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Glycoproteins E and I Facilitate Neuron-to-Neuron Spread of Herpes Simplex Virus

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Two herpes simplex virus (HSV) glycoproteins E and I (gE and gI) form a heterooligomer which acts as an Fc receptor and also facilitates cell-to-cell spread of virus in epithelial tissues and between certain cultured cells. By contrast, gE-gI is not required for infection of cells by extracellular virus. HSV glycoproteins gD and gJ are encoded by neighboring genes, and gD is required for both virus entry into cells and cell-to-cell spread, whereas gJ has not been shown to influence these processes. Since HSV infects neurons and apparently spreads across synaptic junctions, it was of interest to determine whether gD, gE, gI, and gJ are also important for interneuronal transfer of virus. We tested the roles of these glycoproteins in neuron-to-neuron transmission of HSV type 1 (HSV-1) by injecting mutant viruses unable to express these glycoproteins into the vitreous body of the rat eye. The spread of virus infection was measured in neuron-rich layers of the retina and in the major retinorecipient areas of the brain. Wild-type HSV-1 and a gJ$^-$ mutant spread rapidly between synaptically linked retinal neurons and efficiently infected major retinorecipient areas of the brain. gD mutants, derived from complementing cells, infected only a few neurons and did not spread in the retina or brain. Mutants unable to express gE or gI were markedly restricted in their ability to spread within the retina, produced 10-fold-less virus in the retina, and spread inefficiently to the brain. Furthermore, when compared with wild-type HSV-1, gE$^-$ and gI$^-$ mutants spread inefficiently from cell to cell in cultures of neurons derived from rat trigeminal ganglia. Together, our results suggest that the gE-gI heterooligomer is required for efficient neuron-to-neuron transmission through synaptically linked neuronal pathways.

Herpes simplex viruses (HSV) have developed a sophisticated relationship with the human nervous system. Following primary infections of mucosal and submucosal tissues, HSV infects sensory neurons and is transported in axons (11, 23, 31) by microtubule-associated fast axonal transport (30, 46) to sensory ganglia. The virus may transiently replicate and spread to other neurons and glial cells, although, in time, a latent infection is established and there is no virus replication (51). Later, under appropriate conditions, HSV may reactivate from latently infected neurons, and newly replicated virus is transported back to the periphery, producing secondary mucosal infections. Although virus may spread past the sensory ganglia to the central nervous system (CNS), damage to either the peripheral or central nervous system in humans is rare.

The spread of HSV and other alphaherpesviruses through the CNSs of experimental animals follows synaptically linked pathways, suggesting a mechanism of direct transneuronal transport across the synapse (8, 32, 41, 42, 53, 54). Supporting this hypothesis, virus particles in the synaptic cleft have been observed (8, 46). Additionally, the rate of virus spread in the nervous system suggests direct neuron-to-neuron transfer of virus, rather than spread via infected glial cells (39, 53). The spread of HSV between cells, either across cell junctions, synapses, or other structures, is apparently a complex process involving a subset of the viral envelope glycoproteins that are also necessary for entry of virus into cells.

HSV type 1 (HSV-1) encodes at least 11 glycoproteins, and 5 of these glycoproteins, gB, gD, gH, gK, and gL, are essential for productive infections in cultured cells and entry of extracellular virus particles into cells (7, 19, 24, 33, 49). The remaining glycoproteins, gC, gE, gI, gG, gJ, and gM, are not essential for virus infection and replication in cultured cells (2, 3, 35–37, 40, 50, 56). Since it seems unlikely that HSV would conserve nonessential genes, it is probable that these glycoproteins play important roles in vivo in infection of diverse cell types, movement of virus through tissues, or protection from the host immune responses. gE and gI form a complex which acts as a receptor for the Fc domain of immunoglobulin G (IgG) (5, 21, 26). The gE-gI Fc receptor may protect HSV-infected cells from complement-mediated immune lysis by causing IgG aggregation or by reducing the ability of complement components to bind to virus- or cell-associated IgG (1, 16, 20, 21). However, protective effects of this type have not been demonstrated in vivo in an animal model, and there is mounting evidence that the gE-gI heterooligomer is important to facilitate virus spread in vivo, a property that is apparently unrelated to the IgG Fc receptor activity (for a recent review, see reference 58).

HSV-1 mutants lacking either gE or gI are inhibited in their capacity to spread between certain types of cultured cells by the direct cell contact route and the mutants spread poorly in epithelial tissues (4, 13). However, gE$^-$ and gI$^-$ mutants do not display defects in entry of extracellular virus particles into the same cells. Thus, cell-to-cell spread of viruses, apparently across cell junctions, has features that are distinct from entry of extracellular virus. There is also some evidence that the gE-gI heterooligomer is important for cell-to-cell transfer in the nervous system. HSV-1 mutants lacking gE exhibited decreased neurovirulence following intracerebral infections (40), and spread into the nervous system was reduced after infection at the periphery (4, 47). Pseudorabies virus (PrV), another mem-

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TABLE 1. HSV-1 mutants unable to express glycoprotein D, E, I, or J and a wild-type recombinant derived from F-gEβ

Virus	Phenotype and genotype	Reference
F	gD^+ gE^+ gI^+ gJ^+; wild-type HSV-1	17
F-US7kan	gD^+ gE^+ gI^- gJ^+; kanamycin resistance gene inserted into the US7 (gI) gene	26
F-gEβ	gD^+ gE^- gI^+ gJ^+; β-galactosidase gene replaces the US8 (gE) coding sequences	13
F-US5β	gD^+ gE^+ gI^+ gJ^-; insertion of the β-galactosidase gene in the US5 gene (gJ)	59
F-US6kan	gD^- gE^+ gI^+ gJ^+; kanamycin resistance gene inserted into the US6 gene (gD)	27
F-gDβ	gD^- gE^+ gI^- gJ^+; β-galactosidase replaces all gD coding sequences and part of the gI gene	27
wt61	gD^+ gE^+ gI^+ gJ^+; derived from F-gDβ by rescue with plasmid containing wild-type gD and gI genes	52

ber of the *Alphaherpesvirinae* subfamily, similarly depends on gE and gI for cell-to-cell spread in cultured cells and experimental animals (9, 57, 60).

Many previous studies on the spread of HSV and PrV gE^- and gI^- mutants into the nervous system have involved infection protocols in which experimental animals were infected at the periphery. In these investigations, the mutant viruses replicated poorly or spread poorly at the site of primary infection and less virus was produced. Therefore, it was frequently difficult to determine whether the reduced levels of virus produced less infection in the nervous system or whether the gE-gI heterooligomer was required for transmission of virus into the nervous system and transneuronal spread. In this study, we characterized the roles of gE and gI and two other glycoproteins, gD and gJ, in neuron-to-neuron spread of HSV-1. Mutants unable to express gD, gE, gI, or gJ were injected into the vitreous body of the rat eye, and the spread of virus in the neuron-rich tissue of the retina and retinorecipient regions of the brain was characterized. Neuron-to-neuron spread of the viruses was also studied by using cultured rat trigeminal ganglion neurons. The results support the hypothesis that gE and gI promote efficient transneuronal transport of HSV.

MATERIALS AND METHODS

Cells and viruses. Vero (African green monkey kidney) cells were passaged in α-minimal essential medium (α-MEM) containing 5% fetal bovine serum (FBS). VD60 cells (33) were passaged in Dulbecco's MEM lacking histidine supplemented with 5 mM histidinol and 5% FBS. Wild-type HSV-1 strain F (17), the gE deletion mutant F-gEβ (13), the gI^- mutant F-US7kan (26), the gJ^- mutant F-US5β (59), and wt61 (52), were all propagated and titered on Vero cells. The gD^- HSV-1 mutant F-US6kan (27) and the gD^- gI^- mutant F-gDβ (33) were propagated on VD60 cells. For a description of mutant viruses, see Table 1.

Rat neuronal cultures. Trigeminal ganglia from 5- to 7-day-old Wistar rat pups were collected aseptically in Leibovitz's L-15 medium. Pooled ganglia were treated with 0.1% trypsin for 45 min, α-MEM containing 10% FBS was added, and the resulting mixture was repeatedly mechanically triturated through a Pasteur pipette. Connective tissues were allowed to settle, and the supernatant containing dissociated neurons was collected. Neurons were spun down at 300 × *g* for 5 min, resuspended in α-MEM supplemented with 50 ng of 2.5S nerve growth factor (MEMN) (Gibco BRL, Burlington, Ontario, Canada)–10% FBS, and plated on glass coverslips or tissue culture dishes previously treated with 0.1 mg of poly-D-lysine (Boehringer Mannheim) per ml and 10 μg of laminin (Boehringer Mannheim) per ml. The nonneuronal cell population was eliminated by repeated treatments with 10 μM cytosine arabinoside (Sigma) at 2-day intervals.

