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Herpes Simplex Virus Glycoproteins E and I Facilitate Cell-to-Cell Spread In Vivo and across Junctions of Cultured Cells

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Herpes simplex virus (HSV) glycoproteins E and I (gE and gI) can act as a receptor for the Fc domain of immunoglobulin G (IgG). To examine the role of HSV IgG Fc receptor in viral pathogenesis, rabbits and mice were infected by the corneal route with HSV gE$^-$ or gI$^-$ mutants. Wild-type HSV-1 produced large dendritic lesions in the corneal epithelium and subsequent stromal disease leading to viral encephalitis, whereas gE$^-$ and gI$^-$ mutant viruses produced microscopic punctate or small dendritic lesions in the epithelium and no corneal disease or encephalitis. These differences were not related to the ability of the gE-gI oligomer to bind IgG because the differences were observed before the appearance of anti-HSV IgG and in mice, in which IgG binds to the Fc receptor poorly or not at all. Mutant viruses produced small plaques on monolayers of normal human fibroblasts and epithelial cells. Replication of gE$^-$ and gI$^-$ mutant viruses in human fibroblasts were normal, and the rates of entry of mutant and wild-type viruses into fibroblasts were similar; however, spread of gE$^-$ and gI$^-$ mutant viruses from cell to cell was significantly slower than that of wild-type HSV-1. In experiments in which fibroblast monolayers were infected with low multiplicities of virus and multiple rounds of infection occurred, the presence of neutralizing antibodies in the culture medium caused the yields of mutant viruses to drop dramatically, whereas there was a lesser effect on the production of wild-type HSV. It appears that cell-to-cell transmission of wild-type HSV-1 occurs by at least two mechanisms: (i) release of virus from cells and entry of extracellular virus into a neighboring cell and (ii) transfer of virus across cell junctions in a manner resistant to neutralizing antibodies. Our results suggest that gE$^-$ and gI$^-$ mutants are defective in the latter mechanism of spread, suggesting the possibility that the gE-gI complex facilitates virus transfer across cell junctions, a mode of spread which may predominate in some tissues. It is ironic that the gE-gI complex, usually considered an IgG Fc receptor, may, through its ability to mediate cell-to-cell spread, actually protect HSV from IgG in a manner different than previously thought.

Herpes simplex viruses (HSVs) can initiate infection of cultured cells by adsorbing onto the cell surface and then penetrating across cellular membranes so that the nucleocapsid enters the cytoplasm. However, spread of HSV in tissues involves additional features because viruses frequently move across cellular junctions where cells are closely cemented or through extracellular matrix where cells are more distantly separated. Cell-to-cell spread of HSV in monolayers of certain cultured cells can also involve infection of adjacent cells across cell junctions because plaques can be produced in the presence of neutralizing antibodies. Major participants in these processes include the HSV glycoproteins which comprise the protein component of the virion envelope. HSV type 1 (HSV-1) encodes at least 11 glycoproteins, of which gB, gD, gH, gK, and gL are essential for productive infections in cultured cells (10, 18, 40, 46, 60). Virus mutants lacking gB, gD, and either gH or gL, which form a hetero-oligomer (39), are unable to enter cells (10, 18, 44, 60). There is evidence suggesting that gB and gH/gL function directly in the fusion of viral and cellular membranes during virus penetration (8, 9, 10, 21, 23, 34, 56) and that gD binds to cellular proteins (7, 11, 42, 44, 45). In addition, gB and gC probably mediate interactions with heparan sulfate proteoglycans during virus adsorption onto the cell surface (33, 61). The remaining five glycoproteins; gE, gI, gG, gJ, and gM, are considered nonessential in that they are dispensable for virus infection and replication in cultured cells (2, 3, 43, 48, 49, 65).

Since it seems unlikely that HSV conserves unnecessary genes, it is probable that these so-called nonessential glycoproteins play important roles in vivo in the infection of diverse cell types, movement of virus through tissue, or protection from host immune responses. gC functions as a receptor for the complement component C3b (17, 20), and a complex of gE and gI acts as a receptor for immunoglobulin G (IgG) (4, 25, 43). It appears likely that by binding complement or the Fc domain of IgG, these viral proteins may protect HSV-infected cells from complement-mediated immune lysis, in the case of the Fc receptor by causing IgG aggregation or by reducing the ability of complement components to bind to virus- or cell-associated IgG (1, 15, 19, 25).

HSV-1 gE mutants exhibit decreased neurovirulence. Neidhardt et al. (57) reported that an HSV-1 gE mutant was much less neurovirulent than parental strain HSV-1(ANG). Rajcnai et al. (59) found that a gE mutant spread poorly following intraperitoneal inoculation and failed to spread axonally following corneal inoculation. A pseudorabies virus (PrV) mutant deficient in the PrV gE homolog, gI, displayed reduced ability to spread in the rat central nervous system (12). These observations suggested that HSV gE and the PrV homolog function in vivo by facilitating virus spread; however, it has been difficult to interpret some of the in vivo data involving

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gE-negative mutants because it is not known whether IgG Fc receptor activity participates in virus spread, perhaps by reducing immune surveillance or cytolysis of virus-infected cells.

We examined the pathogenicity of HSV-1 gE^- and gI^- mutants after infecting the eyes of rabbits and mice. Mutants unable to express gE or gI produced very small punctate or dendritic lesions in the corneal epithelium, in comparison with the large dendritic lesions produced by wild-type HSV-1. This difference was unrelated to anti-HSV IgG because it was observed before IgG appeared and in mice, in which IgG does not bind to the Fc receptor. Furthermore, we showed that cell-to-cell spread of these mutant viruses was markedly reduced in monolayers of human fibroblasts and epithelial cells. The decreased cell-to-cell spread was not related to differences in the rate or extent of virus entry into these cells. Thus, gE and gI are important in spread of HSV in epithelial tissues and in monolayers of certain cultured human cell types which form cellular junctions.

MATERIALS AND METHODS

Cells and viruses. Vero cells and human R970-5 (here called R970) cells were grown in alpha minimal essential medium (α-MEM) (GIBCO Laboratories, Burlington, Ontario, Canada) supplemented with 5% (vol/vol) heat-inactivated fetal bovine serum (FBS). Normal human fibroblasts were grown in α-MEM supplemented with 10% FBS. HSV-1 strain F (obtained from P. G. Spear, Northwestern University Medical School), F-US7kan (43), 17syn+ (here called 17) (6), *in*1404 (43), and F-gEβ were propagated and titered on Vero cells.

Antibodies. Monoclonal antibody (MAb) 3104, specific for gI (43), and 3114, specific for gE, were gifts of Anne Cross and Nigel Stow (Institute of Virology, Glasgow, United Kingdom). MAbs LP11, which is specific for gH (18), and LP2, specific for gD (55), were gifts from A. C. Minson (University of Cambridge, Cambridge, United Kingdom). MAb DL11, specific for gD (16), was a gift G. Cohen (University of Pennsylvania). Anti-thymidine kinase (anti-TK) polyclonal rabbit serum was generously provided by W. Summers (Yale University). MAb 17βA1, specific for the large subunit of ICP6 (38), was a gift of S. Bacchetti (McMaster University, Ontario, Canada). Rabbit serum against HSV-1 (MacIntyre strain) was purchased from Dako (Dimension Laboratories, Mississauga, Ontario, Canada). Peroxidase-coupled goat anti-rabbit IgG was obtained from Bio-Rad Laboratories (Mississauga, Ontario, Canada). Polyclonal sera specific for gE and gI (R gE/gI) was raised in rabbits inoculated with recombinant adenoviruses AdgE and AdgI (25) by intraperitoneal injection of animals with 10^9 PFU of both AdgE and AdgI.

