HSV-1 Thymidine Kinase Promotes Virulence and Latency in the Mouse

Y. J. Gordon, D. M. Gilden, and Y. Becker

The relationship between thymidine kinase (TK) activity and virulence was studied in the mouse using three HSV-1 strains: (1) NIH TK+ (100% activity), (2) NIH TK+/− (25% TK activity), and (3) NIH TK− (0% TK activity). Following corneal inoculation, keratitis, virus titers (eye, trigeminal ganglia, brain), survival, and latency were determined for each strain. The most virulent strain, NIH TK+ (30% survival) produced the worst keratitis, highest CNS titers, and established latency in 78% of surviving mice. NIH TK+/− demonstrated dose-dependent intermediate virulence (57–90% survival) and established latency in 80% of mice. NIH TK−, the most avirulent strain (93–100% survival) produced eye virus titers equal to the other strains but did not appear to invade the CNS or establish latency. These results indicate that TK gene activity is essential for HSV-1 murine neurovirulence (i.e., efficient CNS invasion, replication, and establishment of latency), but not for ocular replication.


Different strains of HSV-1 have been shown to exist in nature, and to vary in their pathogenicity in animal studies. The enzyme, thymidine kinase (TK), plays an essential role in HSV-1 virus DNA synthesis, and inhibition of this enzyme by antiviral agents (acyclovir, IDU) inhibits virus multiplication. Alteration in the HSV-1 thymidine kinase (TK) gene to produce mutants lacking in thymidine kinase, TK− mutants, appears to reduce virus pathogenicity. The present study will further investigate in the mouse model the correlation between virulence and HSV-1 TK activity. Specifically, the ocular pathogenicity as well as neurovirulence of HSV-1 TK strains will be determined, and the clinical significance of HSV-1 TK− mutants will be considered.

Materials and Methods

Virus Strains

The NIH wild type (wt) strain No. 11124 of HSV-1, grown in BSC-1 monolayers with Eagles MEM plus 10% calf serum, served as a parental strain for the development of a large plaque (LP) (3 mm), and small plaque (SP) (1 mm) derivative. Each of these two strains were plaque-purified and found to be pure, stable, and to have comparable growth curves in tissue culture. A thymidine kinase negative mutant, NIH (TK−) was derived from the LP strain in the standard manner by incubating 100 plaque-forming units (PFU) of LP virus per plate in the presence of 10 mg/ml of bromodeoxyuridine (BUDR) as described by Das Gupta. The three derived strains were assayed for TK activity using an in vitro mouse L(TK−) cell assay previously described. The LP strain was found to have full TK activity (assigned value 100%), and designated NIH TK+. The SP strain had 25% TK activity relative to the LP strain and was designated NIH TK−. The NIH TK− mutant revealed no TK activity at all. All virus strains were frozen in Eagles MEM plus 10% calf serum at −80 F prior to use.

In a series of experiments, 4 to 6-week-old outbred male mice were divided into groups of ten, and each group was inoculated with one of the three experimental NIH TK strains. Following satisfactory sodium pentabarbital IP anesthesia (50 mg/kg), corneal inoculation was accomplished in different experiments by dripping 0.02 ml of 10⁶ PFU/ml coded virus onto both scarified corneas, and massaging the closed lids for 15 seconds. Virulence was evaluated on days 2, 4, 6, and 10 by grading in a masked fashion the severity of keratitis (scale 0–4) using a slit lamp, 0.12% fluorescein eye drops, and a cobalt blue filtered light source.

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Virulence was also measured by determining the eye, trigeminal ganglia (TG), and brain virus titers on days 1 through 6, and day 10 after inoculation. The above organs were removed aseptically and homogenized separately in a Down's Homogenizer in 1 cc of phosphate buffered saline (PBS). Following centrifugation (18,000 rpm for 10 min), serial tenfold dilutions of the supernatant were titrated in BSC-1 monolayers using the method of plaque assay. In a second experiment, virulence of the different virus strains was measured following corneal inoculation with two different virus titers: 0.02 ml of $10^6$ PFU/ml and 0.02 ml of $10^7$ PFU/ml. The mice were examined daily for survival up to 21 days. In a third experiment to detect the presence of latent virus, trigeminal ganglia from each group were explanted 21 days after HSV-1 corneal inoculation (0.02 ml of $10^6$ PFU/ml), washed, minced, and placed on BSC-1 monolayers in tissue culture. These cultures were examined daily for 28 days, and the delayed appearance of cytopathic effect (CPE), characteristic of HSV-1, indicated reactivation of latent virus from the ganglion. Following completion of all experiments, the codes were broken, and the data analyzed statistically using the Fisher Exact Test, chi square and Student's t-test analyses.