Ocular HSV infections. Six-week-old female Wistar rats (two animals per virus) were anesthetized with chloral hydrate and injected with HSV-1 F or HSV-1 mutants (5×10^6 PFU) into the vitreous body of one eye, using a 10-μl Hamilton syringe. At the indicated times, animals were overdosed with chloral hydrate and perfused through the heart with normal saline and then with 4% paraformaldehyde. The brain and injected eye were removed, postfixed overnight in paraformaldehyde, and then equilibrated in 15 or 30% sucrose, respectively. The brain was frozen in 2-methylbutanol cooled to −60°C, and 12-μm-thick serial sections were taken through the coronal plane of the retinorecipient regions. Floating sections were incubated overnight at 4°C with rabbit anti-HSV-1 antibodies (Dako, Dimension Laboratories, Mississauga, Ontario, Canada) diluted 1:100 in phosphate-buffered saline (PBS). Sections were washed three times in PBS for 5 min each and incubated with a 1:100 dilution of fluorescein-conjugated goat anti-rabbit IgG (Terochem Labs, Markham, Ontario, Canada) for 4 h at room temperature, washed three times in PBS for 5 min each, and then mounted on slides with Vectashield mounting medium (Vector Laboratories, Burlingame, Calif.). The cornea and lens were removed from the eye cup, the vitreous fluid was removed with a gentle PBS wash, and the retina was imbedded in OCT compound (Tissue-Tek [Miles Inc., Elkart, Ind.]) and 12-μm-thick serial cross-sections were taken and collected onto gelatin-coated glass slides. Sections were immunostained as described above. Immunofluorescence was detected with a Reichert microscope, and photographs were taken with a Konica camera, Reichert Photostar optic system, and Kodak T-max 400 black-and-white film. Control sections from animals not infected with HSV-1 or processed without primary antibodies or with nonimmune rabbit serum showed no specific immunofluorescence. Immunoreactive cells were observed only in regions of the brain contralateral to the injected eye.

Production of infectious HSV in the retina. Replication of HSV mutants in the eye was tested by anesthetizing rats with chloral hydrate and injecting wild-type F or mutant viruses (5×10^6 PFU) into the vitreous body of both eyes. After 1, 2, or 5 days, rats were overdosed with chloral hydrate and the eyes were removed. The cornea and lens were removed from the eye cup, and the retina was washed with PBS and then frozen at −70°C. Retinas were thawed, suspended in 1-ml portions of α-MEM containing 1% FBS, and homogenized with a small (2-ml) Dounce homogenizer. Homogenates were then titered on Vero cell monolayers.

Production of infectious HSV in neuronal cultures. Cultures of dissociated rat neurons were infected with wild-type HSV-1 (F) or HSV-1 mutants at 1 or 10 PFU/cell (the titers given here were derived with Vero cells; under these conditions, approximately 1 in 10 to 1 in 20 neurons were infected when 1 PFU/cell was used) in α-MEM containing 1% FBS for 2 h. Inoculum was removed, and fresh MEMN–1% FBS was added. For experiments designed to follow virus spread through the cultures, MEMN–1% FBS supplemented with 0.1% human gamma globulin, a source of anti-HSV neutralizing antibodies, was added to the cultures after infection. At appropriate times, cells and media were collected and frozen at −70°C. Samples were thawed and sonicated, and HSV-1 was titered on Vero cells.

Immunofluorescence of neuronal cultures. Neurons were infected with wild-type HSV-1 or HSV-1 mutants at 1 PFU/cell in α-MEM containing 1% FBS for 2 h, and as noted above, these conditions of infection caused only approximately 1 in 10 or 1 in 20 of the neurons to be infected. The virus was removed, and cells were washed and then incubated in MEMN–1% FBS supplemented with 0.1% human gamma globulin, a source of anti-HSV neutralizing antibodies. After 48 h, the medium was removed, and the cells were washed gently with PBS, fixed in 4% paraformaldehyde for 10 min, washed extensively in PBS, and then permeabilized with 0.2% Triton X-100 for 5 min each. Cells were washed with PBS and then incubated in PBS containing 1% normal goat serum, 1% normal horse serum, and 0.2% Tween 20 (GHTPBS) for 30 min. The cells were then incubated simultaneously with mouse anti-160-kDa neurofilament antibodies (Boehringer Mannheim) diluted 1:50 in GHTPBS and rabbit anti-HSV-1 antibodies (Dako) diluted 1:5,000 for 90 min at 37°C. The cells were washed three times with GHTPBS for 5 min each and then incubated with fluorescein-conjugated goat anti-rabbit IgG antibodies and Texas red-conjugated horse anti-mouse IgG antibodies (1:100 diluted; Vector Laboratories) for 90 min at 37°C. Cells were washed three times with GHTPBS for 5 min each and then mounted on microscope slides with Vectashield mounting medium.

RESULTS

HSV gE^-, gI^-, and gD^- mutants spread inefficiently or not at all in the rat retina. Since gE and gI are important for efficient spread of HSV from cell-to-cell and gD is required for virus entry and spread in epithelial cells and fibroblasts, it was of interest to determine whether these glycoproteins were also important for neuron-to-neuron transmission of virus. Initially, we chose to study the spread of HSV-1 mutants after injection into the eye, where virus infection can spread through the neuron-rich layers of the retina and to the retinorecipient regions of the brain.

In all vertebrates, the retina is an inverted structure in which the photoreceptors are located in the outer layers of the eye (most distant from impinging light entering through the lens). Thus, light must pass through most of the retina before it can be detected by photoreceptors, namely, the rods and cones that are present in the bacillary, outer nuclear and outer plexiform

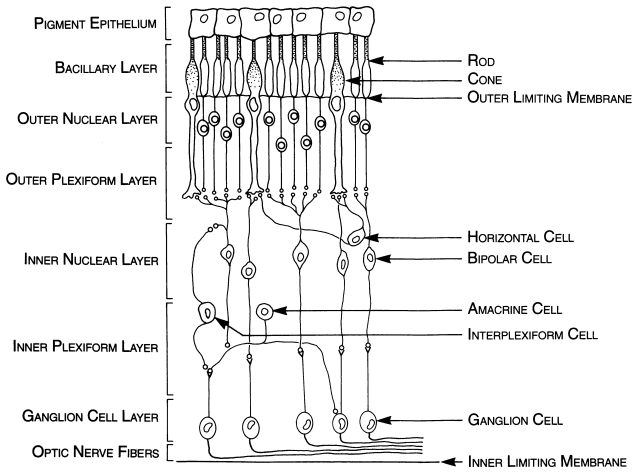

FIG. 1. Cellular organization of the vertebrate retina. The vertebrate retina is composed of eight parallel layers of cells. Light (from the bottom of this figure) impinges on the retina and traverses most of these layers, stimulating photoreceptors (near the top of this figure). The outer layer of the retina, the pigment epithelium, supports and insulates the photoreceptors. The photoreceptors (rods and cones) synapse with bipolar cells located in the inner nuclear layer. Bipolar cells in turn synapse with ganglion cells, located in the ganglion cell layer, which give rise to axons forming the optic nerve. Signals from the photoreceptors in this central pathway are modulated by several different associated neurons which constitute the lateral pathway. Horizontal cells, located in the inner nuclear layer, have synaptic connections with the photoreceptors. Other associated neurons modulating the signal are amacrine cells, located in the inner nuclear and ganglion cell layers, and form synaptic connections with ganglion cells, bipolar cells, and other associated neurons including interplexiform cells. In our experiments, HSV was injected into the vitreous body of the eye, the region adjacent to the inner limiting membrane (at the bottom of the figure).

layers (Fig. 1). Visual signals are conveyed from the photoreceptors through connections linking three distinct types of neurons, making up the central visual pathway of the retina. Hypopolarization of the photoreceptors leads to activation of bipolar cells present in the inner nuclear and inner plexiform layers of the retina. Bipolar cells are in turn connected to ganglion cells, the third type of neuron in the central pathway. Axons from ganglion cells give rise to optic nerve fibers which synapse with neurons located in the visual centers of the brain. Signals conveyed in the central pathway (vertically) from photoreceptors to bipolar cells to ganglion cells are modulated by other neurons, namely, horizontal cells, amacrine cells, and interplexiform cells, which synapse with neurons of the vertical pathway and constitute the lateral signal pathway (Fig. 1). The retina, although very thin, is an intricate system of neuronal connections in which neurons make up a large fraction of the cells. Therefore, the retina provides an excellent model in which to study transneuronal transmission of HSV.

