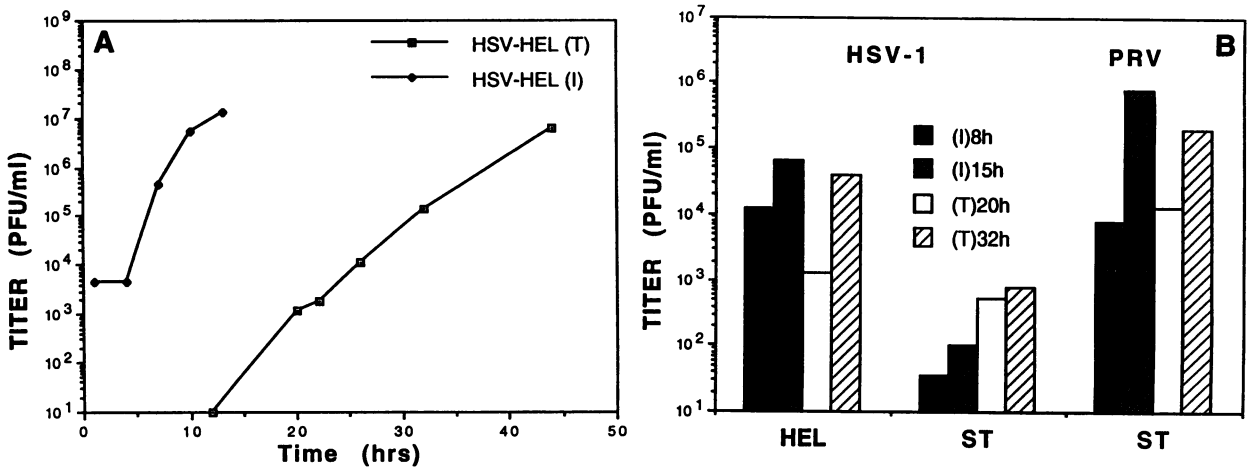

FIG. 6. Yields from HEL or ST cells by infection (I) of HSV-1 or transfection (T) of viral DNA. (A) A one-step growth curve was determined by transfecting ST cells with 5 µg of HSV-1 DNA or with HSV at 1.0 PFU per cell. Cells were harvested at the indicated time points, and yields of infectious progeny were determined in duplicate on Vero cells. (B) ST and HEL cells were transfected with 5 µg of HSV-1, and cells were harvested at 20 and 32 h after addition of DNA with a specific activity of 70 PFU/µg. ST and HEL cells were infected with 350 PFU (total) of virus, and cells were harvested at 8 and 16 h postinfection. All infectious yields were determined in duplicate on Vero cells. Similar experiments were performed in parallel with PRV on ST cells. Replications of the experiment produced similar relative yields from infection and transfection of HEL and ST cells. In a parallel dish, transfection efficiencies were determined from β-galactosidase activity expressed from a plasmid that encoded *lacZ* under cytomegalovirus promoter control.

