The Role of Pseudorabies Virus Thymidine Kinase Expression in Trigeminal Ganglion Infection

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SUMMARY

The role of pseudorabies virus (PRV) thymidine kinase (TK) expression in the pathogenesis of PRV infection of mice was studied with TK-negative (TK-) mutants. Thymidine phosphorylation and arabinosylthymine inhibition of PRV replication and efficiency of plating were used to characterize TK+ and TK- PRV. In addition, a plaque autoradiography procedure was utilized to determine the TK phenotype of individual plaques. TK+ and TK- PRV replicated well in ocular tissues, while TK+ but not TK- did so in ganglion tissue. Mortality was absent after TK- PRV inoculation and widespread after inoculation of similar amounts of TK+ PRV. Latent infection in mice was not detected with either TK+ or TK- PRV. This study indicated the probable importance of PRV TK expression in acute trigeminal ganglion infection.

From investigations with several thymidine kinase-negative (TK-) mutants of herpes simplex virus (HSV) it has become apparent that expression of the virus-specified enzyme is important in the establishment of acute and latent trigeminal ganglion (neuron) infection (Field & Wildy, 1978; Tenser & Dunstan, 1979; Klein et al., 1980). It was hypothesized that herpesviruses such as HSV and varicella-zoster virus, which frequently establish neuronal infections, would require virus-specific TK, whereas cytomegalovirus and Epstein–Barr virus which have a different natural pathogenesis would not (Tenser & Dunstan, 1979). Since experiments to investigate the role of TK expression cannot be performed with varicella-zoster virus, another herpesvirus known to infect the nervous system, pseudorabies virus (PRV), was studied.

PRV causes a naturally occurring infection of swine and bovines and experimentally a severe infection of the nervous system in mice, rats, guinea-pigs and rabbits (McCracken et al., 1973; Field & Hill, 1974; Wittmann et al., 1980). In swine, latent trigeminal ganglion infection may be established (Sabó & Rajcánı, 1976; Gutekunst et al., 1980). In experimental infections, PRV apparently spreads through the nervous system in a sequential pattern very similar to that of HSV. Following peripheral inoculation of PRV, sensory and autonomic ganglion infections ensue, apparently secondary to axonal transport of the virus. Because of the known neural infection produced by PRV in mice and since PRV expresses a virus-specific TK (Hamada et al., 1966), it was of interest to compare TK+ and TK- PRV infections.

Five- to seven-week-old random bred male and female mice (CD-1; Charles River Laboratories, Wilmington, Mass., U.S.A.) were anaesthetized and corneally inoculated bilaterally with 25 μl of virus suspension. TK+ PRV and TK- PRV derived by passage in medium containing arabinosylthymine (ara-T) were obtained from T. Ben-Porat (Vanderbilt University, Nashville, Tenn., U.S.A.). The TK+ stock consisted of infected primary rabbit kidney (RK) cell suspension that had been clarified and diluted to $5 \times 10^5$ p.f.u./0.1 ml. TK-PRV was first plated on RK cells under 0-5% methylcellulose containing ara-T, 150 μg/ml. Experimental TK- stocks were grown from two large plaques in RK cells in medium without
ara-T. These stocks, TK\textsubscript{2} and TK\textsubscript{5}, with titres of $8.5 \times 10^5$ and $3.2 \times 10^5$ p.f.u./0.1 ml respectively, were used for subsequent experiments.

Three methods were used to test the TK phenotype of stock viruses and isolates from animals. Phosphorylation of thymidine (TdR) by lysates of lytically infected TK\textsuperscript{-} mouse cells, and inhibition of TK\textsuperscript{+} and TK\textsuperscript{-} viruses by ara-T was studied as described previously for HSV (Tenser \textit{et al.}, 1981). In addition, viruses were tested by [\textsuperscript{14}C]TdR plaque autoradiography; the procedure was recently described in detail (Tenser \textit{et al.}, 1983). This procedure and ara-T inhibition of efficiency of plaque formation (e.o.p.) were particularly useful for evaluations of the TK phenotypes of animal isolates.

During the acute period of infection (1 h to 5 days after corneal inoculation) cell-free virus was isolated from eye swabs and trigeminal ganglion homogenates. Bilateral eye swabs from each mouse were pooled in medium containing 10\% serum, and both trigeminal ganglia were pooled and homogenized in similar medium. Titrations of ganglion homogenates were performed in RK cells with or without ara-T in the methylcellulose overlay, and autoradiography of plaques was evaluated. At times after infection when latency would be expected to have been established (26 to 41 days after corneal inoculation), isolation of virus was tested by co-cultivation with RK cells. Monolayers were examined periodically and discarded after 4 weeks if negative.