Labelling of cells with [^{35}S]methionine, immunoprecipitations, and gel electrophoresis. Human R970 cells or normal fibroblasts grown on 35-mm dishes were infected with 5 or 10 PFU of either HSV-1 strain F or 17 or mutant F-gEβ, *in*1404, or F-US7kan per cell. After 2 or 5 h, medium was removed and the monolayer was washed twice with medium 199 lacking methionine and then radiolabeled with [^{35}S]methionine (50 μCi/ml; Amersham, Oakville, Ontario, Canada) in medium 199 lacking methionine for 2 or 3 h. Cell extracts were made by using Nonidet P-40 (NP-40)–deoxycholate (DOC) extraction buffer (50 mM Tris [pH 7.5], 100 mM NaCl, 0.5% sodium DOC, 1% NP-40) containing 2 mg of bovine serum albumin per ml and 1 mM phenylmethylsulfonyl fluoride and stored at −70°C. Cell extracts were thawed at room temperature, subjected to a high-speed centrifugation, and mixed with sera or MAbs for 2 h at 4°C, and then protein A-Sepharose (Pharmacia, Dorval, Quebec, Canada) was added for an additional 2 h at 4°C on a rotating wheel. Protein A-Sepharose beads were washed three times with NP-40–DOC buffer, and precipitated proteins were eluted with twofold-concentrated sample buffer (100 mM Tris hydrochloride [pH 6.8], 4% sodium dodecyl sulfate, 4% 2-mercaptoethanol, 20% glycerol, bromophenol blue) and boiled for 5 min. Samples were electrophoresed in a 8.5% *N,N'*-diallyltartardiamide cross-linked polyacrylamide gel as described by Heine et al. (26) at 50 V for 14 to 16 h. Gels were fixed for 1 h, washed twice in distilled water for 2 min, enlightened (Enlightening; Dupont, Mississauga, Ontario, Canada) for 30 min, dried, and placed in contact with Kodak XAR film.

Construction of plasmid pgEβgal and HSV-1 gE^- mutant F-gEβ. All enzymes used to construct plasmids were purchased from Bethesda Research Laboratories (Burlington, Ontario, Canada) or New England Biolabs (Mississauga, Ontario, Canada). Plasmid DNA was prepared by using Qiagen 500 columns (Qiagen Inc., Chatsworth, Calif.). Plasmid pgEβgal was constructed from pSV2XXXgEN (25) by replacing a *Sma*I fragment encompassing the 5′ coding sequences of the gE gene with a 4.3-kb *Bam*HI fragment containing the ICP6::*lacZ* cassette from pD6p (22). Vero cell monolayers (60% confluent 100-mm dishes) were transfected with pgEβgal and viral DNA derived from Vero cells infected with HSV-1 strain F, using the $CaPO_4$ technique (24) as previously described (46). Recombinant viruses which had the gE gene replaced with the β-galactosidase gene were identified as blue plaques following screening with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Boehringer Mannheim Canada, Ltd., Dorval, Quebec, Canada) as previously described (46). Recombinant viruses were plaque purified three times. Viral DNAs were extracted from infected cells (62), and recombination was confirmed by Southern blot analysis (50).

Ocular HSV-1 infections. Six-week-old female A-strain mice (Frederick Cancer Research and Development Center, Frederick, Md.) were anesthetized with 2.0 mg of ketamine hydrochloride (Vetalar; Park-Davis, Morris Plains, N.J.) and 0.04 mg of acepromazine maleate (Aveco Co., Inc., Fort Dodge, Iowa) in 0.1 ml of RPMI 1640 injected intramuscularly. Corneas of anesthetized mice were scarified in a crisscross fashion with a sterile 30-gauge needle, the eyes were infected topically with 3 μl (10^5 PFU) of HSV-1 F, F-US7kan, or F-gEβ, and then the eyelids were rubbed. Two days postinfection (p.i.), the eyes of infected mice were instilled with 3 μl of fluorescein solution and photographed by using a vertically mounted slit lamp with a 27× objective and a cobalt blue light source. After 7 to 14 days, herpes stromal keratitis was observed in animals inoculated with wild-type HSV-1 F as previously described (32).

Single and multistep growth curves of HSV-1 replication. Normal human fibroblasts were seeded in 35-mm dishes and grown to confluency. Cell monolayers were infected with F, F-gEβ, or F-US7kan, using 5 (single-step growth curves) or 0.001 (multistep growth curves) PFU per cell for 2 h, the media were aspirated, the cells were washed once with phosphate-buffered saline (PBS), and fresh media were added. In some instances, the media were supplemented with 0.1% human gamma globulin (HGG) to neutralize the spread of extracellular virus. At various times p.i., cells and media were collected separately and frozen at −70°C. Samples were later thawed, sonicated, and plaqued on Vero cells under standard plaque assay conditions, using 0.1% HGG. After 48 h, the cell monolayers were fixed and then stained with crystal violet and plaques were counted.

Assay for rate of HSV penetration into cells. The low-pH citric acid entry assay, used to measure the rate of viral penetration, was adapted from methods of Huang and Wagner

(37) and Highlander et al. (35). Normal human fibroblasts grown on 12-well dishes were placed at 4°C for 30 min prior to a 2-h adsorption period at 4°C with 100 PFU of HSV-1 F, F-US7kan, or F-gEβ. Viruses were removed, the cells were washed three times with PBS, fresh medium was added, and the dishes were shifted to a 37°C incubator to allow viruses to penetrate. At various times following the temperature shift, the media were replaced with 1 ml of citrate buffer (40 mM citric acid, 10 mM KCl, 135 mM NaCl [pH 3.0]) or PBS for 1 min. These buffers were aspirated, the cells were washed three times with PBS, and fresh medium supplemented with 0.1% HGG and 1% FBS was added. After 48 h, the cell monolayers were fixed and stained with crystal violet and plaques were counted.

Immunoperoxidase staining of fibroblasts. Normal human fibroblasts were seeded on glass coverslips in 35-mm dishes and infected with HSV-1 F, F-US7kan, or F-gEβ at a multiplicity of infection of 0.002. At 2 h p.i., the inoculum was replaced with fresh medium supplemented with 0.1% HGG or with other antibodies. At 12-h intervals, medium was removed and coverslips were washed once with PBS, washed once with water, and air dried. Cells were fixed with 100% acetone (−20°C) for 10 min, washed briefly in water, air dried, and stored at −20°C. Coverslips were incubated with Dako rabbit anti-HSV-1 serum diluted 1:100 in PBST (PBS supplemented with 0.05% Tween 20) and incubated at 37°C for 1 h. Cells were washed three times with PBST and incubated with a 1:100 dilution of goat anti-rabbit IgG conjugated with horseradish peroxidase (Bio-Rad) at 37°C for 30 min. Cells were washed three times in PBST, and peroxidase substrate (equal volumes of 0.02% hydrogen peroxide and 1 mg of DAB [3,3′-diaminobenzidine tetrahydrochloride dihydrate; Bio-Rad] per ml in 100 mM Tris [pH 7.2]) was added for 20 min. Coverslips were washed three times in water, air dried, and mounted on glass slides.

RESULTS

Construction of an HSV-1 mutant, F-gEβ, unable to express gE. HSV-1 mutant *in*1404, which was used in previous studies, was derived from strain 17 and does not express gE because an oligonucleotide was inserted into a *Hpa*I site near the 5′ terminus of the gE coding sequences, producing a stop codon (43). In certain animal experiments, there appeared to be selective pressure for *in*1404 to revert to wild type, perhaps related to the fact that the coding sequences for gE are intact (19a). Therefore, we constructed a second gE-negative mutant in which a large fraction of the gE coding sequence was deleted from HSV-1 strain F so as to simplify comparisons with mutant F-US7kan (43), a gI$^-$ mutant derived from F. Plasmid pgEβgal (Fig. 1) was constructed from pSV2XXXgEN (25) by replacing a *Sma*I fragment with a 4.3-kb *Bam*HI fragment containing the ICP6::*lacZ* cassette of pD6p (22). The *lacZ* insertion begins 4 bp upstream of the ATG initiation codon for gE and deletes approximately 60% of the coding sequences. Vero cells were cotransfected with HSV-1 F viral DNA and pgEβgal, and virus progeny were screened by using agarose overlays containing X-Gal. A recombinant virus, F-gEβ, was isolated, and Southern blot analysis was used to confirm the structure of the viral DNA (data not shown).

To test for expression of gE, human R970 cells were infected with wild-type HSV-1 F or mutant F-gEβ or *in*1404, the cells were labelled with [^{35}S]methionine, and gE was immunoprecipitated. A MAb specific for gE (3114) and a polyclonal serum produced in rabbits inoculated with adenovirus vectors expressing gE and gI (R gE/gI) (25) both precipitated a complex of gE and gI from extracts of cells infected with wild-type HSV-1 F, but no gE was detected in extracts from cells infected with F-gEβ or *in*1404 (Fig. 2). Similarly, the rabbit IgG present in rabbit preimmune serum precipitated the gE-gI complex from extracts of cells infected with wild-type HSV-1 as previously reported (43), but the gE-gI complex was not detected in extracts from F-gEβ- or *in*1404-infected cells. Rabbit polyclonal anti-gE/gI antibodies and MAb 3104, specific for gI, precipitated gI but not gE from extracts of cells infected with F-gEβ and *in*1404. Therefore, F-gEβ does not express gE but expresses gI.