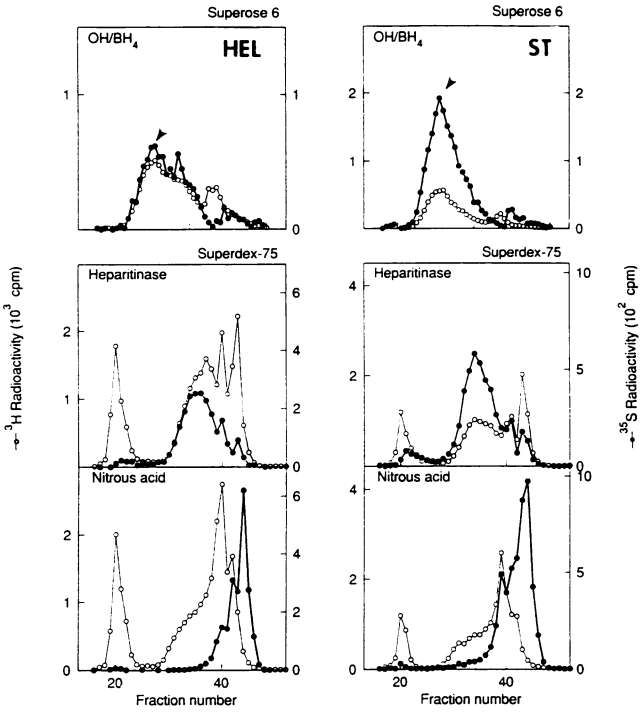

FIG. 7. Characterization of HS chains from HEp-2 and Vero cells. Relative sizes and sulfation patterns of HS chains isolated from cellular HSPG pools of HEL and ST cells are shown. Proteoglycans were extracted from cells radiolabeled with [^{3}H]glucosamine and [^{35}S]sulfate and digested with chondroitinase to remove chondroitin sulfate chains. The remaining HSPG pool was purified by ion-exchange chromatography. The scales of the ordinate axes were adjusted to allow simultaneous comparison of profiles of HS chains (○) and sulfate groups (●). (A) HSPG treated with alkaline borohydride. The arrowheads indicate mature HS chain peaks. Subsequent peaks represent intermediate metabolic HS product oligosaccharides unrelated to HS. (B) HSPG treated with heparitinase or nitrous acid. Peaks represent disaccharides (fractions 42 to 46), tetrasaccharides (fractions 39 to 42), hexasaccharides (fractions 36 to 39), and oligosaccharides (fractions 27 to 36). Fractions 46 to 48 in the nitrous acid profiles contained free sulfate released by cleavage. Fractions 18 to 26 contained oligosaccharides unrelated to HS.

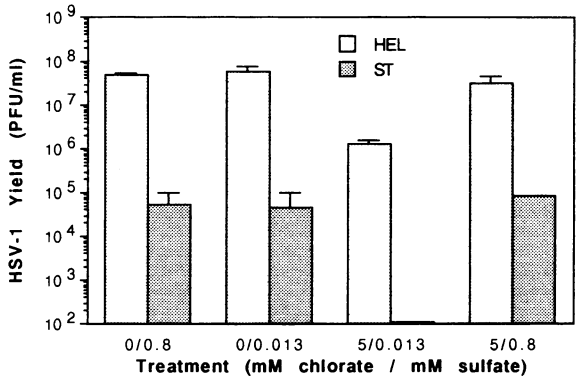

FIG. 8. Effect of sodium chlorate on HSV-1 infection. ST or HEL cells were grown for 16 h before infection in a specially formulated sulfate-free medium (0.013), minimal medium supplemented with 0.8 mM sodium sulfate (0.8), or medium that contained 5.0 mM sodium chlorate (5/0.013) or chlorate supplemented with sulfate (5/0.8). HSV-1 at 3.0 PFU per cell was exposed to monolayers in duplicate in the presence of the same medium. After 90 min, extracellular virus was inactivated and cells were overlaid with DMEM. At 10 h postinfection, virus was harvested and infectious yields were determined on Vero cells.

virus entry. Entry of HSV involves at least two types of attachment that trigger membrane fusion (7, 21, 27). The virus binds to HS (33, 34, 41) and another, non-HS, stable-attachment receptor(s) (7, 17–19, 21, 27). Binding of virus to ST cells (Fig. 5) suggested the presence and activity of at least one of these receptors. We examined ST cells for functional HS or a second receptor(s). Since PRV attaches to HS during entry (30, 33), efficient PRV entry into and infection of ST cells (Fig. 1B, 3A, and 6B) indicated the presence of HS. Moreover, heparin-sensitive HSV-1 binding to ST cells (Fig. 5B) also suggested the presence of functional HS. The residual heparin-resistant binding (Fig. 5B) might result from multivalent attachments of virus glycoproteins to HS or from attachment to a non-HS stable-attachment receptor(s). We tested the hypothesis that ST cells contain functional HS but lack functional amounts of the non-HS stable-attachment receptor.

