Thymidine Kinase-Deficient Herpes Simplex Virus Type 2 Genital Infection in Guinea Pigs

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Received 27 September 1984/Accepted 15 April 1985

In guinea pigs, thymidine kinase-producing strains of herpes simplex virus type 2 replicated to high titer in the vagina and spinal cord, and animals developed severe clinical disease. Infection with thymidine kinase-deficient virus resulted in similar vaginal virus titers; however, animals exhibited little or no clinical illness and only low titers of virus were detected in spinal cord homogenate cultures. Neural and extraneural latent infection as well as recurrent infection were noted in animals inoculated with either thymidine kinase-producing or -deficient viruses. These data suggest that neural pathways are important in the pathogenesis of genital herpes and that virus-coded thymidine kinase may influence virulence but is not required for latency.

During the course of initial herpes simplex virus (HSV) infection, specific immune responses occur which limit productive viral replication but permit the establishment of a latent viral state. Thus, most infections with HSV appear self-limited but, in fact, the virus persists within the host (3, 37). Although viral latency is incompletely understood, it is thought that recurrent herpetic infection is a consequence of reactivation of virus latent within host cells (25, 28, 38, 44). Although HSV disease is rarely life-threatening, its recurrent nature and the associated discomfort cause many patients to seek medical treatment.

One prescription drug, acyclovir, is presently available for the treatment of initial oral and genital herpes. Acyclovir and several experimental antiviral agents are selectively phosphorylated by HSV-coded thymidine kinase (TK). The triphosphate form of acyclovir competitively inhibits the utilization of normal substrates by the viral DNA polymerase and is also incorporated terminally at the 3’ ends of growing DNA chains stopping DNA synthesis at that point. Treatment with acyclovir, however, has resulted in the emergence of drug-resistant HSV mutants, some of which are deficient in viral TK (TK−) (6, 8, 35). The rising incidence of HSV type 2 (HSV-2) genital infections (5) and the increasing use of antiviral agents for the treatment of genital herpes are likely to result in increased human exposure to TK− mutants of HSV-2.

HSV-coded TK is distinct from mammalian cell TK (17) and may facilitate HSV replication in nondividing cells (16). Because HSVs are neurotropic viruses normally capable of both productive and latent infection in nondividing neural cells, it has been hypothesized that TK− virus may be less neurovirulent than wild-type TK-producing (TK+) strains (11). For example, HSV type 1 (HSV-1) TK− mutants produce less clinical disease and lower viral titers in neural tissues, and they may be incapable of producing latent ganglionic infection in animal models of nongenital HSV infections (11, 14, 21, 30, 41). Viral TK therefore would appear to influence HSV neurovirulence. Although acute and subsequently latent HSV-2 infection of neural tissues occurs as a consequence of initial genital infection (29, 33, 36), the importance of neural infection in initial and recurrent genital herpes is incompletely understood and complicated by the observation that latent virus may also reside in extraneural tissues, such as skin at the site of initial infection (15, 34, 36, 43). The role of viral TK in initial genital infection, as well as in persistent infection at neural and extraneural sites, has not been established. In this study we examined the influence of viral TK on the pathophysiology of HSV-2 genital infection.

MATERIALS AND METHODS

Animals. Female strain 2 (Children’s Hospital Research Foundation Vivarium, Cincinnati, Ohio) or Hartley guinea pigs (Charles River Breeding Laboratories, Wilmington, Mass.) weighing ca. 150 to 200 g were used in these studies. Their vaginal closure membrane was ruptured and the vaginal vault was swabbed with a premoistened calcium alginate-tipped swab (Spectrum Diagnostics, Glenwood, Ill.). One hour later the animals were inoculated with 0.1 ml of virus instilled into the vaginal vault by use of a syringe and a 20-gauge plastic catheter (Abbott Hospitals, North Chicago, Ill.).

Cells and viruses. First- through third-passage rabbit kidney (RK) cells were prepared from the kidneys of 3-week-old, Pasturella-free, female New Zealand white rabbits (Hazleton-Dutchland Inc., Denver, Pa.) and maintained with Eagle basal medium containing heat-inactivated (56°C, 30 min) fetal bovine serum, penicillin (100 U/ml), streptomycin (50 µg/ml), and L-glutamine (2 mM). The preparation of human foreskin fibroblast cells and fetal guinea pig tissue cultures has been previously described (27). Clarified pools of the MacIntyre strain of HSV-1 (ATCC VR-539), the MS (ATCC VR-540) and 333 (19) strains of HSV-2, and the bromodeoxyuridine-resistant mutant of strain 333 (20) prepared in RK cells and stored frozen at −70°C. The bromodeoxyuridine-resistant mutant of strain 333 fails to induce detectable TK activity (TK−) in TK-deficient cells but enhances the resident TK activity of biochemically transformed cells (7, 18).