F-US7kan and F-gEβ display a reduced pathogenicity in the mouse eye. Rabbits and mice were infected by the corneal route with gE$^-$ or gI$^-$ mutant or wild-type HSV, and disease was evaluated as previously described (28). Only the results for mice will be described, although very similar results were also obtained for rabbits. In two identical experiments, three groups of four mice (6 weeks old; A strain) were topically infected with each virus by corneal scarification. Two days later, epithelial lesions were examined and photographed by using a slit lamp following instillation of a fluorescein dye. A marked difference between the ability of wild-type HSV-1 F and the mutants F-gEβ and F-US7kan to produce epithelial lesions was observed (Fig. 3 and Table 1). Wild-type HSV-1 produced very large lesions with an amorphous center and dendritic projections, the gE$^-$ mutant produced barely perceptible, punctate keratopathies, and the gI$^-$ mutant, F-US7kan, produced smaller dendritic lesions, but these were clearly distinct from those produced by wild-type F (Fig. 3). At 7 to 14 days p.i., herpes stromal keratitis was observed in mice infected with wild-type F but not in mice inoculated with either mutant (Table 1). All mice infected with F succumbed to encephalitis and were sacrificed, whereas no signs of viral encephalitis were observed in mice infected with F-US7kan or F-gEβ. Patterns of disease illustrated in Table 1 and Fig. 3 were uniformly observed within each group of mice. Large differences in epithelial disease were observed by 2 days postinoculation and would, therefore, not be related to the IgG Fc receptor activity of the gE-gI complex, since it appears highly unlikely that anti-HSV IgG could appear in this short period of time. In the rabbit eye, anti-HSV IgG or IgA cannot be detected until 5 to 13 days p.i. (13, 27). In addition, mouse IgG binds poorly or not at all to the HSV Fc receptor (41). In other experiments (not shown), rabbits or mice infected with wild-type HSV-1 strain 17 displayed large dendritic lesions, whereas the lesions produced by the gE-negative mutant *in*1404 (43) were small, barely detected, punctate lesions. In these experiments performed for 2 to 5 days, there was insufficient time for *in*1404 to revert to wild type. Therefore, these differences in pathogenicity were observed with two independently isolated gE mutants and a gI mutant, supporting the premise that this phenotype was related to the absence of the gE-gI hetero-oligomer rather than to mutations in other genes.

Mutants unable to express gE or gI produce small plaques on monolayers of human fibroblasts. To investigate the basis for the reduced pathogenicity of HSV-1 gE or gI mutants, we characterized the replication of mutant viruses in a number of different cultured cells. On several nontransformed fibroblasts derived from human skin biopsies, we observed a dramatic decrease in the size of plaques produced by F-gEβ and F-US7kan relative to those produced by wild-type HSV-1 F (Fig. 4). Similarly, *in*1404 produced much smaller plaques than parental wild-type strain 17 on fibroblast monolayers, and several other gI-negative mutants displayed the small-plaque phenotype (not shown). Since these other mutant viruses were

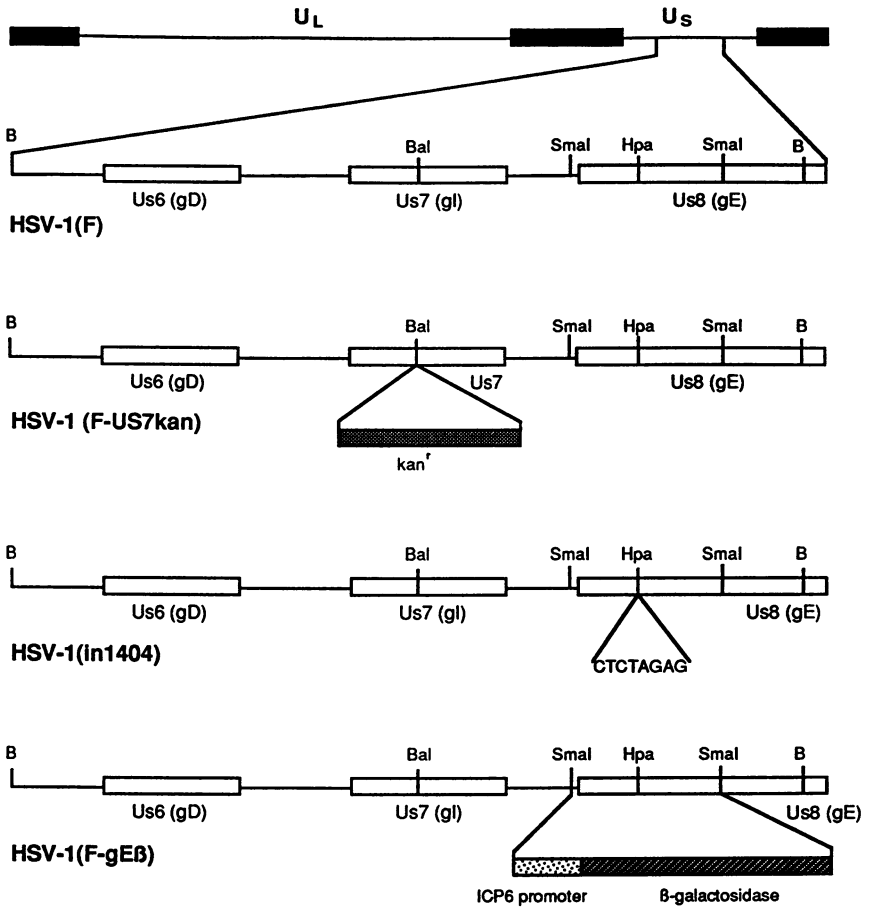

FIG. 1. Schematic representations of DNA sequence rearrangements in HSV-1 gE$^-$ and gI$^-$ mutant viruses. The region of HSV-1 DNA which includes the *Bam*HI J fragment containing the US6 (gD), US7 (gI), and US8 (gE) genes (51) is depicted for the HSV-1 recombinants F-US7kan, *in*1404, and F-gEβ. F-US7kan was derived from F-gDβ after gI coding sequences were disrupted by the insertion of a kanamycin resistance gene cassette into a unique *Bal*I site (43). *in*1404 was derived from HSV-1 strain 17 by insertion of an 8-bp *Xba*I linker into an *Hpa*I site near the 5′ end of the US8 coding sequences (43). In F-gEβ, an ICP6::β-galactosidase cassette (22) replaces 60% of gE coding sequences. B, *Bam*HI; Bal, *Bal*I; Hpa, *Hpa*I; UL, unique long region of HSV genome; US, unique short region.

independent isolates, it is unlikely that mutations other than those in gE or gI contributed substantially to the small-plaque phenotype. There was a small but significant difference in the size of plaques produced on Vero cells by F-gEβ versus those produced by F on Vero cells; however, this difference was not normally observed until the plaques were relatively large (Fig. 4). The plaque phenotype of F-US7kan was partially syncytial on Vero cells, perhaps because F-US7kan was derived from F-gDβ, which produces syncytial plaques on complementing VD60 cells (46). However, we did not observe fusion of human fibroblasts infected with F-US7kan, and thus the syncytial mutation is not likely to contribute to the differences in plaque sizes on fibroblasts. In addition, a wild-type revertant of F-gDβ, wt 61, which expresses gD and gI (62a), produced normal large plaques on fibroblasts, whereas other gI$^-$ mutants derived from F-gDβ produced small plaques (not shown), suggesting that any secondary mutations in F-US7kan which produce a syncytial phenotype on Vero cells did not affect plaque morphology on fibroblasts and confirming a role for gI in the small-plaque phenotype. In other experiments, we detected a similar small-plaque phenotype when F-US7kan, *in*1404, and F-gEβ were plated on monolayers of human fibroblasts which had been transformed with plasmids contain-

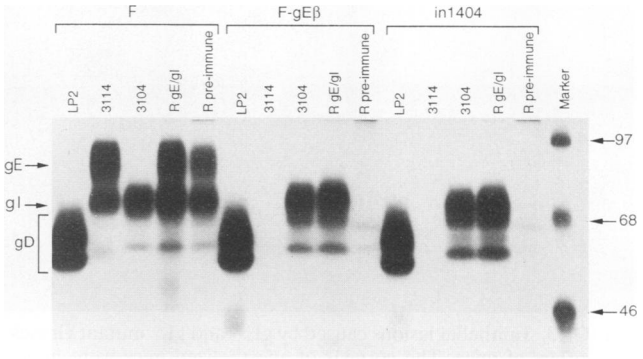

FIG. 2. Expression of gD, gI, and gE in cells infected with gE mutants F-gEβ and *in*1404. Human R970 cells were infected with F, F-gEβ, or *in*1404, and the cells were radiolabelled with [^{35}S]methionine from 5 h until 7 h p.i. Cell extracts were immunoprecipitated with anti-gD MAb LP2, anti-gE MAb 3114, anti-gI MAb 3104, rabbit polyclonal anti-gE/gI serum (R gE/gI), or a rabbit preimmune serum (R pre-immune). Immunoprecipitated proteins were subjected to electrophoresis through 8.5% polyacrylamide gels. The positions of gD, gE, and gI as well as molecular size markers (Marker) of 97, 68, and 46 kDa are indicated.