Results

Figure 1 summarizes the keratitis produced by the three HSV-1 TK strains after corneal inoculation with 0.02 ml of $10^6$ PFU/ml virus suspension. The observed differences in keratitis on days 4, 6, and 10 were not statistically significant. However, on day 2, the mean corneal score produced by the NIH TK+ strain (1.20) was significantly higher ($P < 0.001$) than the NIH TK+/- strain (0.26) and higher ($P < 0.005$) than the NIH TK- strain (0.38). While the NIH TK+ strain produced the variable sized dendrites (Fig. 2A) in 75% of inoculated eyes, the NIH TK+/- and NIH
TK− strains produced only punctate keratitis (Fig. 2B) in 35–50% of inoculated eyes. Significant differences in keratitis were observed on day 2 even though all three HSV-1 strains infected all eyes and produced statistically comparable eye virus titers on days 2, 4, 6, and 10 (Table 1). However, only the NIH TK− strain proved to be highly invasive and pathogenic (Table 2). The NIH TK+ strain infected 90% of trigeminal ganglia (mean virus titer 9.01 × 10^3 PFU/ml) which was significantly higher \( (P < 0.001) \) than the NIH TK−/− strain that infected only 20% of trigeminal ganglia (mean virus titer 1.70 × 10^3 PFU/ml). Similarly, the NIH TK+ strain infected 90% of brains (mean virus titer 3.17 × 10^3 PFU/ml), which was significantly greater \( (P < 0.002) \) than the NIH TK−/− strain that infected no brains. The NIH TK+ strain was also significantly more neurovirulent \( (P < 0.002) \) than the NIH TK−/− mutant, which did not appear to invade and/or multiply in the mouse CNS (negative TG and brain virus titers).

Table 2 summarizes the virulence of the different virus strains as shown by survival 21 days after corneal inoculation at two different virus titers. At the lower inoculating titer (0.02 ml of 10^6 PFU/ml), the NIH TK+ strain again proved to be the most virulent \( (P < 0.001) \) with 29% survivors vs 90% survivors for NIH TK−/−, 93% for NIH TK−, and 93% for media controls. However, the NIH TK−/− strain did demonstrate a dose-dependent increased virulence \( (P < 0.001) \) at the tenfold higher inoculating titer (0.02 ml of 10^7 PFU/ml) with 57% survivors compared to 90% survivors at the lower inoculating titer. The NIH TK− strain proved to be avirulent; no increased virulence was found at the higher inoculating titer, 95% survivors vs 93% survivors at the lower titer.

Finally, latency (Table 4) was demonstrated in seven of nine surviving mice (78%) in the NIH TK+ group, 24 of 30 mice (80%) in the NIH TK−/− group, and 0 of 27 mice (0%) in the NIH TK− group. It is significant \( (P < 0.001) \) that latent virus was readily recovered from TG of mice infected with HSV-1 strains demonstrating full or partial TK activity, NIH TK+ and NIH TK−/− respectively; whereas no latent virus could be detected in the TG of mice infected with the mutant totally lacking in TK activity, NIH TK−.

**Discussion**

In the present mouse model, virulence and latency correlated with the viral TK activity of the HSV-1 strains tested. The amount of viral TK activity appeared to influence (1) the severity of keratitis, (2) peripheral virus uptake, invasion, and multiplication in the CNS, and (3) the establishment of latency. The NIH TK+ (100% TK activity) produced the most severe keratitis, highest TG and brain titers, lowest survival, and established latency in 78% of survivors. The NIH TK−/− (25% TK activity) demonstrated a dose-dependent virulence and established latency in 80% of survivors. The NIH TK− (0% TK activity) multiplied well in the eye, but did not appear to invade or multiply in the CNS, establish latency, or kill any animals. The present study characterized the ocular pathogenicity of HSV-1 TK strains, and thereby extended the results of Field and Wildy, and

**Table 1. Mean eye virus titers (pfu/ml) N = 20 eyes/group**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Day 2*</th>
<th>Day 4*</th>
<th>Day 6*</th>
<th>Day 10*</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIH TK+</td>
<td>1.52 ± 1.75 × 10^2</td>
<td>1.74 ± 5.36 × 10^2</td>
<td>1.05 ± 2.13 × 10^2</td>
<td>0</td>
</tr>
<tr>
<td>NIH TK−/−</td>
<td>1.48 ± 2.82 × 10^2</td>
<td>2.05 ± 5.96 × 10^2</td>
<td>1.82 ± 4.01 × 10^2</td>
<td>0</td>
</tr>
<tr>
<td>NIH TK−</td>
<td>1.61 ± 1.54 × 10^2</td>
<td>3.78 ± 6.21 × 10^2</td>
<td>0.91 ± 3.38 × 10^2</td>
<td>0</td>
</tr>
</tbody>
</table>

* *P < 0.01 compared to NIH TK+*
† *P < 0.002 compared to NIH TK+*

<table>
<thead>
<tr>
<th>Virus</th>
<th>Trigeminal ganglia (N = 20) (%)</th>
<th>Brain (N = 10) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIH TK+</td>
<td>9.01 × 10^2 (90)</td>
<td>3.17 × 10^3 (90)</td>
</tr>
<tr>
<td>NIH TK−/−</td>
<td>1.70 × 10^6 (20)*</td>
<td>0 (0)f</td>
</tr>
<tr>
<td>NIH TK−</td>
<td>0 (0)f</td>
<td>0 (0)f</td>
</tr>
</tbody>
</table>