Wild-type HSV-1 and mutant HSV-1 unable to express gD, gE, gI, or gJ (for description of mutant viruses, see Table 1) were injected into the vitreous body of the rat eye, and the spread of virus in the retina was assessed after 2 and 5 days by immunofluorescence. In retinas from animals infected with wild-type HSV-1, distinct foci of infection which extended from the ganglion cell layer to the outer nuclear layer were evident after 2 days. This result suggested that virus had infected ganglion cells and then spread through the central visual pathway connecting the ganglion cells to the photoreceptors (Fig. 2 and 3). Foci of infection were observed 2 days after infection with wild-type HSV-1 and with mutants unable to express gE (F-gEβ), gI (F-US7kan), or gJ (F-US5β), and the numbers of foci produced by the mutants did not appear to differ from those produced by the wild type, although no careful statistical analysis was performed. These observations suggest that the primary infection of retinal ganglion cells was not affected by the loss of gE, gI, or gJ. Confirming this, gE^-, gI^-, or gJ^- HSV-1 mutants produced as much infectious virus after 1 or 2 days as was produced by wild-type HSV-1 (Fig. 4). Therefore, gE^-, gI^-, and gJ^- mutants were able to initiate infection and replicate in retinal neurons as efficiently as wild-type HSV-1. No immunostaining was observed when sections were incubated with rabbit nonimmune sera, and thus, the immunostaining was not altered by HSV Fc receptor activity.

When retinas were infected with the gD^- mutant (F-US6kan) or the gD^- gI^- mutant (F-gDβ), infected foci were observed and the number of these foci appeared to be similar to those produced by wild-type HSV-1 (Fig. 3). However, with both the gD^- mutants, the spread of virus infection to the outer layers of the retina containing the photoreceptors was reduced. Infection of neurons other than the ganglion cells was observed with gD^- mutants but was more restricted than with wild-type HSV-1 and could be related to initial infection of neurons other than ganglion cells or to a limited amount of virus spread in the absence of gD. Since gD is essential for entry of HSV into all the cultured cells tested to date, infections with these two gD^- viruses involved virus stocks produced on complementing VD60 cells, which express gD (33). A recombinant virus, wt61, which was derived from F-gDβ by rescuing the gD and gI coding sequences, also produced equal numbers of foci of infection and replicated as well as wild-type F (Fig. 3 and 4). Therefore, gD^- viruses, when supplied with gD in *trans*, were able to initiate infections of retina neurons, but in the absence of gD, the viruses were severely restricted in their ability to spread between neurons.

After 5 days, retinas infected with the gE^- mutant (F-gEβ) or the gI^- mutant (F-US7kan) displayed much less HSV-specific staining than retinas infected with wild-type HSV-1 (Fig. 2). The wild-type HSV-1 (F) spread throughout the various neuronal cell layers of the retina, infecting the majority of the retinal neurons. By contrast, the infection with F-US7kan or F-gEβ was markedly restricted in this spread. Lateral spread of these two mutants within the retina appeared reduced, so that columns of neurons in the central visual pathway were infected, but there was not broad infection throughout the retina, as was observed with wild-type virus. In addition, the yields of infectious virus produced by the gE^- and gI^- mutants after 5 days were 10-fold lower than that produced by wild-type HSV-1, yet the yields of virus produced after 1 and 2 days were not different (Fig. 4). There was not a substantial increase in the amount of infectious wild-type HSV-1 produced between 2 and 5 days in the retina, even though higher levels of viral antigens were observed by immunofluorescence after 5 days. These results may be related to inefficient production of infectious HSV-1 in retinal neurons. HSV-1 replicates poorly in rodent cells, and this effect may be more pronounced in retinal neurons. However, it is clear from these results that the gE^- and gI^- viruses replicate less efficiently in the retina than wild-type HSV-1, and this result appears to be related to decreased cell-to-cell spread and not to an inability to produce an initial infection of the retina.

In contrast to the gE^- and gI^- mutants, the gD^- mutant (F-US6kan) and the gD^- gI^- mutant (F-gDβ) failed to spread in a detectable fashion from the initial point of infection. After 5 days, expression of viral antigens was difficult to detect and it appeared that these two mutant viruses were largely cleared (Fig. 3). We did not attempt to detect infectious viruses in the retina 5 days after infection with F-US5kan or F-gDβ, since these viruses, lacking gD, would not enter and infect most cultured cells (33), and because there is persuasive evidence

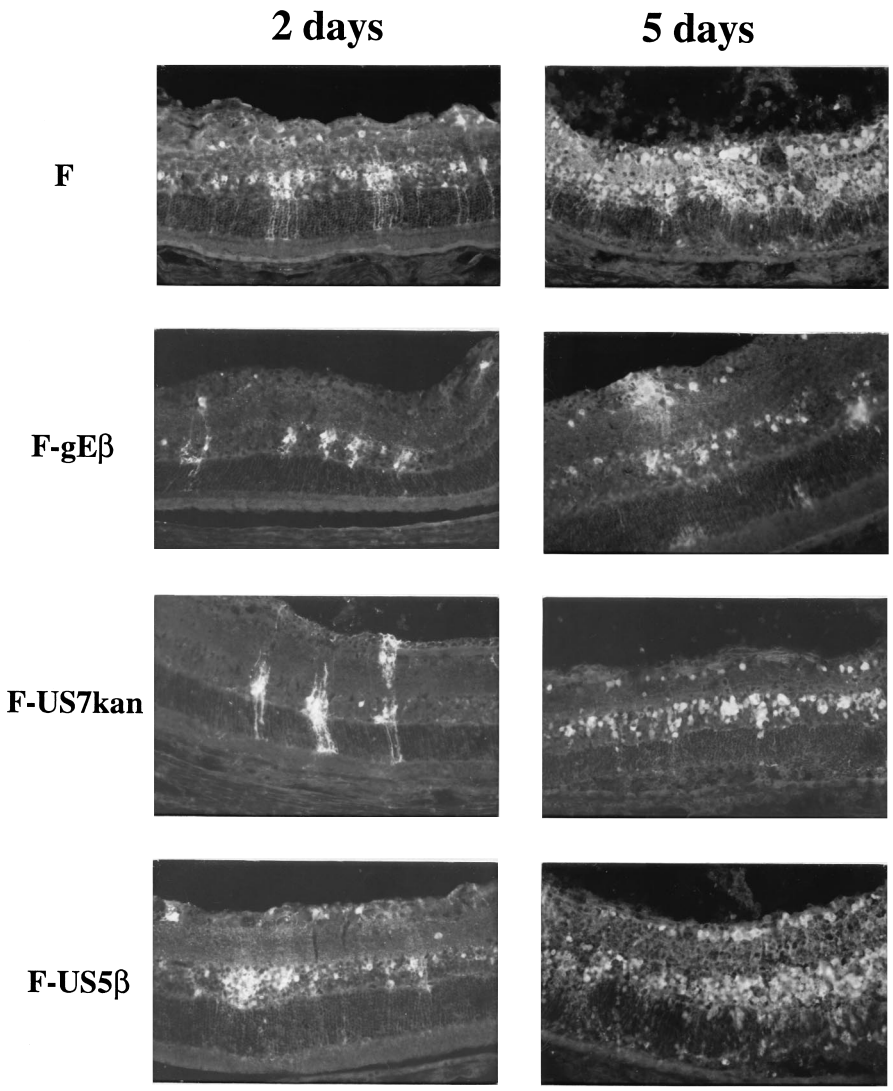

FIG. 2. Infections of the rat retina by gE$^-$, gI$^-$, and gJ$^-$ mutant viruses. The wild-type HSV-1 strain F, the gE$^-$ mutant (F-gEβ), the gI$^-$ mutant (F-US7kan), or the gJ$^-$ mutant (F-US5β) was injected into the vitreous body of one eye of each anesthetized rat, using 5×10^6 PFU of each virus. Two or five days after infection, anesthetized animals were perfused with 4% paraformaldehyde, and then the retinas were postfixed overnight in 4% paraformaldehyde and equilibrated in 30% sucrose. Retinas were imbedded in OCT compound and sectioned, and sections mounted on gelatin-coated glass slides were stained with rabbit anti-HSV antibodies and then with FITC-conjugated anti-rabbit antibodies. No detectable staining was observed with rabbit nonimmune serum. Sections were photographed with a Reichert fluorescence microscope and camera and Kodak T-max 400 film.

that the viruses were not being transmitted from neuron to neuron in the retina. wt61, which was rescued from F-gDβ, spread throughout the retina after 5 days and produced levels of infectious virus similar to those observed with wild-type F at both early and late times (Fig. 3 and 4). The gJ$^-$ mutant, F-US5β, also behaved like wild-type HSV-1 (F). Thus, gD is essential for the spread of HSV-1 in the retina, but gJ is not.