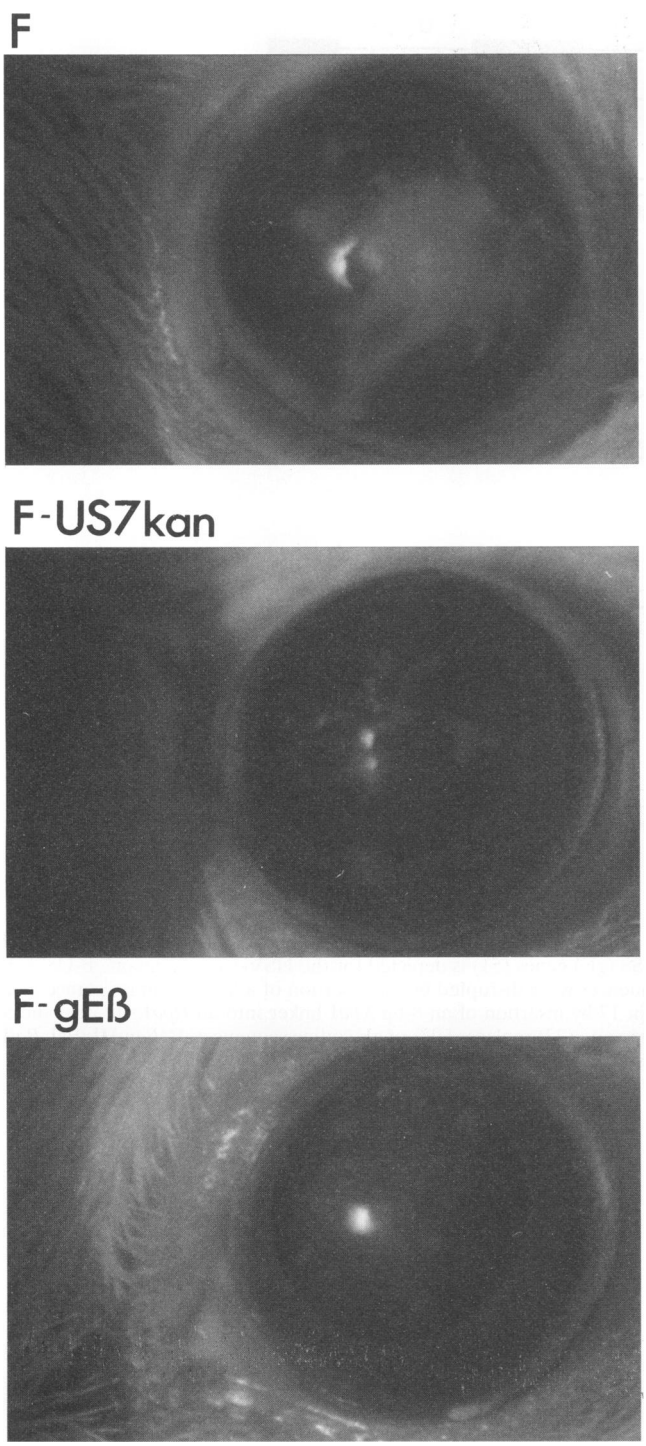

FIG. 3. Epithelial lesions caused by gE$^-$ and gI$^-$ mutant viruses in the cornea of mice. The corneas of anesthetized mice were infected with either wild-type HSV-1 strain F, the gI$^-$ mutant F-US7kan, or the gE$^-$ mutant F-gEβ. The corneas were examined and photographed 2 days after infection by instilling fluorescein and using a slit lamp with a cobalt blue light source.

ing the simian virus 40 large T antigen and with some but not all epithelial cells (not shown). Since both the gE$^-$ and gI$^-$ mutant viruses produced small plaques on cells displaying a flattened, well-spread morphology with extensive cell contacts and there were often no differences between wild-type and mutant viruses with more rounded cells possessing fewer cell junctions, we suspected that these differences were related to cell morphology or intercellular junctions.

TABLE 1. Pathogenicity of HSV-1 gE$^-$ and gI$^-$ mutants in mice infected by the corneal route

HSV strain	Presence of disease			
	Epithelium[a]	Skin[b]	HSK[c]	Encephalitis[d]
F	+++	Severe	++	+
F-US7kan	++	None	None	−
F-gEβ	+	None	None	−

[a] Epithelial lesions were observed 2 days after infection by instilling fluorescein and using a slit lamp. Lesions were graded as mild (+), moderate (++), or severe (+++).

[b] Periocular skin disease was measured 14 days after infection.

[c] Herpes stromal keratitis (HSK) was measured 14 days after infection and was graded as mild (+), moderate (++), severe (+++), or perforated cornea (++++).

[d] Mice began to die as a result of encephalitis by 14 days p.i. and were sacrificed. Mice infected with F-US7kan and F-gEβ showed no signs of encephalitis.

To more accurately quantify the spread of virus infection in monolayers of human fibroblasts, we stained cell monolayers infected with wild-type and mutant viruses, using anti-HSV antibodies and an anti-IgG peroxidase conjugate (Fig. 5). At 36 h p.i., plaques produced by wild-type F contained over 200 stained cells per plaque whereas plaques produced by F-gEβ and F-US7kan contained an average of 56 cells per plaque, and these differences were statistically significant (Student unpaired t test, $P < 0.005$). After 60 h of infection, all cells in dishes infected with wild-type F were stained by immunoperoxidase, whereas defined regions of infection were observed with F-gEβ and F-US7kan. In these experiments, the numbers of plaques produced on fibroblast monolayers by mutant and wild-type viruses were not different, and similar results were obtained when *in*1404 and 17 were compared (results not shown). Therefore, it appears that cell-to-cell spread of wild-type HSV-1 is more efficient than that of the gE$^-$ or gI$^-$ mutant viruses in human fibroblasts.

HSV-1 mutants unable to express gE or gI replicate normally on human fibroblasts but spread poorly from cell to cell. The most likely explanation for reduced plaque sizes by gE$^-$ and gI$^-$ mutant viruses was that certain aspects of virus replication were inhibited in human fibroblasts so that progeny viruses were produced more slowly or less efficiently. To test this hypothesis, a single round of HSV replication was followed by infecting fibroblasts at a multiplicity of infection of 5 PFU per cell and harvesting cells and culture media independently. No differences were detected in the yields of infectious virus either in the media (Fig. 6A) or associated with cells (Fig. 6B) in three independent experiments. Similarly, no differences in yields of infectious viruses were obtained following infection of fibroblasts with wild-type strain 17 and gE mutant *in*1404 (not shown).