Virus detection. Swab samples of vaginal secretions were
collected with a premoistened calcium alginate-tipped swab. The swab samples were placed in 1 ml of Eagle basal medium containing penicillin (100 U/ml), streptomycin (50 µg/ml), gentamicin (50 µg/ml), and amphotericin B (2.5 µg/ml). During initial infection, groups of animals were sacrificed daily and their lumbar sacral dorsal root ganglia and spinal cords were aseptically removed. Ten percent (wt/vol) homogenates were prepared from the tissue samples. Swab specimens and supernatants from the tissue homogenates were stored at −70°C until assayed. Virus was titrated by plaque assay in RK cells employing a 1% methylcellulose overlay. Five weeks after intravaginal inoculation, animals were killed and the external genital skin, uterine cervix, and lumbosacral dorsal root ganglia were aseptically removed. A portion of each tissue was homogenized, and the clarified supernatant was cultured on RK cells for 10 days to evaluate for the presence of infectious HSV-2. A second portion of each tissue was finely minced and explanted onto semiconfluent RK cell monolayers. Additional RK cells were periodically added to the explant cultures and observed for cytopathic effect for at least 6 weeks. The remaining tissue, stored at −70°C, was analyzed for HSV DNA by the dot-blot DNA hybridization technique we have previously described (27). Briefly, DNA extracted from infected and uninfected animal tissues was spot hybridized on nitrocellulose filters with 32P-labeled HSV-2 DNA probes prepared from nucleocapsids (4, 27, 39). Hybridization was conducted in 50% formamide at 42°C for 16 to 24 h (27). The extent of binding was analyzed by autoradiography.

Infectious virus was defined as virus recovered from cell-free homogenate cultures. Latent virus was defined by failure to recover HSV from cell-free homogenate cultures.

![Image](http://jvi.asm.org/)

**FIG. 1.** Course of vaginal HSV-2 replication after intravaginal viral inoculation. Vaginal viral replication was measured by plaque titration of vaginal swab samples. (A) Six weaning Hartley guinea pigs infected at a variety of inocula of either wild-type (TK*) strain 333 or its TK- mutant. (B) Eighteen weaning Hartley guinea pigs inoculated with −6.5 log10 PFU of either TK* strain MS, TK* strain 333, or TK* strain 333 HSV-2.

**RESULTs**

**Primary infection.** Intravaginal inoculation of HSV-2 resulted in viral replication in vaginal tissues. Vaginal HSV-2

**TABLE 1.** Incidence of clinical disease in HSV-2-infected weaning female guinea pigs

<table>
<thead>
<tr>
<th>Clinical feature</th>
<th>Incidence (%) with the following virus:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MS/TK*</td>
</tr>
<tr>
<td>Any genital skin lesions</td>
<td>24/25 (96)</td>
</tr>
<tr>
<td>Urinary retention</td>
<td>21/25 (84)</td>
</tr>
<tr>
<td>Hindlimb paralysis</td>
<td>3/25 (12)</td>
</tr>
<tr>
<td>Death</td>
<td>6/25 (24)</td>
</tr>
</tbody>
</table>

* Animals were defined as infected if virus was recovered from at least two vaginal swab samples collected at 24-h intervals beginning 24 h after intravaginal HSV-2 inoculation (4.5 to 6.5 log10 PFU). Clinically significant urinary retention was said to be present if, on at least two occasions 24 h apart, more than 2 ml of urine could be expressed by Crede’s maneuver on the bladder.
replication, as reflected by the titer of virus in vaginal swab samples, was greatest in the first 2 days after inoculation and then declined steadily to low or undetectable levels by day 10. The onset, magnitude, and duration of vaginal virus replication in strain 2 animals were comparable after inoculation with either TK+ or TK– strain 333 HSV-2 delivered at inocula ranging from 4.5 to 6.5 log10 PFU (Fig. 1a). The pattern of vaginal HSV-2 replication after intravaginal inoculation of Hartley guinea pigs with 6.5 log10 PFU of the MS strain was similar to that observed for TK+ and TK– HSV-2 333 strain (Fig. 1b).