Spread of HSV-1 mutants from retinal ganglion cells to the major visual centers in the brain. Axons of retinal ganglion cells project to six regions in the brain (for reviews, see references 43 and 48). Following an intravitreal inoculation of the rat eye and infection of the retina with HSV, several of these retinorecipient nuclei become infected. This is thought to involve anterograde intra-axonal transport of virus from retinal ganglion cells whose axons project to recipient neurons in the CNS (42). We followed the spread of the HSV-1 mutants from retinal ganglion cells to three retinorecipient nuclei in the brain: (i) the suprachiasmatic nuclei (SCN), located in the hypothalamus, which is involved in controlling circadian rhythms; (ii) the lateral geniculate nucleus (LGN), subdivided into dorsal and ventral lateral nuclei (dLGN and vLGN, respectively), which relays information to the visual cortex and to several subcortical areas; and (iii) the superior colliculus (SC), also known as the optic tectum, which facilitates shifts in gaze (for a review, see reference 43) (Fig. 5). Two or five days after retinal infection, animals were anesthetized and then perfused with 4% paraformaldehyde, and the brains were removed and prepared for cryosectioning. Serial sections through the coronal plane were taken from the regions of the brain corresponding to the SCN, SC, and LGN (Fig. 5). Sections were immunostained with anti-HSV rabbit polyclonal antibodies and then with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit antibodies, and infected neurons were visualized by immunofluorescence.

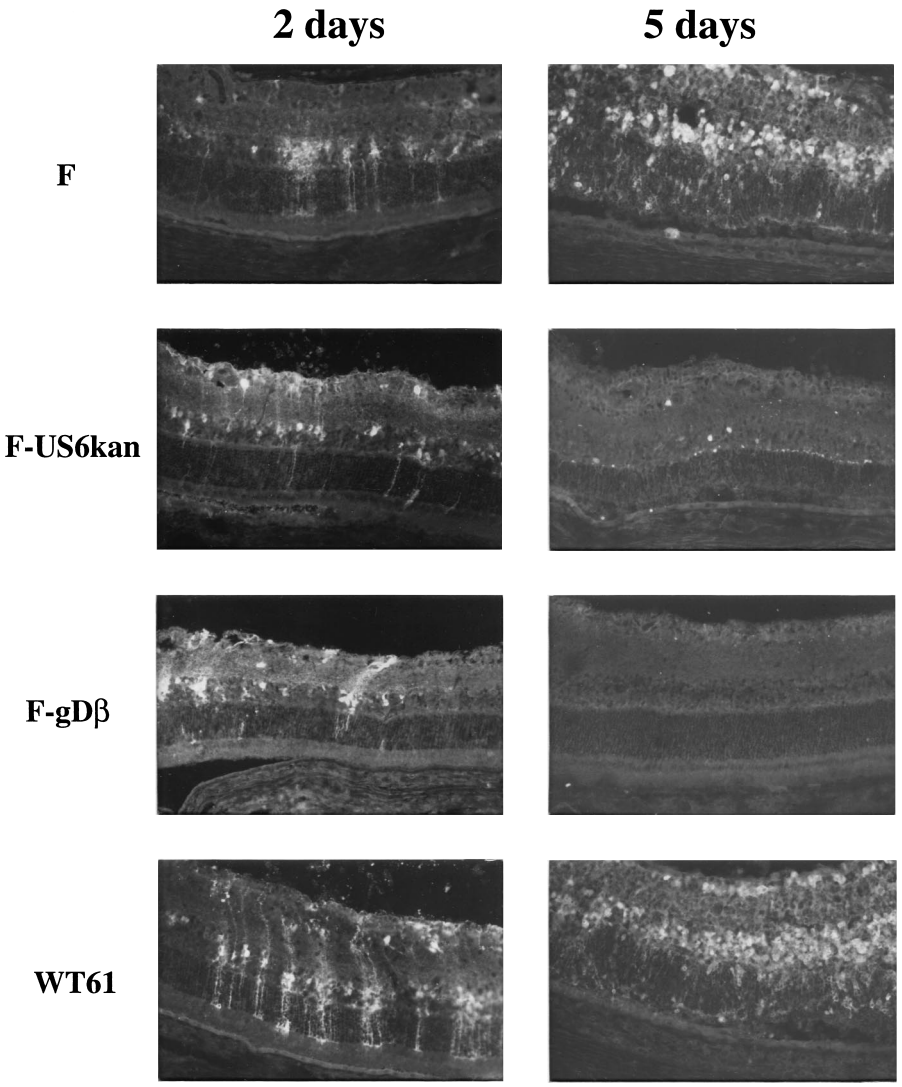

FIG. 3. Infections of the rat retina by gD$^-$ mutant viruses. The wild-type HSV-1 strain F, the gD$^-$ mutant (F-US6kan), the gD$^-$ gI$^-$ mutant (F-gDβ), or wt61 was inoculated into the vitreous body of one eye of each anesthetized rat, using 5×10^6 PFU. Stocks of both gD$^-$ mutants were derived from complementing VD60 cells, which supply gD in *trans*. Two or five days after infection, the eyes were removed and the retinas were fixed and stained as described in the legend to Fig. 2.

In rats infected with wild-type HSV (F), some immunostaining was evident in the SCN after 3 days (data not shown) and was more pronounced by 5 days (Fig. 6). In the LGN, HSV-specific immunostaining first became evident 4 days after retinal infection (data not shown) and was more extensive by day 5 (Fig. 6). The majority of labeled cells in the LGN were in the dLGN, although there were a few HSV-infected cells scattered throughout the vLGN. Immunostaining in the SC became evident after 3 days and progressively became more intense over the next 2 days (data not shown and Fig. 6). The SC sections shown include the superficial layers of the colliculus which are retinorecipient neurons. It was conceivable that virus leaking from the eye could infect oculomotor neurons which project to the deeper layers of the colliculus. However, sections through these layers failed to indicate the presence of viral antigens (data not shown). In animals infected with either the gI$^-$ mutant (F-US7kan) or the gE$^-$ mutant (F-gEβ), immunostaining in the SCN and LGN was markedly less (fewer cells were labeled) than that observed in rats infected with wild-type HSV-1 (F) (Fig. 6). While the degrees of immunolabeling throughout the LGN for gE$^-$ and gI$^-$ mutants were reduced from that of the wild type, the majority of HSV-specific labeling was distributed in the dLGN in a manner similar to that of the wild-type virus. Similar amounts of immunolabeling were observed in the SCs of animals infected with either wild-type HSV-1 or the gE$^-$ and gI$^-$ viruses after 5 days. Therefore, mutant viruses unable to express gE or gI are restricted in their ability to spread to some retinorecipient areas of the brain.

The gJ$^-$ mutant, F-US5β, infected all three retinorecipient nuclei as well as wild-type HSV-1 did (Fig. 6). As with wild-type HSV-1, immunostaining in the LGN in F-US5β-infected animals was largely restricted to the dLGN, rather than the vLGN. Together, these data suggest that gJ is not required for efficient transneuronal spread from retina to brain. In contrast, no immunostaining of any retinorecipient region of the brain was observed 5 days after infection with the gD$^-$ mutant (F-US6kan) or the gD$^-$ gI$^-$ mutant (F-gDβ). The recombinant wt61, in which gD and gI expression were rescued, produced staining patterns very similar to that observed with wild-type F (Fig. 7). These results demonstrate that gD is essential for

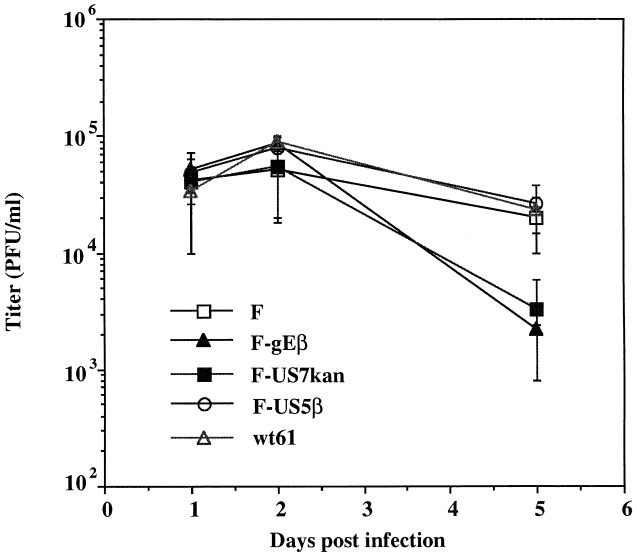

FIG. 4. Production of infectious viruses by HSV-1 gE$^-$, gI$^-$, and gJ$^-$ mutants in the rat retina. Retinas were infected with either wild-type HSV-1 (F), the gE$^-$ mutant (F-gEβ), the gI$^-$ mutant (F-US7kan), the gJ$^-$ mutant (F-US5β), or wt61. After 1, 2, or 5 days, the retinas were removed, washed in PBS, frozen, thawed, and then homogenized. Homogenates were titered on Vero cell monolayers.

transneuronal spread of HSV to retinorecipient neurons in the brain.