Since we observed marked differences in the plaque sizes produced by mutant and wild-type viruses on human fibroblasts, we expected that the yields of infectious viruses produced after multiple rounds of infection (initiated by using 0.001 PFU per cell) would also be different. In this assay, F-gEβ and F-US7kan produced approximately 10- to 20-fold less infectious virus in the cell culture supernatant than was produced by F after 48 and 60 h of infection (Fig. 7A). The amount of cell-associated virus produced in fibroblasts was reduced by 5- and 10-fold in cells infected with the gE$^-$ and

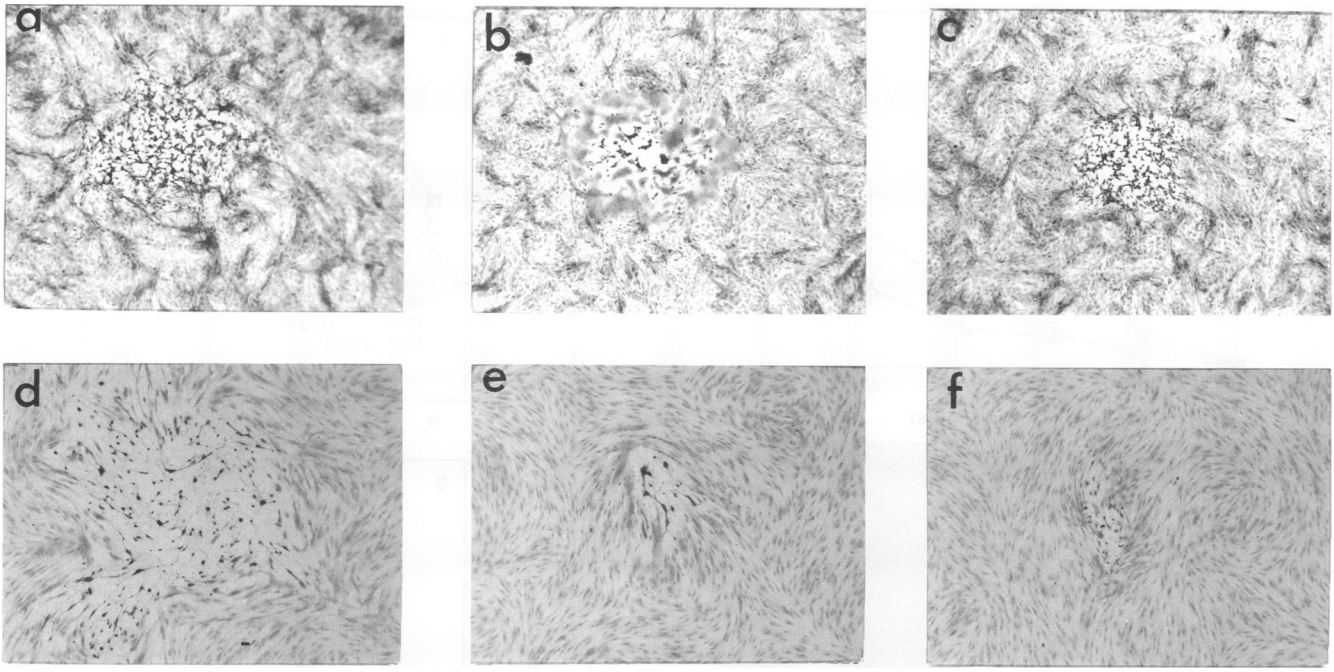

FIG. 4. Plaques produced by F-US7kan and F-gEβ on monolayers of Vero cells and normal human fibroblasts. Monolayers of Vero cells (a to c) or normal human fibroblasts (d to f) were infected with wild-type F (a and d), F-US7kan (b and e), or F-gEβ (c and f) at 0.001 PFU per cell. At 2 h p.i., cell monolayers were washed once with PBS and α-MEM supplemented with 1% FBS, and 0.1% HGG was added for a further 48 h. The cells were fixed and then stained with crystal violet.

gI$^-$ mutants relative to wild-type HSV-1 (Fig. 7B). However, there was a much greater difference (100- to 200-fold) in the yields of infectious virus produced by wild-type versus mutant virus when HGG, a source of neutralizing antibodies, was kept present in the culture medium (Fig. 7C). Moreover, there was little difference in the titers of virus from wild-type HSV-1-infected cells incubated with or without HGG, whereas there were dramatic (20- to 100-fold) differences in yields of infectious virus produced by gE$^-$ and gI$^-$ mutant viruses with and without HGG (compare Fig. 7B and C). Again, similar results were obtained when *in*1404 and wild-type 17 were compared (not shown). These results suggest that a primary mode of spread of wild-type HSV-1 in these cells is from cell to cell, by mechanisms which do not place the virus in contact with neutralizing antibodies. Apparently, gE$^-$ and gI$^-$ mutants function poorly in this form of direct cell-to-cell transmission.

F-US7kan, F-gEβ, and wild-type F enter cells with similar kinetics. To examine the kinetics of virus entry, we used a low-pH inactivation assay previously described by Huang and Wagner (37) and adapted for HSV by Highlander et al. (35). Cells were incubated with virus at 4°C, allowing virus adsorption onto cells; then the cells were moved to 37°C to allow virus penetration into cells and at various times briefly treated with a low-pH citrate buffer to cause unpenetrated viruses to be released or inactivated. Rates of entry were determined by quantifying plaques produced on monolayers incubated for various times, using untreated cells as controls. F and F-gEβ entered fibroblasts with very similar kinetics (Fig. 8); in other experiments, a second gE$^-$ mutant, *in*1404, entered fibroblasts with kinetics identical to those of wild-type strain 17 (not shown). F-US7kan entry reproducibly lagged behind that of F and F-gEβ at early time points (Fig. 8), while at later times, e.g. 60 and 90 min, entry into cells had been completed and the final extents of entry for all three viruses were similar or identical. The slower rate of entry observed with F-US7kan appears not to explain the observed differences in cell-to-cell spread of gI$^-$ and gE$^-$ viruses because the final extent of entry measured at 60 min was similar for all three viruses and because the two gE$^-$ mutants, which also formed small plaques, entered with kinetics similar to those of wild-type strains F and 17.

To further study entry of the mutant viruses into cells, we infected fibroblasts with 5 PFU per cell and quantified an HSV-1 immediate-early protein, ICP6, and an early protein, TK, although only the results for TK are shown. Similar quantities of TK were immunoprecipitated from extracts of cells infected with F-gEβ, F-US7kan, and wild-type F (Fig. 9). Similar results were observed when ICP6 was quantitated and when *in*1404 and wild-type 17 were compared (not shown). Thus, gE$^-$ and gI$^-$ mutant viruses and wild-type HSV-1 caused equal quantities of early viral proteins to be produced, and the lag in F-US7kan penetration (Fig. 8) does not affect the production of immediate-early and early HSV-1 proteins in these cells. In other experiments, fibroblasts were infected with wild-type and mutant viruses at 1 PFU per cell, and again equal quantities of TK were produced by mutant and wild-type viruses (not shown).

The small-plaque phenotype of gE$^-$ and gI$^-$ mutants is not explained by loss of Fc receptor activity. In the plaque assays described above, cell monolayers were incubated with virus for 2 h and then overlaid with medium supplemented with pooled HGG, which contains anti-HSV antibodies, to inhibit formation of satellite plaques. Since gE$^-$ and gI$^-$ viruses are deficient in Fc receptor activity, it was formally possible that the differences in plaque size might be related to an inability to bind human IgG. To test this possibility and to extend these analyses, fibroblasts were infected with HSV-1 and then incubated without antibodies or with a panel of mouse and rabbit