In contrast to vaginal virus shedding, clinically apparent initial genital infection due to TK– HSV-2 was less severe and of shorter duration than infection produced by either TK+ strain (Fig. 2). First lesions with all three strains were observed on day 4 after inoculation at the time when vaginal virus titers were beginning to decline. In the TK+–infected animals, lesions began as erythematous macules which rapidly progressed to vesicles and then to a severe ulcerative stage. The majority of these animals had concomitant urinary retention; 10 to 20% developed hindlimb paralysis, and ca. 25% of the guinea pigs died (Table 1). Surviving guinea pigs demonstrated complete resolution of illness by the end of week 2. In contrast, TK–infected animals seldom exhibited overt disease, and those that did develop lesions exhib-
ited only a small number of discrete vesicles which did not progress to the severe stage. None of the TK-infected animals died.

The reduced clinical severity of TK- HSV-2 genital infection, despite equivalent vaginal virus titers, prompted us to investigate the magnitude of viral replication at other sites. Both the magnitude and the duration of productive TK- HSV-2 replication in spinal cord homogenates were less than those observed for TK+ viruses (Fig. 3). Additionally, virus was detected by day 3 in homogenate cultures of lumbosacral dorsal root ganglia, and the titers were lower in TK-infected animals (mean titer, 1.4 log10 PFU/g of tissue) than in 333 TK+infected animals (mean titer, 2.7 log10 PFU/g of tissue).

Recurrent infection. After recovery from initial infection, survivors were examined for evidence of recurrent herpetic disease. Brief, isolated vesicular recurrences appeared on the external genital skin and were observed in both TK- and TK+infected animals. The lesions were 1 to 2 mm in diameter with an erythematous base. Recurrences were not associated with systemic illness, lasted ca. 24 h, and were not observed in mock-infected guinea pigs. In addition, guinea pigs were noted to shed HSV-2 from the vagina in the absence of demonstrable lesions (asymptomatic shedding).

Latent infection. Approximately 5 weeks after intravaginal HSV-2 inoculation, the genital skin, uterine cervix, and lumbosacral dorsal root ganglia were examined for evidence of persistent viral infection. To evaluate for the presence of infectious HSV-2, a portion of each tissue was homogenized and the supernatant was cultured for 10 days on RK cells. No infectious virus was recovered from any of the tissue homogenate cultures. The presence of persistent HSV DNA was sought in tissues by dot-blot DNA hybridization. The blot pattern in Fig. 4a shows homology between the HSV-2 DNA probe and both HSV-1 and HSV-2 DNA extracted from human fibroblasts. There was no detectable homology for guinea pig cellular DNA, or guinea pig cytomegalovirus (ATCC VR-682) and guinea pig herpes-like virus (ATCC VR-543) DNAs. In the accompanying blot (Fig. 4b), the probe bound to extracted HSV-2 DNA (positive control) but not to DNAs from uninfected tissue controls. This probe also bound DNA extracted from dorsal root ganglia, external genital skin, and uterine cervix of six infected animals, demonstrating the presence of HSV DNA in the tissues of both TK- and TK+infected animals.

Because the probe we utilized cannot discriminate the TK+ or TK- phenotypes, the possibility existed that the latent virus in TK-infected animals represented a wild-type revertant. To address this question, latently infected tissues were finely minced and cocultivated with low-passage RK. Latent virus often required up to 6 weeks of cocultivation but was detected in the lumbosacral dorsal root ganglia, external genital skin, and uterine cervical specimens of guinea pigs infected with either TK+ or TK- viruses (Table 2). HSV isolates were then characterized by enzyme assays of cytosol extracts of infected cells and by thymidine plaque autoradiography. The viruses reisolated from guinea pigs that had been infected with parental TK+ HSV-2 induced high levels of TK activity, but the viruses reisolated from animals infected with the TK- mutant did not have detectable TK-inducing activity in TK-deficient mouse fibroblast [LM(TK-)] cells (Table 3). A typical thymidine plaque autoradiographic experiment is shown in Fig. 5. The experiment shows that all of the plaques formed by inoculating TK+ HSV-2 (333) onto TK+ RAB(BU) cells incorporated labeled thymidine into DNA, but that none of the plaques formed in the RAB(BU) cells by virus reisolated from the dorsal root ganglia or cervix of TK- HSV-2-infected animals incorporated labeled thymidine into DNA. HSV-2 isolates from five dorsal root ganglia, two cervix, and four genital skin specimens from six guinea pigs infected with TK+ viruses were TK+. Isolates from four dorsal root ganglia, three cervix, and four genital skin specimens from eight animals infected with TK- virus were TK- (Table 2).