Cell-to-cell spread of gE$^-$, gI$^-$, and gD$^-$ mutants in cultured neurons. To further investigate neuron-to-neuron spread of HSV-1, we tested the ability of the mutants to spread in cultures of primary rat sensory neurons derived from trigeminal ganglia. By culturing the neurons on a poly-D-lysine and laminin substrate, which is known to stimulate neuritic growth (22), it was possible to produce a complex array of cell-cell and cell-neurite contacts or synapses. Neurons were grown for several weeks in the presence of a mitotic inhibitor, cytosine arabinoside, so that fibroblasts and glia were killed, producing cultures that were greater than 95% neurons. This system provided an ideal model to study the spread of HSV-1 between neurons where accessory cells were absent and input multiplicities of virus could be controlled.

HSV was plated on the cultured neurons at a low multiplicity of infection so that over the course of several days, viruses spread from the initial infected cells to neighboring neurons and through the dense network of neurites. It should be noted that the virus stocks used in these experiments were titered on Vero cells and when the cells were infected with 1 PFU per neuron, less than 10% of the neurons displayed viral antigens after 12 h. All the neurons in the culture could be infected when higher multiplicities of virus were used (10 PFU per neuron) or when virus was allowed to spread through the cultures. Following 48 h of infection with either wild-type or mutant viruses, cells were fixed and then immunostained for HSV antigens or for the 160-kDa neurofilament protein. The spread of HSV through the culture was assayed by counting the number of individual cells labeled with both anti-HSV and antineurofilament antibodies (Fig. 8A). Wild-type HSV-1 (F) and wt61, infected in excess of 600 neurons after 48 h (Fig. 8B). In contrast, the gE$^-$ mutant (F-gEβ) or the gI$^-$ mutant (F-US7kan) infected less than half this number of neurons. The gD$^-$ mutant, F-US6kan, infected still fewer neurons, although there was some spread of virus infection in the absence of gD. However, in the absence of both gD and gI, F-gDβ did not spread beyond a single infected neuron. These results are similar to those we previously described for fibroblast monolayers in which there was some virus spread in the absence of gD and no virus spread in the absence of both gD and gI (6).

Replication of both mutant and wild-type HSV-1 in neuronal cultures was also assessed by infecting the cultures and then measuring infectious virus after various times. When cells were infected with higher multiplicities of HSV-1 (10 PFU per cell), all the neurons in the culture expressed HSV antigens after a single round of replication. Under these conditions, wild-type HSV-1 and gE$^-$, gI$^-$, and gJ$^-$ mutant viruses all produced equal quantities of infectious virus (Fig. 9A). Therefore, gE$^-$, gI$^-$, and gJ$^-$ mutant viruses infect these neurons and produce infectious virus normally. However, when lower multiplicities of infection were used, so that only approximately 1 in 10 to 1 in 20 of the neurons were initially infected, production of infectious viruses in cultures infected with the gE$^-$ and gI$^-$ viruses was reduced by approximately 10-fold (Fig. 9B). Under these conditions, production of infectious virus was dependent on neuron-to-neuron spread of virus, especially after 24 h, since anti-HSV antibodies were present to neutralize any infectious virus released into the medium. Together, these data, along with our observations that gE$^-$ and gI$^-$ viruses spread poorly in the retina (Fig. 2), support the hypothesis that the gE-gI heterooligomer facilitates neuron-to-neuron spread. Again, we did not measure replication of the gD$^-$ viruses in these latter assays, because the viruses cannot enter most cultured cells and because immunofluorescence experiments (Fig. 8) indicated that these viruses could not spread.

DISCUSSION

There is good evidence that the HSV gE-gI heterooligomer functions to bind the Fc domain of IgG (for a recent review, see reference 58). However, more recently, there has been substantial evidence presented that this glycoprotein complex plays an important role in allowing HSV to spread from cell to cell in certain cultured cells (4, 13), in epithelial and other tissues in vivo (4, 13), and in the nervous system (40, 47). No role has been demonstrated for the Fc receptor in protecting HSV from the immune system in vivo, the gE-gI binds IgG relatively weakly, and other alphaherpesviruses have gE-gI ho-

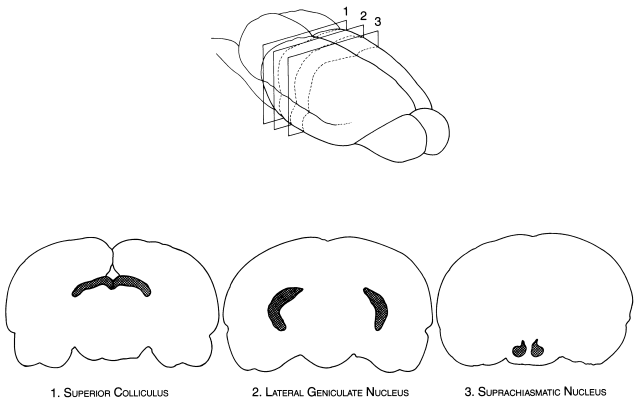

FIG. 5. Retinorecipient regions of the rat brain. Three coronal sections through the brains of rats, which had been infected in the eye with HSV-1 mutants, were taken. These sections correspond to the stereotaxic locations of the SC (section 1), or optic tectum, which is located in the midbrain, LGN (section 2), which is present in the thalamus, and SCN (section 3), which is in the hypothalamus. Each of these nuclei is highlighted and, for clarity, has been enlarged, so that they are not to scale with other regions of the brain.

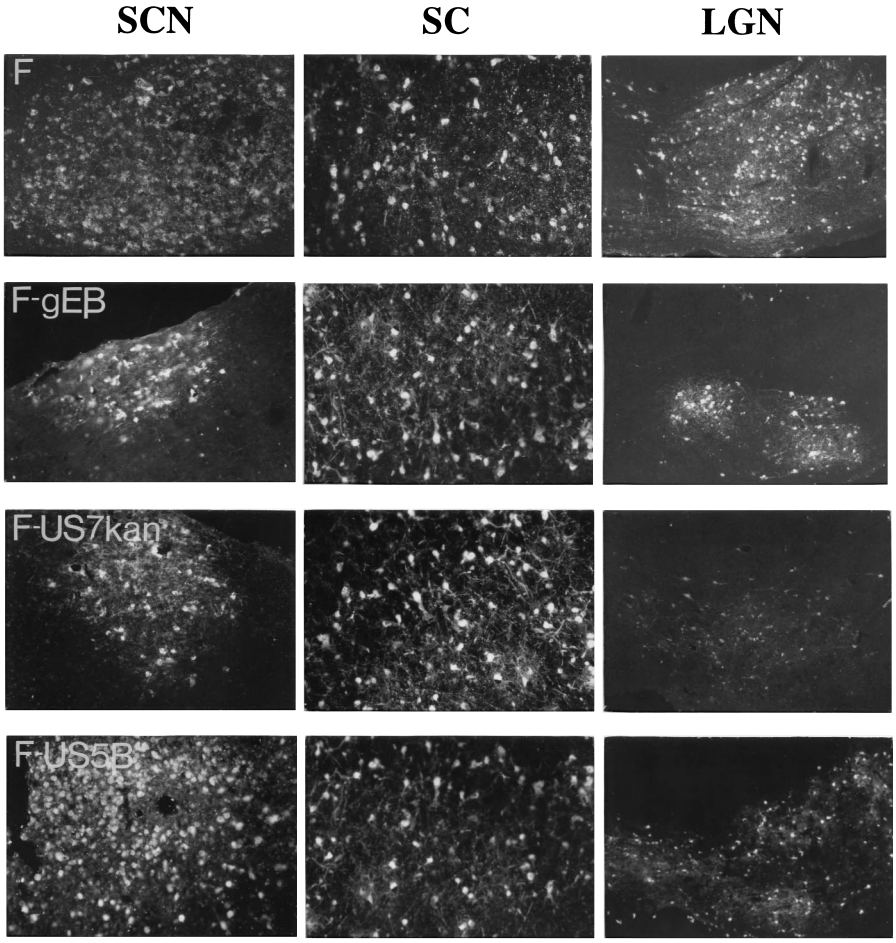

FIG. 6. Infection of retinorecipient regions of the rat brain by HSV-1 gE$^-$, gI$^-$, and gJ$^-$ mutants. Rats were infected in the vitreous body of one eye with wild-type HSV-1 (F), the gE$^-$ mutant (F-gEβ), the gI$^-$ mutant (F-US7kan), or the gJ$^-$ mutant (F-US5β). Five days later, the animals were anesthetized and perfused with 4% paraformaldehyde. The brains were removed, postfixed overnight in 4% paraformaldehyde, equilibrated in 15% sucrose, and sectioned. The sections were stained with rabbit anti-HSV antibodies and then with FITC-conjugated anti-rabbit antibodies, and immunostaining was observed only in the contralateral regions of the brain. The sections shown are the SCN, SC, and LGN, which includes both the dLGN (shown on the right side of the micrograph) and vLGN (left side).