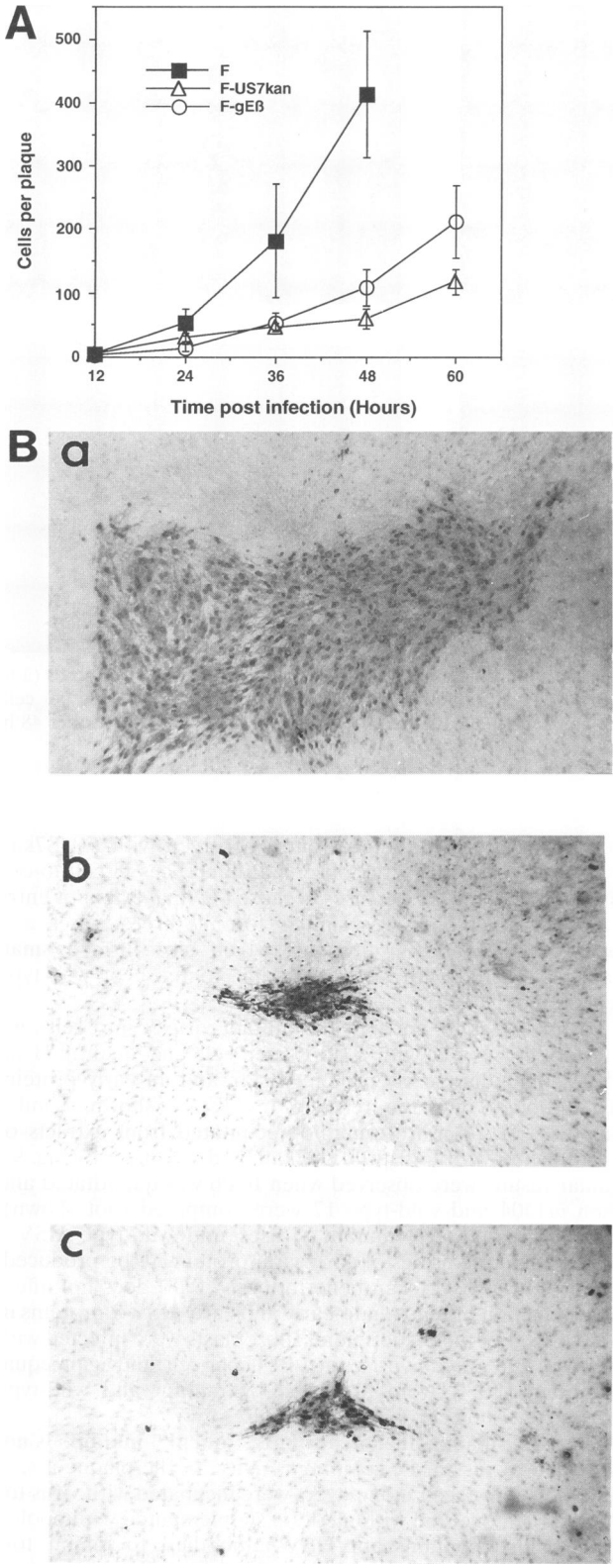

FIG. 5. Immunoperoxidase staining of infected cells in plaques produced by gE$^-$ and gI$^-$ mutant viruses. (A) Monolayers of normal fibroblasts seeded on glass coverslips were infected with 0.001 PFU per cell and maintained in α-MEM supplemented with 1% FBS and 0.1% HGG. At 12-h intervals, the cells were fixed with acetone and stained with rabbit anti-HSV antibodies, peroxidase-conjugated anti-rabbit antibodies, and peroxidase substrate. The numbers of infected cells in 10 representative plaques at each time point were counted and averaged. At 36 h p.i., the number of cells infected by wild-type F is significantly different from the number of cells infected by F-gEβ or F-US7kan (Student unpaired t test, $P < 0.005$). (B) Representative immunoperoxidase-stained plaques produced 48 h p.i. with wild-type F (a), the gI$^-$ mutant F-US7kan (b), or the gE$^-$ mutant F-gEβ (c).

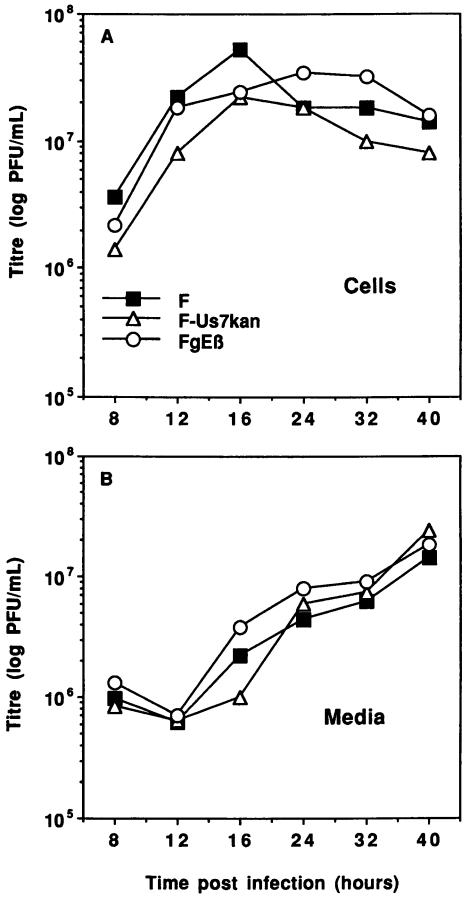

FIG. 6. Production of infectious viruses by HSV-1 gE$^-$ and gI$^-$ mutants infecting human fibroblasts. Normal fibroblasts growing in six-well dishes were infected with F, F-US7kan, or F-gEβ at 5 PFU per cell. At the indicated times after infection, cells (A) and media (B) were collected and frozen at −70°C. Samples were thawed, sonicated, and titered under standard plaque assay conditions on Vero cell monolayers. The results shown are representative of three separate experiments, but individual points shown are the results of a single determination because of the relatively large number of samples.

HSV-specific antibodies, both neutralizing and nonneutralizing. When no antibodies were present, plaques produced by wild-type F were large; in many cases, by 48 h of infection, it was difficult to count the numbers of cells infected, whereas plaques produced by F-gEβ or F-US7kan were much smaller (Fig. 10, no serum). Two strongly neutralizing mouse anti-gD MAbs, LP2 and DL11, reduced the size of plaques produced by both wild-type and mutant viruses in a fashion similar to HGG; however, again there were significant differences between the sizes of plaques produced by the mutant viruses and those produced by wild-type viruses. It has been difficult or impossible to detect binding of mouse IgG to the HSV Fc

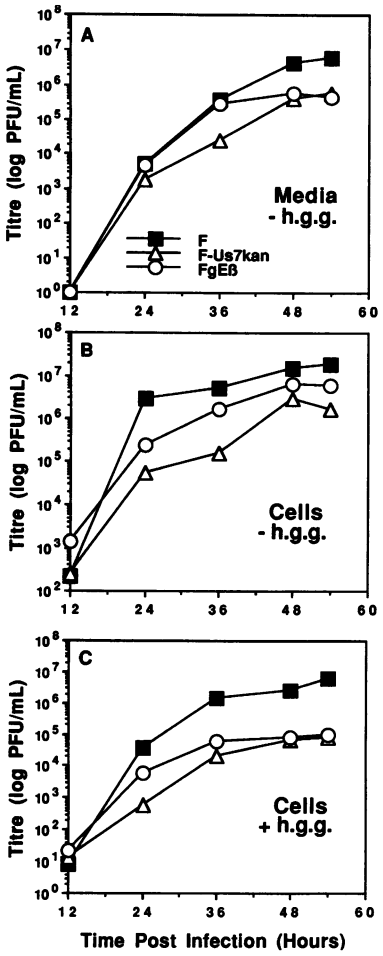

FIG. 7. Spread of gE$^-$ and gI$^-$ mutant viruses through fibroblast monolayers. Normal human fibroblasts growing in 35-mm dishes were infected with F, F-US7kan, or F-gEβ at 0.001 PFU per cell; after 2 h, the monolayers were washed with PBS, and α-MEM–1% FBS (A and B) or α-MEM–1% FBS supplemented with 0.1% HGG (C) was added. At the indicated times, media (A) or cells (B and C) were collected and frozen at −70°C. In panel C, cells were washed twice with medium twice to remove HGG before being collected. Samples were later thawed, sonicated, and titered by plaque assay on Vero cell monolayers. The results shown are representative of three separate experiments, but individual points shown are the results of a single determination because of the relatively large number of samples.

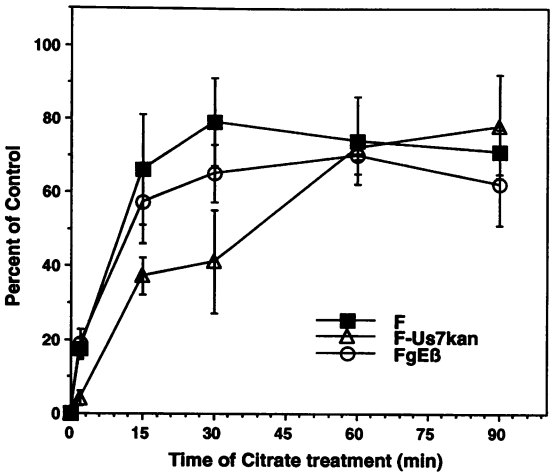

FIG. 8. Kinetics of penetration of gE$^-$ and gI$^-$ mutant viruses into human fibroblasts. Confluent monolayers of human fibroblasts growing in 12-well dishes were incubated with approximately 100 PFU of either F, F-US7kan, or F-gEβ for 2 h at 4°C. Following removal of the viruses, cells were washed three times to remove unbound virus, and the temperature was shifted to 37°C. At various times, cell monolayers in triplicate were treated for 1 min with citrate buffer (pH 3.0) or with PBS. The cell monolayers were then washed three times with PBS and incubated for 2 days in α-MEM–1% FBS supplemented with 0.1% HGG. Cells were fixed and then stained with crystal violet, and plaques were counted. At each time point, the average number of plaques produced on citrate-treated monolayers was divided by the average number of plaques produced on PBS-treated monolayers and expressed as a percentage. Error bars represent standard deviations.

receptor (41, 43); thus, these mouse MAbs would not be expected to alter spread of virus by interacting with the Fc receptor. Other mouse MAbs, e.g., LP11, which is specific for gH, decreased wild-type and mutant HSV-1 plaque sizes to a lesser extent; however, again there were marked differences in the sizes of mutant and wild-type plaques. Rabbit antibodies, which can bind to the HSV Fc receptor, either neutralizing (e.g., rabbit anti-gD serum) or nonneutralizing (e.g. anti-gE/gI), gave similar results. In other experiments, these differences between mutant and wild-type HSV-1 were also observed when fibroblasts were overlaid with methylcellulose (not shown). The results showed clearly that gE$^-$ and gI$^-$ mutants spread poorly through fibroblast monolayers whether antibodies capable of neutralizing HSV or binding to the Fc receptor were present or not; thus, this effect was unrelated to the Fc receptor activity of the gE-gI hetero-oligomer.