**DISCUSSION**

Genital infection produced by TK- HSV-2 was clinically mild despite vaginal virus replication comparable to that produced by TK+ viruses. The milder illness, however, was associated with reduced viral replication in lumbosacral

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**TABLE 2. Recovery of latent HSV-2 from guinea pig tissues**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Recovery (%) of the following virus:</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>MS/TK+</td>
</tr>
<tr>
<td>Dorsal root ganglia</td>
<td>41/61 (67)</td>
</tr>
<tr>
<td>Genital skin</td>
<td>16/34 (47)</td>
</tr>
<tr>
<td>Uterine cervix</td>
<td>10/20 (50)</td>
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*Animals were sacrificed and tissue specimens were aseptically collected 35 to 67 days after intravaginal HSV-2 inoculation. A portion of the tissue was homogenized, and the supernatant was cultured on RK cells for infectious virus. All homogenate cultures were negative. The remaining tissue was finely minced and the explants were cocultivated with RK cells. Explant cultures were maintained for 6 weeks. Selected isolates from 333 TK- and 333 TK- infected animals were then confirmed to be the TK phenotypes of the inoculum virus by enzymatic assay and plaque autoradiography (18, 20, 42).*

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**TABLE 3. Induction of TK activity after infection of LM(TK+) cells with HSV-2 (333) strains reisolated from virus-infected guinea pigs (gps)**

<table>
<thead>
<tr>
<th>Expt no.</th>
<th>Source of reisolated virus</th>
<th>TK activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>No virus</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>TK+ virus used for intravaginal inoculation of gp</td>
<td>6.0</td>
</tr>
<tr>
<td></td>
<td>DRG2 of gp no. 254 inoculated with TK- HSV-2</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>Cervix of gp no. 254 inoculated with TK- HSV-2</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>Cervix of gp no. 257 inoculated with TK+ HSV-2</td>
<td>9.4</td>
</tr>
<tr>
<td></td>
<td>DRG of gp no. 257 inoculated with TK+ HSV-2</td>
<td>6.4</td>
</tr>
<tr>
<td>2</td>
<td>No virus</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>TK+ virus used for intravaginal inoculation of gp</td>
<td>10.3</td>
</tr>
<tr>
<td></td>
<td>DRG of gp no. 251 inoculated with TK+ HSV-2</td>
<td>19.1</td>
</tr>
<tr>
<td></td>
<td>DRG of gp no. 253 inoculated with TK+ HSV-2</td>
<td>11.8</td>
</tr>
<tr>
<td></td>
<td>Cervix of gp no. 253 inoculated with TK+ HSV-2</td>
<td>18.2</td>
</tr>
<tr>
<td></td>
<td>DRG of gp no. 256 inoculated with TK+ HSV-2</td>
<td>0.04</td>
</tr>
</tbody>
</table>

*Confident monolayer cultures of LM(TK+) cells were infected at a multiplicity of infection of about 2 to 8 PFU per cell for 6 h. TK was extracted from the cytosol fraction and assayed as described previously (18-20).

† Picomoles of [3H]thymidine per min at 38°C per microgram of protein.

‡ DRG, Dorsal root ganglia.
dorsal root ganglia and spinal cord. These data imply that in producing genital infection, TK− HSV-2 is less virulent than wild-type (TK+) HSV-2. Similarly, TK− HSV-1 has been reported to have reduced pathogenicity in animal models of herpes encephalitis (11), herpes keratitis (30, 41), and herpes labialis (21). Additionally, studies with temperature-sensitive mutants of HSV-2, some of which were TK−, showed that these mutants were less pathogenic in a model of genital infection but also apparently replicated less well in vaginal tissue than did the wild-type parental strain (1). The mechanism by which virus-coded TK influences the pathogenicity of HSV may relate to reduced virulence in neural tissues (11, 21, 23, 30, 41). We have previously postulated that virus in lumbosacral dorsal root ganglia or spinal cord or both is the source of HSV-2 responsible for genital skin lesions during initial infection (36). Thus, after vaginal replication, virus transmitted via peripheral nerves replicates in central neural tissues and then is transported via peripheral nerves to genital skin where local HSV replication produces vesicular skin lesions. Reduced amounts of TK− HSV-2 in neural tissues could be a consequence of reduced transmission of virus to the neural sites or reduced replication in neural tissues. In either case, the correlation between low TK− HSV-2 titers in neural tissues and mild genital skin disease supports the hypothesis that replication of HSV in neural tissue is an essential component in the pathogenesis of genital herpes.