The amounts, chain lengths, and sulfation levels of HS on ST and HEL cells were determined biochemically by column chromatography of glucosaminoglycans radiolabeled in sulfate or glucosamine. The analyses (Fig. 7) indicated that ST cells contain levels of HS similar to or slightly higher than those in HEL cells (35 and 21%, respectively). Moreover, the average lengths of glucosaminoglycan chains (73 kDa for HEL cells and 84 kDa for ST cells) and the levels of sulfation incorporated into these chains (47% for HEL cells and 50% for ST cells) were very similar. Thus, ST cells contain HS on the cell surface that is similar in structure to the HS on highly susceptible HEL cells (Fig. 7) and also to that on HEp-2 and Vero cells (27). From these biochemical analyses of HS, PRV infection, and heparin-sensitive binding of HSV, we conclude that ST cells contain functional amounts and types of HS that should allow initial HSV-1 attachment.

Function of receptors on HEL and ST cells. To further test the hypothesis that ST cells contain functional HS but lack functional non-HS stable-attachment receptors, we determined the effect of undersulfation of HS on infectious yields of HSV from ST and HEL cells. HSV does not bind well to undersulfated HS (12, 24, 28, 34). Therefore, if the binding observed and limited progeny from ST cells were mediated through HS interactions, sodium chlorate treatment of ST cells to undersulfate HS should remove this entry pathway and abolish the small amounts of virus infection observed. In contrast, for HEL cells that contain both functional HS for initial attachment and a functional non-HS receptor(s) for stable attachment, undersulfation by sodium chlorate should have a different effect. Since HS on HEL cells seems to be important but not essential for HSV entry (12, 28), loss of HS in the presence of a functional stable-attachment receptor should reduce but not abolish HSV entry and infection. We predicted that if a stable-attachment receptor(s) were present on HEL cells but absent on ST cells, substantially different HSV yields would result from sodium chlorate treatment.

Figure 8 shows infectious-virus yields obtained under conditions optimized for the minimum amounts of sodium sulfate that allow cell growth and virus production. Minimum sulfate (0.013 mM) that did not change cell metabolism (28) was sufficient for production of HSPG used by HSV, since virus yields from cells were equivalent in the presence (0.8 mM) or absence (0.013 mM) of excess sodium sulfate. This illustrates the high efficiency of cellular enzymes for performing sulfation

TABLE 1. Virus yields after chlorate treatment

Cell line	Relative (%), total (PFU/ml) HSV-1 yields[a]	
	Minimal sulfate (13 μM)	Chlorate (5 mM)
HEL	100, 4.95×10^7	2.95, 1.46×10^6
ST	0.17, 8.4×10^4	0.0002, 1.1×10^2

[a] Compared with yields obtained from HEL cells.

(23). Also at minimum sulfate, virus yields are several orders of magnitude lower on ST cells than on HEL cells. Addition of sodium chlorate (5/0.013 in Fig. 8) to compete for sulfation enzymes reduces sulfation by 80% (28) and decreases virus yields from highly susceptible HEL cells from 10^7 to 10^6. However, on poorly susceptible ST cells, HSV yields are reduced from 10^4 to almost undetectable levels. These effects could be completely reversed by addition of excess sodium sulfate (5/0.8 in Fig. 8). The difference in HSV yields on ST and HEL cells in the absence of functional sulfated HSPG supports the hypothesis that while HEL cells contain both functional HS and stable-attachment receptors, ST cells contain only functional HS that allows initial attachment but only some minimal virus entry into a few cells. Loss of HS sulfation virtually eliminates virus infection of ST cells. In contrast, on HEL cells, the non-HS receptor is capable of mediating virus entry in the absence of sulfated HS. Table 1 compares relative virus yields as percentages of that normally produced in highly susceptible HEL cells. These results show that ST cells contain HS but lack functional non-HS receptors. They agree with results of other studies (12, 28) which suggest that HS is important but not required or sufficient for efficient HSV-1 entry and infection.