mologs that do not bind IgG, so it has been proposed that gE-gI primarily functions in vivo to promote cell-to-cell spread of alphaherpesviruses (58). Previous studies had indicated that HSV gE$^-$ or gI$^-$ mutants are defective in cell-to-cell spread in epithelial tissues of experimental animals and spread poorly into the nervous system. However, since these mutants frequently replicated poorly at the periphery and produced substantially less infectious virus, it was not clear whether gE$^-$ and gI$^-$ viruses had difficulty gaining entry into neurons or were defective in transneuronal spread. Our studies were designed to address this question and, specifically, to evaluate neuron-to-neuron spread of HSV-1 mutants unable to express gE or gI, as well as two glycoproteins, gD and gJ, encoded by neighboring genes.

We chose to examine the spread of HSV-1 mutants in the retina and into the retinorecipient areas of the rat brain for a number of reasons. First, the retina is a dense matrix of neuronal connections which resembles the gray matter of the brain, and this neuronal circuitry is well characterized in the rat (for a review, see reference 43). Second, the retina can be readily infected by HSV, without significant injury to the tissue, and can be readily dissected and stained. Similar injections into the mouse eye are technically more challenging and less reproducible. Third, by using the rat visual system, we could more readily compare our observations to previous results involving HSV (41, 42) and PrV (9, 10, 18, 57). The central visual pathway in the retina consists of three types of neurons aligned in series. The visual signal originates with the photoreceptors, rods and cones, found at the back of the retina in the bacillary and outer nuclear layers (Fig. 1). This signal is conveyed by bipolar cells, located in the outer plexiform and inner nuclear layers of the retina, to retinal ganglion cells in the innermost layer of the retina. Axons from ganglion cells project to the visual centers of the brain. This synaptically linked pathway is modulated by lateral connections with horizontal cells in the outer plexiform layer and with amacrine cells in the inner plexiform layer. This complex yet well-ordered architecture of cellular and synaptic connections within the retina provides an ideal model to study neuron-to-neuron transmission of HSV without many of the complications associated with peripheral or intracerebral infections.

Two days after infection with wild-type HSV-1, we observed distinct foci of infection primarily in the inner and outer nuclear and ganglion cell layers, apparently involving columns of infected neurons, suggesting that infection spreads through direct synaptic connections of the central retinal pathway.

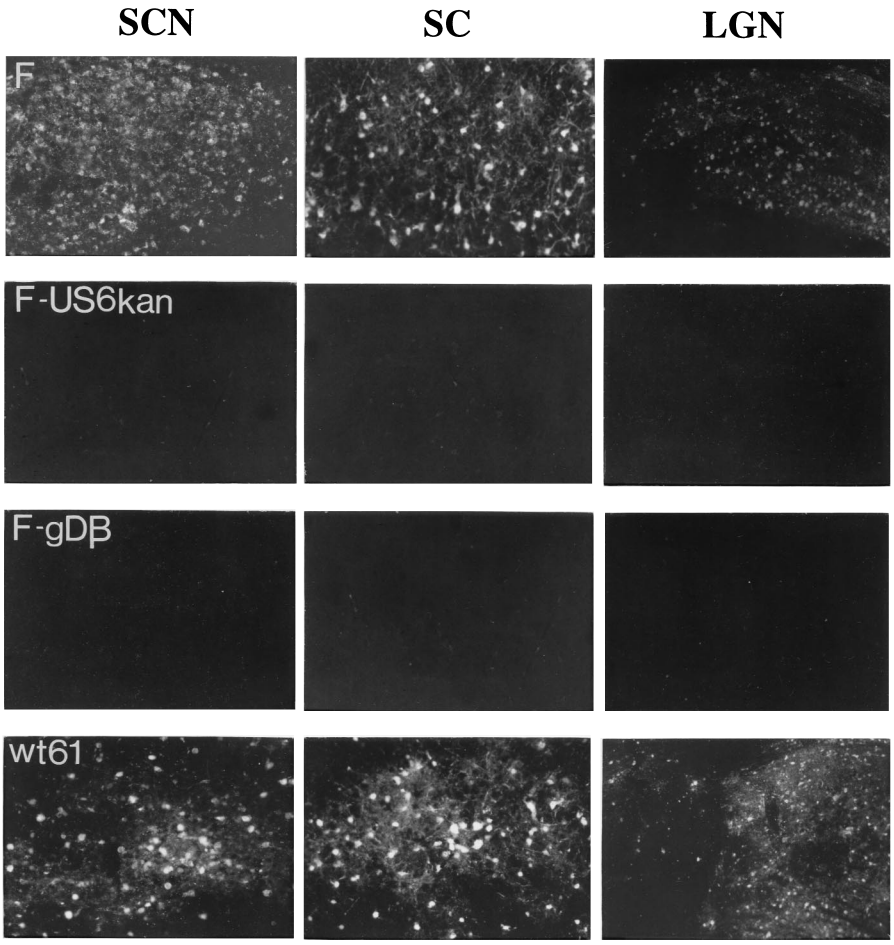

FIG. 7. Infection of retinorecipient regions in the brain by HSV-1 gD$^-$ mutants. Rats were infected in one eye with wild-type HSV-1 (F), the gD$^-$ mutant (F-US6kan), the gD$^-$ gI$^-$ mutant (F-gDβ), or wt61. Five days later, the animals were anesthetized and perfused with 4% paraformaldehyde. The brains were removed, postfixed, sectioned, and stained as described in the legend to Fig. 6.

These data are consistent with previous observations involving HSV and PrV (10, 41). The ensuing lateral spread of HSV through the retina, observed after 5 days, apparently involved infection of horizontal and amacrine cells. These cells form synaptic connections with neurons of the central pathway and also form gap junctions with other amacrine or horizontal cells (for a review, see reference 12), facilitating rapid lateral cell-to-cell spread of HSV. Müller fibers, a type of glial cell which forms tight connections with retinal neurons, may also be involved in lateral spread of HSV in the retina (28).

HSV-1 mutants unable to express gE or gI initiated infection of neurons in the rat retina, in a manner comparable to that observed with wild-type HSV-1; equal numbers of foci of infection were observed in the retina after 2 days. Therefore, in agreement with our previous observations involving cultured fibroblasts (13), it appears that the gE-gI heterooligomer is not required for entry of extracellular virus into neurons, in this case primarily neurons of the inner layers of the retina including ganglion cells. However, by 5 days after infection, it was clear that gE$^-$ and gI$^-$ mutants were defective in virus spread through the retinal microcircuitry; this was especially true of the lateral spread of virus. Consistent with the immunocytochemical analysis of the retina, we found that 10-fold-fewer infectious HSV-1 gE$^-$ or gI$^-$ mutant virus was produced after 5 days. Therefore, the gE-gI heterooligomer is required for efficient cell-to-cell spread in the retina. Mutants lacking gD also established primary infections in the retina, although the virus particles in this case were derived from complementing cells, so that gD was present. However, the gD$^-$ viruses did not spread significantly, and by 5 days there was little expression of viral antigens.

We also investigated the roles of glycoproteins D, E, I, and J in transneuronal spread of HSV-1 from retinal ganglion cells to retinorecipient areas of the brain. The SC, or optic tectum, located in the midbrain, was infected equally well by gE$^-$ and gI$^-$ mutants and wild-type HSV-1. By contrast, the gE$^-$ and gI$^-$ mutants infected the LGN and SCN poorly, so that many fewer cells were infected by the mutants than by the wild-type parent strain F.