In assays of TdR phosphorylation by lysates of TK\textsuperscript{-} cells lytically infected with TK\textsuperscript{+} and TK\textsuperscript{-} PRV, optimal results were obtained by infection at an m.o.i. of 5 for 24 h in assays run at pH 6 at 37°C. In such assays TdR phosphorylation by TK\textsuperscript{+} PRV was 11.2 pmol/mg protein/20 min (mean of three assays). For the TK\textsubscript{2} and TK\textsubscript{5} viruses, values were 1\% and 0\% respectively of the TK\textsuperscript{+} value. In assays at 33°C the TK\textsuperscript{+} value decreased slightly (to 76\%). In assays at pH 8 and 7 values were similar to the maximum (96\% and 92\% respectively), but at pH 5 TdR phosphorylation dropped markedly (to 6\%).

Testing for ara-T inhibition of TK\textsuperscript{+} PRV replication with 50 μg/ml of the drug showed inhibition by 23-fold. Inhibition was increased to 96-fold with 100 μg/ml and to 2000-fold with 150 μg/ml. Ara-T (150 μg/ml) inhibition of TK\textsuperscript{-} PRV replication was slight (2.5- to 3.8-fold) for the two viruses tested. In e.o.p. assays the inhibition of TK\textsuperscript{+} PRV by ara-T (150 μg/ml) was 510-fold, and for the TK\textsuperscript{-} viruses inhibition was minimal (0.3- to 0.4-fold). The e.o.p. assays using 150 μg/ml of ara-T were performed to characterize virus isolated from mice after TK\textsuperscript{-} PRV inoculation.

Fig. 1 shows plaque autoradiography results obtained with stock TK\textsuperscript{+} PRV and TK\textsuperscript{5} PRV. TK\textsuperscript{+} plaques are clearly seen in Fig. 1 (a), and all 282 plaques in the cell monolayer appeared on the autoradiogram; no TK\textsuperscript{-} virus was present. On the other hand, in Fig. 1 (b) all plaques (approx. 1000) were TK\textsuperscript{-} since [\textsuperscript{14}C]TdR uptake had not occurred. From plaque autoradiograph assays, the reversion frequencies of the TK\textsubscript{2} and TK\textsubscript{5} viruses were <10\textsuperscript{-3}.

Replication of TK\textsuperscript{+} and TK\textsuperscript{-} PRV in ocular tissues and in trigeminal ganglion during the period of acute infection is seen in Fig. 1 (c, d). After inoculation of similar amounts of virus the peak mean titre in ocular tissues for TK\textsubscript{2} PRV (1.3 × 10\textsuperscript{5} p.f.u./eye) was slightly less than that for TK\textsuperscript{+} PRV (4.2 × 10\textsuperscript{5} p.f.u./eye) (Fig. 1c). The peak titre for TK\textsubscript{5} was similar (9.0 × 10\textsuperscript{4} p.f.u./eye). Evidence of \textit{in vivo} replication was apparent. Because of the importance of quantities of ocular virus in interpreting trigeminal ganglion titres, a group of mice were corneally inoculated with smaller amounts of TK\textsuperscript{+} PRV. Results are shown with the virus inoculum (2 × 10\textsuperscript{4} p.f.u./0.1 ml) which resulted in the survival of 30 to 50\% of mice. The rate of virus replication was rapid, and on days 1 and 2 titres were similar to those for the other viruses. On all days ocular virus was undetectable in at least one animal, which raises the possibility that these mice were never infected. However, the percentages of mice without detectable ocular virus on days 1, 2, 3 and 5 increased from 12.5\% (1 of 8 mice) to 33\% (3 of 9) to 57\% (4 of 7) to 100\% (6 of 6). These increasing percentages, and since no mice were lost through death prior to 3 days post-inoculation, indicate that most mice negative for ocular virus on days 3 and 5 probably had cleared the virus. It is probable that 12.5\% is a maximal estimate of the proportion of uninfected mice.

As seen in Fig. 1 (d), TK\textsuperscript{+} PRV replicated in trigeminal ganglion to high titre (4.7 × 10\textsuperscript{4} p.f.u./mg), but titres of PRV TK\textsubscript{2} and TK\textsubscript{5} were minimal (7.5 × 10\textsuperscript{3} and 3 × 10\textsuperscript{3} p.f.u./mg).
Fig. 1. (a, b) Plaque autoradiography with [14C]TdR of 3-day-old PRV plaques in 150 mm culture dishes. (a) Stock TK+ virus: all 282 plaques counted in the monolayer culture from which this autoradiogram was obtained are TK+. (b) Stock TK− virus: all of the approximately 1000 plaques in the monolayer culture are TK−; evidence of isotope incorporation is not present. Many plaques can be seen and although their rims are slightly denser than their centres, they are easily distinguished from TK+ plaques. (c, d) Replication of TK+ and TK− PRV in ocular tissues and trigeminal ganglia of mice. Three to six mice per point were used; means and standard errors are shown. ••••, TK+ PRV; ○○○○, TK− PRV at low dose; ●●●●, TK7 PRV; ○○○○, TK8 PRV. (c) Virus replication in ocular tissues as detected in eye swabs. (d) Virus replication in trigeminal ganglion tissue in the same mice detected in ganglion homogenates. □□□□, ara-T titration results of TK7 and TK8 respectively. To calculate means, eye swab and ganglion titrations with undetectable virus were scored as zero and are indicated by ↓ under the plotted values.
Table 1. Survival and latent trigeminal ganglion infection after corneal inoculation of TK+ and TK− PRV