Latent HSV within the host is felt to be the source of virus responsible for recurrent herpetic disease (25, 28, 38, 44). In the past 50 years, both direct and indirect evidence has been amassed to support the suggestion of Goodpasture (13) that neurons in the sensory ganglia harbor latent virus. More recently, it has been demonstrated that HSV may establish a latent infection in extraneural tissues, including the guinea pig vagina and footpad (34) and the mouse vulvovagina (43) and ear pinna (15). Our observation that latent virus may also be recovered from external genital skin and uterine cervix after recovery from HSV-2 genital infection suggests that extraneural latent virus may play a role in the pathophysiology of recurrent HSV infection and possibly in the evolution of cervical carcinoma (2, 24). Currently, it is unknown whether there are differences between virus latent in skin and virus latent in ganglia, or whether the latent virus in extraneural tissue resides in neural or nonneural elements. The relative importance of virus latent in neural and extraneural sites in producing recurrent genital disease and cervical carcinoma remains to be defined.
The ability of a TK- HSV mutant to establish a latent infection appears related to whether or not the mutant replicates in neural tissues. Thus, studies in which virus could not be detected in ganglia during acute infection also reported no recovery of latent TK- HSV (14, 23, 30). In contrast, when TK+ virus has been recovered from ganglia during acute infection, as we observed, it has often been possible to demonstrate latent TK+ HSV infection (10, 11, 41, 42). These studies suggest that viral replication at neural sites may be necessary for the establishment of latency.

Although viral TK- is important for replication in neural tissues, it is not an obligatory requirement for establishment, maintenance, or reactivation of latent HSV. For example, Field and Lay (10) have recently characterized latent infections in mice inoculated with an HSV-1 strain that was clinically resistant to acyclovir. They reported that although latency was uncommonly established, latent TK- HSV-1 could be isolated from some ganglia. Similarly, our studies of genital HSV-2 infection of guinea pigs demonstrate that viral TK is not an essential requirement for latent HSV-2 infection in either neural or extraneural tissues. In addition, our data suggest that only low levels of virus replicating in neural tissues are necessary to establish latent infection. Such a hypothesis is supported by the observation that although acyclovir treatment of infected mice reduced HSV replication in neural tissues, the establishment of latent infection was not prevented (9).

The frequency with which we detected latent TK- virus was higher than that reported by other investigators (10, 11, 41, 42). Most studies that have explored the role of TK in latency have utilized HSV-1 mutants with eye or skin inoculation of mice (10, 11, 14, 21, 30, 41, 42). Several factors reported to be important in the establishment of latency may have contributed to the differences between our findings and those of others. These factors include virus strain (12, 31), route of inoculation (26, 31), and animal species (32). A possible explanation for the higher frequency with which we detected latent virus compared with investigators in other laboratories is the difference in methods used to search for virus. We used sensitive DNA hybridization techniques to probe for HSV DNA. Additionally, because TK- viruses do not replicate in nondividing cells as well as TK+ HSV (16), to facilitate recovery of latent TK- virus, cells in the log phase of growth were frequently added to the flask used for explant cocultivation. With these techniques, the frequency of detection of latent TK- HSV-2 from guinea pig tissues was similar to that of TK+ virus. Other investigators have reported the recovery of TK+ virus after infection with a TK- HSV mutant (1, 30). In the present study, however, the enzyme assays (Table 3) and plaque autoradiographic studies (Fig. 5) demonstrated that the viruses reisolated from the TK- HSV-2-infected guinea pigs had no detectable TK activity and lacked the ability to induce TK activity. Thus, the recovery of latent TK- HSV-2 in our experiments is not attributable to residual enzyme-inducing activity in the TK- HSV-2, the presence of TK+ virus in the virus pools used to infect the guinea pigs, or formation in vivo of TK+ revertants.

These studies suggest that viral replication in neural tissues may play an important role in the pathogenesis of symptomatic HSV-2 genital infection. The absence of viral TK may alter the neurovirulence, and hence the pathogenesis, of acute HSV-2 genital infection, but may affect viral latency only indirectly. Viral TK, however, is not an essential requirement for latent infection in either neural or extraneural tissues.

**ACKNOWLEDGMENTS**

This work was supported in part by funds from the Children’s Hospital Medical Center, Cincinnati, Ohio, now celebrating its centennial anniversary. L.R.S. is the recipient of a John A. and George L. Hartford Fellowship. We thank Jackie Brody for technical assistance and Vicki Rieder for manuscript preparation.

**LITERATURE CITED**