There are at least two possible explanations for the defects in the spread of the gE$^-$ and gI$^-$ mutants to the SCN and LGN. First, since we observed reduced spread of the mutant viruses within the retina, fewer retinal ganglion cells were infected. We expect that this more restricted pattern of HSV infection in the retina might cause fewer retinorecipient neurons in the CNS to be infected. In the rat, more than 90% of all retinal ganglion cells project to the contralateral SC (34), whereas a much smaller fraction of the ganglion cell population project to the SCN and LGN. Therefore, we suspect that the more restricted spread of gE$^-$ and gI$^-$ mutants in the

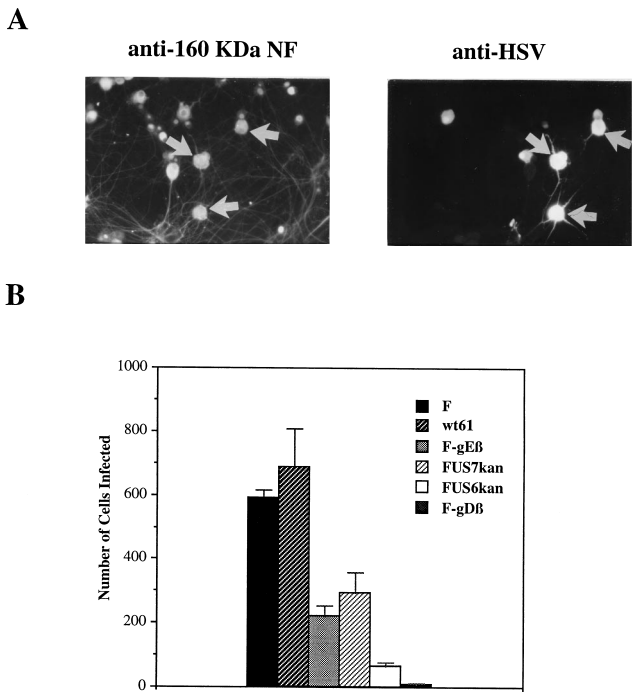

FIG. 8. Spread of HSV-1 gE$^-$ gI$^-$, and gD$^-$ mutant viruses in cultures of dissociated rat sensory neurons. Approximately 2,000 neurons derived from dissociated rat trigeminal ganglia were plated on coverslips coated with laminin and poly-D-lysine. Cultures were treated with a mitotic inhibitor (cytosine arabinoside) which killed fibroblasts and glial cells so that cultures were produced which were greater than 95% neurons. Neuronal cultures were infected with wild-type HSV-1 (F), the gE$^-$ mutant (F-gEβ), the gI$^-$ mutant (F-US7kan), the gD$^-$ mutant (F-US6kan), the gD$^-$ gI$^-$ mutant (F-gDβ), or wt61 at a low multiplicity of infection so that approximately 1 in 10 to 1 in 20 of the neurons were infected. After 2 h, the cultures were washed with PBS and overlaid with MEMN–10% FBS supplemented with 0.1% human gamma globulin (a source of anti-HSV neutralizing antibodies). After 48 h, cells were fixed briefly with 4% paraformaldehyde, incubated with both rabbit polyclonal anti-HSV-1 antibodies and a mouse monoclonal antibody directed against the 160-kDa neurofilament protein and then with FITC-conjugated anti-rabbit antibodies and rhodamine-conjugated anti-mouse antibodies. (A) Representative micrographs of neuronal cultures infected with wild-type HSV-1. (Left) Cells were stained with anti-160-kDa neurofilament (NF) antibodies. (Right) The same cells were stained simultaneously with anti-HSV antibodies. The arrows indicate cells which were stained with both anti-HSV-1 antibodies and anti-160-kDa neurofilament. (B) Cells stained with both antineurofilament and anti-HSV antibodies were counted after 48 h. The resulting values are the means of 10 separate cultures that were independently infected, stained, and counted. Error bars represent standard deviations.

retina, coupled with fewer projections to the SCN and LGN, combined to produce less infection in these regions of the brain. A second explanation, which is not mutually exclusive of the first, is that gE$^-$ and gI$^-$ mutants infect the SCN and LGN less efficiently because these viruses spread poorly by anterograde axonal transport to the brain or infect secondary neurons in the brain less efficiently. There is no evidence that gE$^-$ or gI$^-$ mutants possess defects in anterograde transport, and since there is evidence that the viral nucleocapsids and glycoproteins move independently in axons (46), it seems unlikely that the gE-gI heterooligomer would affect axonal transport (discussed further below). Reduced expression of HSV antigens in the SCN and LGN could be attributed to fewer infectious virus particles reaching these regions from retinal ganglion cells, as well as less efficient spread of virus within the SCN and LGN. It was previously reported that an HSV gE$^-$ mutant spread poorly after inoculation into the brain (40). However, we favor the notion that fewer gE$^-$ and gI$^-$ viruses reach the SCN and LGN because we could show that there was less virus present in the retina to initiate such infections and also because the differences were observed relatively early after infection in the brain. We can draw similar conclusions about the work of Balan et al. (4), who studied HSV infection of the peripheral nervous system. HSV gE$^-$ and gI$^-$ mutants produced a more limited infection in the mouse ear than did wild-type HSV-1, and there was a dramatic reduction in virus spread to sensory ganglia, apparently, at least in part, because less virus was produced in the skin.

To further address the issue of whether the gE-gI hetero-

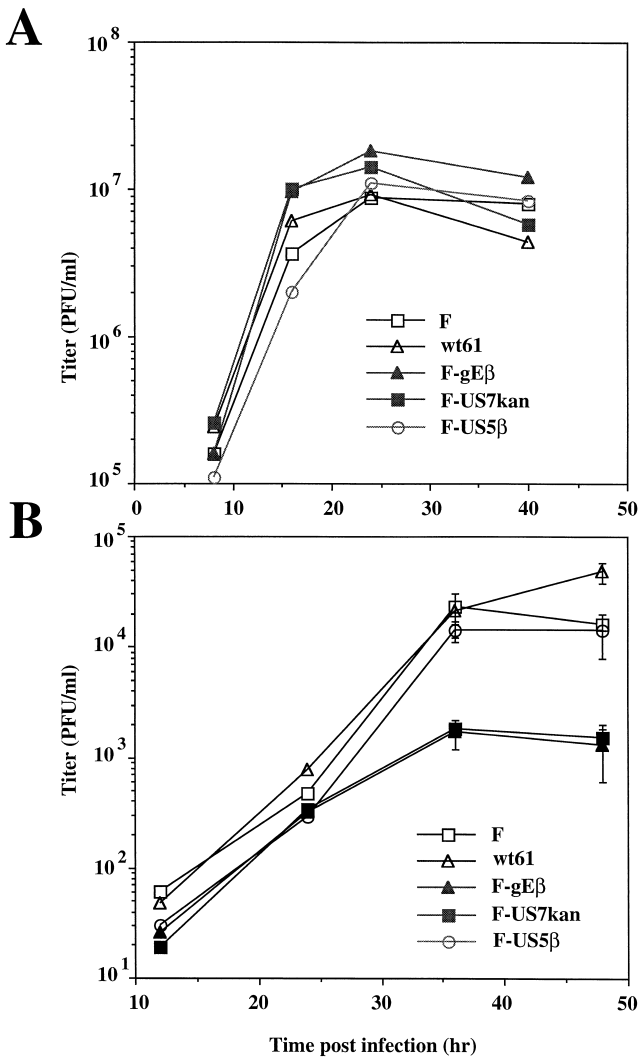

FIG. 9. Production of infectious viruses by HSV-1 gE$^-$, gI$^-$, and gJ$^-$ mutants after infection of rat sensory neurons in culture. Neuronal cultures were established and then the cells were infected with wild-type HSV-1 (F), the gE$^-$ mutant (F-gEβ), the gI$^-$ mutant (F-US7kan), the gJ$^-$ mutant (F-US5β), or wt61, using 10 PFU/cell in panel A or a low multiplicity of infection in panel B, so that only 1 in 10 or 1 in 20 neurons were infected initially. In panel A, 24-well dishes, containing approximately 4×10^4 neurons per well were used; in panel B, 24-well dishes, containing 2×10^3 neurons per well were used. After 2 h, the monolayers were washed with PBS and incubated in MEMN–10% FBS (A) or MEMN–10% FBS supplemented with 0.1% human gamma globulin (B) for the indicated times. At the indicated times, medium and cells (A) or cells alone (B) were collected and frozen at −70°C. In panel B, cells were washed twice with medium to remove neutralizing antibodies prior to being collected; note that only a fraction of the cells were infected after 48 h. Samples were thawed, sonicated, and titered on Vero cells.