DISCUSSION

These experiments were initiated to investigate the protective effects of the HSV IgG Fc receptor in rodent corneal models of HSV pathogenesis. Our premise was that the gE-gI hetero-oligomer acting as an Fc receptor might protect HSV-1 from immune surveillance or cytolysis. Mutants lacking either gE or gI were used because full Fc receptor activity requires both gE and gI (4, 25, 43). Marked differences in the epithelial lesions caused by wild-type and mutant viruses were observed in both rabbits and mice; however, to our surprise, these

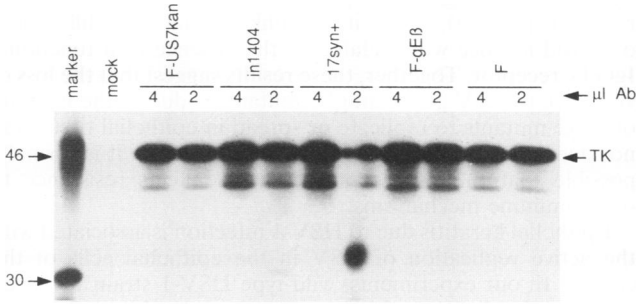

FIG. 9. Expression of HSV-1 TK in fibroblasts infected with gE$^-$ and gI$^-$ mutants. Human fibroblasts were infected with F, F-US7kan, F-gEβ, 17, or *in*1404 at 5 PFU per cell and labelled with [^{35}S]methionine from 5 h until 7 h p.i. Cell extracts were made, and TK was immunoprecipitated with either 2 or 4 µl of rabbit anti-TK serum. Note that in one of the 17syn+ lanes, the anomaly is due to the presence of an air bubble in the gel. Positions of TK and molecular mass markers of 46 and 30 kDa are indicated.

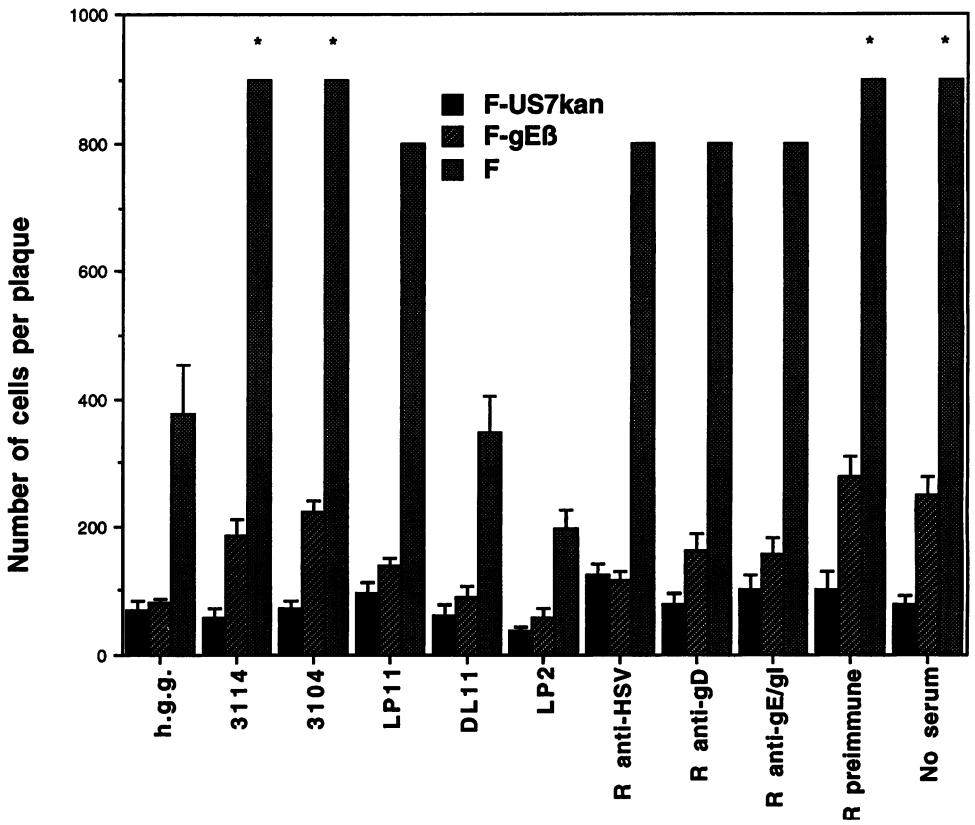

FIG. 10. Effects of murine and rabbit IgG on plaque sizes. Normal human fibroblasts were seeded on glass coverslips and infected with F, F-US7kan, or F-gEβ at 0.001 PFU per cell. At 2 h p.i., cell monolayers were washed with PBS and incubated with α-MEM supplemented with 1% FBS and either 0.1% HGG or one of the murine or rabbit antibodies; mouse ascites fluids and rabbit serum were diluted 1:1,000. The cells were fixed with acetone 48 h p.i. and stained with anti-HSV–immunoperoxidase. The total number of infected cells per plaque was counted for 10 representative plaques and expressed as an average. Error bars represent standard deviations. Bars representing monolayers infected with F and treated with MAb LP11, R anti-HSV serum, R anti-gD serum, or R anti-gE/gI serum represent estimates of approximately 800 cells per plaque, although these were difficult to count. The asterisks over bars representing F-infected monolayers treated with no serum, R preimmune serum, and MAbs 3114 and 3104 refer to instances when HSV-1 F infected all cells in the monolayer (we estimate >1,500 cells per plaque); thus, it was impossible to definitively determine the numbers of cells per plaque.

differences were observed well before IgG appeared in the eye, i.e., 2 or 3 days after inoculation. Detectable levels of anti-HSV IgG or IgA were not observed in the rabbit eye until 5 to 13 days after infection of nonimmunized animals (13, 27). Although we know of no quantitative studies of murine IgG in the eye, murine IgG binds poorly or not at all to the HSV Fc receptor (41, 43); thus, it is unlikely that the differences observed in mice were related to the absence of a functional IgG Fc receptor. Together, these results suggest that the loss of virulence of HSV gE^- and gI^- mutants is due to the inability of these mutants to replicate or spread in epithelial tissue and not due to loss of IgG binding activity, although it is formally possible that the Fc receptor complex affects resistance to other immune mechanisms.

Epithelial keratitis due to HSV-1 infection is associated with the active replication of HSV in the epithelial cells of the cornea. In our experiments, wild-type HSV-1 strain F caused severe epithelial keratitis characterized by large dendritic lesions and extensive stromal disease, and all of the infected mice developed encephalitis. By contrast, gE^- and gI^- mutant viruses produced small punctate epithelial lesions and no stromal disease, and the animals displayed no encephalitis. It appears likely that the restricted stromal disease caused by mutant viruses is an outcome of reduced virus spread in the epithelium so that less virus reaches the stroma. However, there is also evidence that certain virus isolates can cause extensive stromal disease while producing little epithelial disease (14, 63). Stromal disease is thought to be largely due to immunopathological responses to viral antigen and does not occur in T-cell-deficient mice (28, 31, 53), and virus and host factors contribute to progression from epithelium to stroma (14, 29, 30, 54, 63, 64). The relationship between epithelial and stromal disease in the cornea is not clear. For instance, some HSV-1 strains produce large epithelial lesions, establish latent infections in the neurons of the trigeminal ganglia, but fail to reactivate from latency and also fail to induce stromal disease. In contrast, other strains produce very little epithelial disease but cause severe stromal keratitis (27). Until stromal inflammation is better understood, we cannot speculate on the role of the gE-gI hetero-oligomer in this process. We also note that these results are not limited to ocular models of HSV infection because wild-type HSV-1 F produced easily detected lesions in female guinea pig vaginal epithelium, yet no lesions could be detected in F-US7kan-infected animals (5).