<table>
<thead>
<tr>
<th>Virus inoculated</th>
<th>Titre (p.f.u./0-1 ml)</th>
<th>No. survived/ No. inoculated (%)</th>
<th>No. latently infected*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRV TK+</td>
<td>5 × 10^5</td>
<td>0/10 (0)</td>
<td>-†</td>
</tr>
<tr>
<td></td>
<td>1 × 10^5</td>
<td>0/5 (0)</td>
<td>-†</td>
</tr>
<tr>
<td></td>
<td>5 × 10^4</td>
<td>1/6 (17)</td>
<td>0/1</td>
</tr>
<tr>
<td></td>
<td>1 × 10^3</td>
<td>11/11 (100)</td>
<td>0/11</td>
</tr>
<tr>
<td>PRV TK−</td>
<td>9 × 10^5</td>
<td>11/11 (100)</td>
<td>0/11</td>
</tr>
<tr>
<td>PRV TK−</td>
<td>3 × 10^5</td>
<td>11/11 (100)</td>
<td>0/11</td>
</tr>
</tbody>
</table>

* Latent ganglion infection was determined by evidence of virus reactivation following co-cultivation with primary RK cells.
† No animals survived to the period of latency.

respectively). Little if any inhibition of the e.o.p. of the latter ganglion isolates by ara-T indicated their probable TK− phenotype. The TK phenotype of these ganglion isolates was further evaluated by plaque autoradiography. Of 201 trigeminal ganglion plaques derived from TK−-inoculated mice and tested by autoradiography, all were TK−.

In mice inoculated with the lesser amount of TK+ PRV, replication in trigeminal ganglion was apparent although to slightly lower titres compared to the larger inoculum. Three days after inoculation of the lesser amount of TK+ PRV, 17% of ganglion homogenates were negative for virus; this is an estimate of the proportion of mice that did not develop neural infections and is similar to the 12.5% of mice estimated to be uninfected from ocular isolation results. Ganglion titres after TK+ PRV inoculation were much greater than after inoculation of the TK− viruses. The very similar ocular titres but very dissimilar trigeminal ganglion titres indicate the probable importance of the viral TK for replication in ganglion. Clinically, while TK+ inoculated mice showed considerable discomfort and scratching, TK−-inoculated mice showed no unusual behaviour, despite high ocular virus titres.

As seen in Table 1 all mice survived corneal inoculation with the TK− viruses, but none survived inoculation with similar amounts of TK+ virus. Survival was obtained after inoculation of diluted TK+ PRV. In view of the minimal replication of virus in the ganglion during the acute period of infection, it was not surprising that latent trigeminal ganglion infection was not detected after TK− inoculation. However, latent infection was also not detected in survivors of TK+ PRV inoculation.

From the results it appeared that expression of PRV TK was important for virus replication in trigeminal ganglion tissue but not for replication in ocular tissues. Due to the probable axonal route of virus transport from cornea to trigeminal ganglion (McCracken et al., 1973; Field & Hill, 1974), the restriction of TK− PRV replication in trigeminal ganglion tissue is equivalent to restriction of replication in ganglion neurons. The small amount of TK− PRV in ganglion tissue 3 days after corneal inoculation probably reflected axonal transport of ocular virus, although a low level of ganglionic replication cannot be excluded. Inoculation of a group of mice with the smaller inoculum of TK+ PRV was performed to decrease the amount of ocular virus and to determine the effect of trigeminal ganglion titres. On days 2 and 3 the similar ocular titres but very different trigeminal ganglion titres for the TK− viruses and low inoculum TK+ virus indicate the probable important role for PRV TK expression in ganglion (neuron) infection. These results support the hypothesized importance of TK expression for herpesviruses that infect neurons.

Since mortality from PRV appears to be due to nervous system disease and since TK− PRV probably did not replicate in trigeminal ganglion (neurons), it was not surprising that there were no mortalities after corneal inoculation with TK− PRV. In addition, the lack of TK− latent infection was not surprising. What was surprising, however, was the lack of latent trigeminal ganglion infection in mice that survived acute infection after TK+ PRV inoculation. It appeared that mice either died with acute infection or survived and were not latently infected. At this
point it can only be stated that latent infection as detected by co-cultivation could not be detected. It would be of interest to study tissues by other methods such as *in situ* hybridization as has been performed for HSV (Galloway et al., 1982; Tenser et al., 1982).

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REFERENCES


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