oligomer is necessary for neuron-to-neuron spread of HSV, it was advantageous to examine the infection of neurons cultured in vitro, conditions that made it possible to control virus multiplicities and study neurons in isolation from accessory cells. Cultures of rat trigeminal ganglion neurons were established, and the cultures were treated with drugs to kill nonneuronal cells. The neurons formed an extensive network of neurites, and once infected with HSV-1, virus could spread from cell to cell without exposure to neutralizing antibodies present in the medium. Thus, we could study virus spread through a two-dimensional neuronal network of cell-cell, cell-neurite, and neurite-neurite contacts. Mutants unable to express gE or gI infected approximately 50% fewer neurons than did wild-type HSV-1 in a 48-h period, under conditions in which only a small fraction of the neurons were initially infected and viruses spread from neuron to neuron. After immunocytochemical staining, foci of virus infection could be observed in the neuron cultures, and after 24 h, the gE^- and gI^- viruses produced the same number of foci of infection as that produced by wild-type HSV-1. Moreover, the production of infectious viruses did not differ significantly when neuronal cultures were infected at a higher multiplicity of infection, so that every cell was infected. In contrast, when neurons were infected at a lower multiplicity of infection and spreading of virus through the medium was prevented with neutralizing antibodies, the gE^- and gI^- viruses produced 10-fold-less virus than wild-type HSV-1 did. These data strongly support the hypothesis that the gE-gI heterooligomer is required for efficient neuron-to-neuron spread of HSV and yet is not necessary for entry of extracellular virus particles or production of infectious virus. Note that many of the junctions formed between the cultured neurons were not synaptic junctions; however, such junctions are likely to play important roles in virus spread within the retina and brain. Nevertheless, these results, coupled with the in vivo results, are consistent with the hypothesis that gE-gI also facilitates transsynaptic spread of HSV.

The gD^- mutants (F-US6kan, which lacks gD alone, and F-gDβ, which lacks gD and gI) were severely compromised in their ability to spread from neuron to neuron. Although a few neurons were infected by F-US6kan, F-gDβ did not spread beyond a single infected neuron. Therefore, we can conclude that gD is required for neuron-to-neuron spread, although there is some limited spread in its absence, as was observed with cultured human fibroblasts (6).

Our results must also be considered in light of previous detailed observations on the spread of PrV infection from the retina to the brain. Card et al. (9) observed that a gE^- PrV spread poorly from the retina to certain retinorecipient regions of the rat brain, the SC, also denoted the optic tectum, and LGN. The SCN, in the hypothalamus, was infected equally well by wild-type and gE^- viruses. A subsequent study indicated that a gI^- mutant had a similar phenotype (57). It appeared that the gE^- PrV infected fewer neurons in the retina (9), as we found here with HSV-1 gE^- and gI^- mutants. Both wild-type PrV, as well as gE^- and gI^- mutants, infected only a fraction of the retinal ganglion cells and the researchers concluded that reduced spread of PrV to the SC and LGN was related to the inability of gE^- or gI^- viruses to infect a subset of the ganglion cells that project to the SC and LGN. However, it was not clear from these studies whether PrV gE^- and gI^- mutants produced less-infectious progeny in the retina than wild-type PrV did. Even if this were the case, these observations cannot be explained by relating the results to the observation that approximately 90% of retinal ganglion cells project to the contralateral SC, because the SC was inefficiently infected by the gE^- and gI^- PrV. Enquist et al. (18) concluded that PrV gE and gI are not necessary for the primary infection in the retina, as we concluded for HSV gE and gI. Our studies involving cultured trigeminal ganglion neurons suggested that HSV gE^- or gI^- mutants could infect all of the neurons in culture when a high multiplicity of infection was used or when the virus was given sufficient time to spread in the culture. Thus, we found no evidence for selective infection of a subset of trigeminal ganglion neurons, although it is possible that there was selective growth of certain neurons in these cultures. Furthermore, we found no evidence that in the retina gE^- and gI^- viruses lack the ability to infect specific neurons, rather our data supports the notion that HSV gE^- and gI^- mutants are defective in the efficiency in which they are transmitted from any infected neuron to another neuron. However, PrV appears to differ from HSV in that the spread of virus into selected neurons in the retina or regions of the brain apparently requires gE-gI. It should also be noted that PrV gE^- mutants also have defects in the spread to the CNS following intranasal infection of pigs (25, 29, 32a).

There are several important differences between HSV and PrV that must also be considered. HSV infects the rat nervous system less efficiently than does PrV, so that our studies necessitated 5- to 50-fold-more virus than was previously used in the PrV studies. Moreover, unlike PrV, which produces significant pathogenesis frequently leading to death, in our experiments, no animals infected with wild-type HSV-1 showed any overt symptoms of infection and the animals survived with apparently normal functions. There are also notable differences in the mechanisms by which PrV and HSV spread from cell to cell. Our laboratory has previously demonstrated that gD is essential for cell-to-cell spread in certain cultured cells (33), corneal epithelium (14), and the retina (this study). However, we note that there is a small amount of cell-to-cell spread in the absence of gD (6) (Fig. 8). By contrast, the PrV gD^- mutants display little or no defects in virus spread in cultured cells and in the nervous systems of mice following either injection into a nerve or subcutaneous or intraperitoneal injection (1a, 44, 45).

It is by no means clear how the gE-gI heterooligomer functions to increase the efficiency of HSV cell-to-cell transmission between fibroblasts, epithelial cells, or neurons. The existing data suggest at least two mechanisms by which the gE-gI heterooligomer could affect the spread of HSV between neurons or other cell types. One possibility is that gE-gI may bind cellular proteins which act as viral receptors and which are concentrated at cell junctions or, in this case, at neuronal synapses. Thus, by binding to such proteins, gE-gI might promote cell-to-cell transmission of HSV but would be unnecessary for entry of extracellular virus particles into cells. There is evidence for transfer of HSV and other alphaherpesviruses across synaptic junctions. Electron micrographs of neurons infected in vitro or in vivo show alphaherpesviruses at synaptic junctions (8, 46), and although the viruses are not always released there, it appears that a reasonably large fraction of virus particles are released into the synaptic cleft.

There is good evidence for anterograde transport of nucleocapsids combined with independent transport of viral glycoproteins and envelopment of capsids at axon terminals (46). Preferential sorting of viral glycoproteins to the axon terminal (15) could promote envelopment and exocytosis at these sites. Penfold et al. (46) observed nodules along HSV-infected axons corresponding to patches of viral glycoproteins, suggesting exocytosis of viruses at or near axon terminals. There is also evidence that HSV can attach preferentially to synaptosomes derived from rodent brain and to glial cells but less efficiently to neuronal perikarya (55). The velocity of HSV spread

through secondary and tertiary neuronal networks in vivo (50 to 100 mm/day) strongly supports virus transmission across synapses (39, 41) but does not exclude other mechanisms of transmission. Therefore, gE-gI may promote virus entry into afferent neurons by promoting interactions between virus particles and cellular receptors concentrated at synapses. McGeoch (38) noted that gI appears to belong to a family of U_S-encoded proteins, including gD and gG, and possibly gE, with similar positioning of cysteine residues and suggested that these glycoproteins might be derived from a common ancestor. Therefore, since there is good evidence that gD facilitates virus entry into cells by interacting with a limited set of cellular receptors (for a recent review, see reference 6), gE-gI may similarly facilitate cell-to-cell spread by promoting interactions with cellular receptors concentrated at cell junctions or synapses. However, it is also important to note that in our neuronal cultures many of the cell contacts were nonsynaptic cell junctions. In the retina, spread across nonsynaptic junctions would be less likely.

The second mechanism by which the gE-gI heterooligomer could affect cell-to-cell spread of HSV involves sorting or targeting of HSV particles to cell junctions or, in the case of neurons, to axon terminals or synapses. Although it is not known whether gE and gI are sorted to specific regions of polarized cells or neurons, these glycoproteins could promote the appearance of enveloped virus particles near cell or synaptic junctions. The recent results of Penfold et al. (46) support the view that HSV nucleocapsids and glycoproteins are independently transported to axon terminals. Therefore, it is unlikely that gE-gI specifically target capsids to axon terminals. However, these glycoproteins may promote assembly of enveloped particles or sorting of the particles to synaptic junctions by unknown processes. We are currently investigating these two potential mechanisms by which gE-gI could promote cell-to-cell spread of HSV.

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ADDENDUM IN PROOF

We have recently restored gE expression in F-gEβ by recombination of a wild-type gE gene. This virus, F-gEβR, produced large plaques on human fibroblasts and behaved similarly to wild-type virus in the rat visual system.

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