\* \*P < 0.01 compared to NIH TK+*
\† P < 0.002 compared to NIH TK+*

**Table 3. Survival (Day 21)**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Lower inoculum* 0.02 ml of 10^6 pfu/ml</th>
<th>Higher inoculum 0.02 ml of 10^7 pfu/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Survivors/group (%)</td>
<td>Survivors/group (%)</td>
</tr>
<tr>
<td>NIH TK+</td>
<td>30/104 (29)</td>
<td>N.D†</td>
</tr>
<tr>
<td>NIH TK−/−</td>
<td>112/125 (90)</td>
<td>17/30 (57)†</td>
</tr>
<tr>
<td>NIH TK−</td>
<td>98/113 (93)</td>
<td>19/20 (95)†</td>
</tr>
<tr>
<td>Media Control</td>
<td>70/75 (93)</td>
<td></td>
</tr>
</tbody>
</table>

* *P < 0.001 for observed differences among HSV-1 strains at lower inoculum.*
† *P < 0.001 compared to NIH TK−/− at the lower inoculum.*
‡ *Not done.*
CNS (TG) when the eye is inoculated with a mixture employed. More sensitive hybridization techniques provides the missing TK, which then enables TK metabolism due to its rapid turnover rate. In the present study, epithelial cells supported virus multiplication of all three HSV-1 TK strains tested. The NIH TK+ and NIH TK+/− strains multiplied well because they produce their own TK, which promotes virus multiplication, while the NIH TK− virus probably used available cellular TK to facilitate its own replication. In contrast, nondividing neural tissue in the CNS is characterized by low levels of thymidylate metabolism, and only HSV-1 strains capable of producing enough of their own TK (NIH TK+ at 10^6 PFU/ml inoculum, and NIH+/− at 10^7 PFU/ml inoculum) are able to multiply in these tissues. HSV-1 strains lacking in TK (NIH TK−) are unable to multiply in neural tissue, and therefore demonstrate reduced pathogenicity. Further support for this concept is offered by Tenser who has demonstrated in complementation studies that TK− mutants are able to multiply in the CNS (TG) when the eye is inoculated with a mixture of TK+ and TK− viruses. Presumably, the TK+ virus provides the missing TK, which then enables TK− virus to invade and multiply in neural tissue.

In the present study, the failure to demonstrate small amounts of TK− virus in the TG or brain during both the acute and latent stages of infection may also reflect a limitation in the sensitivity of the methods employed. More sensitive hybridization techniques are currently being used to look for HSV-1 TK− mutants in the TG.

The biology of HSV-1 TK− mutants has significant clinical relevance. Firstly, acyclovir, a promising antiviral agent, has been known to select readily for TK− mutants both in vitro and in man following parenteral drug therapy. Since acyclovir is currently being evaluated for the treatment of ocular herpes, it is important to understand the pathogenicity of emergent TK− mutants. The present study in the mouse suggests that TK− mutants induce a minimal, self-limited keratitis. However, additional studies are indicated to continue and extend this observation. Secondly, TK− mutants appear to demonstrate a striking CNS avirulence that suggests possible use as a herpes vaccine. Our preliminary studies show that following corneal inoculation, the NIH TK− mutant will produce the mouse against corneal challenge by the virulent NIH TK+ parental strain.

In conclusion, HSV-1 TK expression did appear to correlate with virulence and establishment of latency in the murine model, and further investigation into the nature of HSV-1 TK− mutants should be pursued.

Key words: HSV-1, thymidine kinase negative mutants, keratitis, neurovirulence, latency

References


Table 4. Recovery of latent virus from trigeminal ganglia (Day 21)

<table>
<thead>
<tr>
<th>Virus</th>
<th>Positive mice*/group†</th>
<th>(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIH TK+</td>
<td>7/9</td>
<td>(78)</td>
</tr>
<tr>
<td>NIH TK+/−</td>
<td>24/30</td>
<td>(80)</td>
</tr>
<tr>
<td>NIH TK−</td>
<td>0/27</td>
<td>(0)</td>
</tr>
</tbody>
</table>

* At least one positive ganglion/mouse.
† P < 0.001 for observed differences among HSV-1 strains.

Tenser and Dunstan who reported that TK− strains of HSV-1 have reduced CNS pathogenicity when compared to the TK+ parent strains.

One possible explanation of the observed experimental results is based on the level of thymidylate metabolism of the infected host cell. Ocular epithelium presumably has a high level of thymidylate metabolism due to its rapid turnover rate. In the present study, epithelial cells supported virus multiplication of all three HSV-1 TK strains tested. The NIH TK+ and NIH TK+/− strains multiplied well because they produce their own TK, which promotes virus multiplication, while the NIH TK− virus probably used available cellular TK to facilitate its own replication. In contrast, nondividing neural tissue in the CNS is characterized by low levels of thymidylate metabolism, and only HSV-1 strains capable of producing enough of their own TK (NIH TK+ at 10^6 PFU/ml inoculum, and NIH+/− at 10^7 PFU/ml inoculum) are able to multiply in these tissues. HSV-1 strains lacking in TK (NIH TK−) are unable to multiply in neural tissue, and therefore demonstrate reduced pathogenicity.

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