DISCUSSION

We showed that ST cells are poorly susceptible to infection by HSV-1 because of a defect in virus entry (Fig. 1 to 6). The low levels of virus found were produced by less than 0.5% of the ST cell population. Heterogeneity of the ST cell line has been confirmed by establishment of nonsusceptible and more highly susceptible swine cell clones (32). On ST cells, HS is present, allows some virus binding (Fig. 7), and contributes to the low level of virus produced since undersulfation of HS can eliminate most of this infectious virus (Fig. 8 and Table 1). ST cells seem to lack a functional non-HS cell surface component that mediates stable attachment and penetration for efficient entry of HSV. Virus penetration and subsequent replication can be induced by cell surface treatment with PEG (Fig. 2 and 3). Since HSV-1 replication also occurs in cells when virus DNA is transfected (Fig. 6), ST cells are suitable for replication of HSV-1 if the virus or its genome can successfully enter. Poor susceptibility to HSV-1 infection also seems to occur with other swine cell lines (Fig. 2B; 32) and extends to poor susceptibility to HSV-2 (Fig. 3A; 38). These findings are consistent with a model of neutral-pH entry of HSV by several phases of attachment in a cascade of virus-cell interactions (7, 27, 35) and with the presence of a non-HS receptor that is required for HSV-1 penetration (7, 18, 21, 27, 28, 35). Characterization of the defect in entry into ST cells establishes a critical and novel cell line for isolation and study of the HSV stable-attachment receptor(s).

Poor susceptibility of ST cells to HSV-1, the presence of functional HS, and the absence of a functional stable-attachment receptor(s) are consistent with at least two types of attachment required for efficient HSV-1 entry into susceptible cells (7, 21, 27). Initial attachment to HS can occur on ST cells and is likely responsible for a great part of the virus binding observed. However, lack of penetration, as assayed by virus protein synthesis (Fig. 3) and Southern blot analysis (Fig. 4), shows that this HS binding does not facilitate efficient entry. Reduction of HS sulfation on both HEL and ST cells and the effects on infectivity (Fig. 8 and Table 1) suggest that HS is important for efficient entry. However, it is not required on sodium chlorate-treated HEL cells or sufficient on untreated ST cells to mediate efficient penetration and infectious entry. Further examination of the requirement and role of HS is reported elsewhere (28). However, the observations reported here are consistent with evidence that HS enhances HSV-1 entry (12, 28) but is not the primary cell surface component which determines susceptibility and tropism.

In experiments reported elsewhere (32), we found that the minimal susceptibility of swine cells to HSV infection can be increased by transfection of genomic DNA from human cells. Transfer of susceptibility with human DNA indicates that ST cells can be used to determine the nature of and clone the HSV receptor, or other components, lacking in ST cells. These studies are in progress. Although transfer of a gene(s) that could encode a cell surface protein, such as a stable attachment receptor for HSV, fits well with evidence that ST cells lack a functional stable-attachment receptor(s), it is possible that human DNA transfers a gene that results in alteration of HS, or another cell surface product, or a DNA sequence or gene product that affects an intracellular factor which enhances HSV-1 replication. In either case, availability of poorly susceptible ST cells will be important for isolating and identifying the gene(s) or its product and for understanding HSV entry and tropism for swine and human cells.

HSV-1 neutral-pH entry through a cascade of virus-cell interactions that trigger cell surface fusion (7, 35, 41) is consistent with the presence of at least two types of receptors on susceptible cells, such as HEL, HEp-2, and Vero cells (18, 21, 27), but lack of a critical stable-attachment receptor(s) on ST cells. Susceptibilities of other human and swine cells lines (32, 37) and of humans (15, 16) or infant pigs (38) to HSV and PRV indicate that tropism of these alphaherpesviruses for cultured ST and HEL cells may represent species tropism. It has been well established that PRV, an alphaherpesvirus native to swine that is very similar to HSV, does not infect or replicate in humans (15, 16). We have shown (37) that PRV does not replicate well in most human cells, particularly primary cells, because of a post-entry defect in regulation of immediate-early gene expression. We have also found that under conditions in which PRV caused the death of infant pigs in several days, the pigs survived with no evidence of HSV replication or cytopathic effects (38). These studies (this work; 15, 16, 32, 37, 38) suggest that the susceptibilities of cultured cells are likely relevant to natural animal tropism of these viruses. The ubiquity of HSV infection of a broad range of animal cells (35) indicates that either the required stable-attachment receptor(s) is also quite ubiquitous on cells or that there are a variety of cell surface proteins that can be used to mediate stable attachment and entry. The variety of host tissues that HSV infects suggests that both of these possibilities may occur. Further understanding of HSV interaction with poorly susceptible ST cells and use of these cells to identify the human gene product that increases susceptibility provide exciting developments in understanding of HSV entry, tropism, and pathogenesis.

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