To study further the role of the gE-gI complex in HSV infection, replication of the mutant viruses was examined in a number of cultured rodent and human cell types. HSV is normally propagated in transformed monkey or human cells which are often morphologically and metabolically different from the cells normally infected in vivo (epithelial cells,

fibroblasts, keratinocytes, neurons, etc.). Mutants unable to express gE or gI produced small plaques on monolayers of normal human fibroblasts and certain epithelial cells. The interpretation of these results was initially complicated by the fact that our standard plaque assays included human antibodies which can neutralize HSV and prevent production of satellite plaques. However, in the absence of antibodies, wild-type HSV-1 also spread through these monolayers much more efficiently than did gE$^-$ and gI$^-$ mutant viruses. Similar results were obtained when monolayers were overlaid with mouse antibodies, nonneutralizing antibodies, or methylcellulose. In considering the relationship between the observations that gE$^-$ and gI$^-$ mutants produce small plaques in human fibroblast monolayers and small lesions in the eye, we note that Stulting et al. (63) found a correlation between the ability of an HSV-1 strain to replicate in mouse primary fibroblasts and keratinocytes and its ability to induce stromal keratitis.

The small-plaque phenotype and decreased virulence of gE$^-$ and gI$^-$ mutants might have resulted from defects in a number of aspects of virus replication, including adsorption, penetration, production or egress of infectious particles, or cell-to-cell spread of the viruses. Viral mutants and wild-type HSV-1 applied to human fibroblasts, using either 1 or 5 PFU per cell, entered the cells normally and produced equivalent quantities of viral proteins and infectious viruses which were shed normally into the cell culture supernatant. Other studies (not shown) involving electron microscopy indicated that mutant and wild-type virus particles were distributed in an identical fashion in infected cells and reached the cell surface. However, when fibroblast monolayers were infected with gE$^-$ and gI$^-$ mutants under conditions in which only a fraction of the cells were infected and neutralizing antibodies were kept present in the overlay medium, the production of infectious viruses over several rounds of replication was 100- to 200-fold lower than that produced by wild-type virus. Moreover, the yields of gE$^-$ and gI$^-$ mutant viruses were 20- to 100-fold lower when neutralizing antibodies were present than observed in the absence of antibodies; by contrast, neutralizing antibodies had little or no effect on the production of wild-type HSV-1. From these data, we concluded that viruses lacking gE or gI spread poorly from cell to cell across cellular junctions; furthermore, the results support the notion that direct cell-to-cell transmission across cell junctions can be a primary mechanism for spread of HSV-1 in some cell types. In transformed monkey Vero cells, the effect of these mutations was much less evident, although on careful examination, gE$^-$ mutants F-gEβ and *in*1404 produced slightly smaller plaques than their respective wild-type parents did, especially when infected cell monolayers were incubated for additional hours.

One important concern with HSV mutants engineered by transfection of viral DNA is that secondary mutations can arise which may cause unwanted phenotypic changes. This concern is particularly acute when HSV mutants are propagated in cultured cells and tested in animal models. To confirm that the observed phenotype is related to mutations in a particular gene, virus revertants are often characterized or, alternatively, multiple mutants can be characterized. In these studies, we found that two independently isolated gE mutants, F-gEβ and *in*1404, and a gI mutant, F-US7kan, produced smaller epithelial lesions than did their wild-type parents and produced microscopic plaques on fibroblasts. In all subsequent experiments designed to elucidate differences between mutant and wild-type viruses, *in*1404, F-gEβ, and F-US7kan behaved identically or similarly. Furthermore, a number of other independently isolated gI-negative mutants also produced small plaques on human fibroblasts (not shown). Since gE and gI are extensively complexed and dependent on one another for function (43), it appears highly likely that these phenotypes, i.e., inability to spread in vivo and in vitro, are caused by the absence of gE or gI and not by secondary mutations; however, it is formally possible that other mutations were acquired during the construction of these virus mutants. Our observations with these HSV-1 gE$^-$ and gI$^-$ mutants further strengthen the hypothesis that gE and gI function as a complex, whether to bind IgG or to facilitate cell-to-cell spread by an unknown mechanism.

During the course of these studies, a report describing effects of a mutation in the PrV virus gI protein, a homolog of HSV-1 gE, appeared; in this study, it was found that the mutant virus was deficient in direct cell-to-cell transmission (66). The mutant virus replicated normally in cells but was less able to form syncytia and spread primarily by adsorption of released virus to uninfected cells, whereas wild-type virus spread primarily by direct cell-to-cell transfer. Another PrV gI$^-$ mutant virus was restricted in its ability to spread in the rat nervous system (12). Together, these results for PrV, previous results for HSV-1 gE mutant viruses (52, 57, 59), and the observations described here involving both gE and gI strongly support a general role for the gE-gI complex in cell-to-cell spread in a variety of human and rodent tissues.

The spread of HSV through tissue is likely to be a complicated series of events mediated by the large repertoire of viral membrane glycoproteins. It is quite apparent that HSV can spread from an infected cell to an uninfected neighbor by either of two distinct pathways. Viruses can detach from infected cells and then adsorb onto and enter uninfected cells, or virus particles can be transferred across junctions between cells so that the viruses do not come in contact with neutralizing antibodies. It appears that HSV is infrequently released from infected cells because electron micrographs of HSV-infected cells frequently show large numbers of virus particles encrusting the cell surface and the quantities of infectious HSV released by cultured cells are often low. HSV-1 gE and gI appear to be unimportant in entry of extracellular virus into cells, but both proteins play important roles in direct cell-to-cell spread across cell junctions. This latter mode of virus spread apparently leads to infection of neighboring cells through a set of receptors different from those used for entry of exogenous virus, and it appears that the gE-gI heterooligomer engages these receptors. Another subset of herpesvirus glycoproteins are apparently required for entry of extracellular virus but not for cell-to-cell transmission. For example, PrV mutants unable to express gp50, a homolog of HSV gD, were found to be severely compromised in their ability to enter cells but after entering a host cell can spread from cell to cell and produce plaques without gp50 (58). Similarly, we have found that HSV-1 gD-negative mutants can produce small plaques on human fibroblasts when the infections are initiated with complemented virus (14a), supporting the notion that gD is not absolutely required for cell-to-cell spread. In these experiments, F-gDβ, which is unable to express gD and gI, could not produce plaques on fibroblasts, again confirming the requirement for gE and gI in this cell-to-cell spread.

The propensity to spread directly from cell-to-cell across cell junctions appears to be very much dependent on the cell type. Cell contacts are frequently formed between fibroblasts and epithelial cells, and both of these cell types form diverse junctional complexes, including adherens junctions, desmosomes, and tight junctions. However, other cells, e.g., Vero and MDBK cells, also possess cell junctions and can spread in monolayers of these cells (in the presence of neutralizing HSV antibodies), and yet the phenotypes of gE$^-$ and gI$^-$ mutants

were much less obvious with these cells. A second type of direct cell-to-cell transmission of HSV involves fusion of infected cells with uninfected cells which can occur with wild-type HSV but is more conspicuous with syncytial mutants. Again, HSV-induced cell fusion is very much dependent on cell type and appears to involve mechanisms similar to those involved in cell-to-cell transmission of virus whereby cells do not fuse. To date, few efforts have been made to characterize interactions between HSV and components of cellular junctions; however, these studies have been initiated and should provide valuable information as to how herpesviruses spread in tissue.

At the present time, we have only hints as to how gE and gI might function to mediate cell-to-cell spread of HSV across cellular junctions. HSV mutants lacking gE or gI can spread in the presence of neutralizing antibodies and in rodent eyes, although much less efficiently than wild-type viruses, suggesting that alternate pathways for cell-to-cell spread exist. An important caveat here is that a double mutant lacking both gE and gI was not tested, and thus it is possible that such a mutant would display a more absolute phenotype. However, we suspect that the gE-gI hetero-oligomer functions to facilitate cell-to-cell transmission of HSV by interacting with cellular receptor molecules localized to cell junctions. In this regard, it is interesting to note that HSV gE-gI binds only weakly to the Fc domain of IgG, and it has been difficult or impossible to demonstrate IgG binding to the gE-gI homologs in PrV (67) and varicella-zoster virus (47). Therefore, it is tempting to speculate that the HSV gE-gI heterooligomer functions not by binding to IgG but by binding to other cell surface proteins which are members of the IgG supergene family. It is particularly interesting that the gE polypeptide contains a 14-amino-acid region which is homologous with other cell adhesion proteins known to bind sulfated glycoconjugates, e.g., thrombospondin (36).

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