INTRODUCTION

Herpes simplex virus (HSV) thymidine kinase (TK) activates a number of highly effective and specific antiviral drugs, such as acyclovir, that are widely used for prophylaxis and treatment of viral infections. However, resistance to acyclovir is frequently observed in the clinic and is a particularly serious problem to immunocompromised patients (Christophers et al., 1998; Englund et al., 1990). Many acyclovir-resistant clinical isolates have been reported to lack detectable TK activity, often due to mutations that prevent the synthesis of full-length polypeptide (Gilbert et al., 2002). Interestingly, laboratory strains engineered to lack TK activity are not observed to replicate in mouse ganglia and do not reactivate from latently infected mouse ganglia (Coen et al., 1989b; Efstathiou et al., 1989; Thompson & Sawtell, 2000). Given their substantial attenuation in animal models, understanding how TK-negative (TK−) mutants cause disease in humans is an important clinical question. There are several lines of evidence that may help to answer this question. One is that many clinical acyclovir-resistant isolates express low levels of active TK that are sufficient for ganglionic replication and reactivation (Griffiths & Coen, 2003; Hwang et al., 1994). Alternatively, there appear to be viruses circulating within the population that do not absolutely require TK to be pathogenic (Griffiths et al., 2003; Horsburgh et al., 1998). It has also been suggested that another mechanism exists, in which a population of virus that is predominantly TK− may benefit from TK activity provided in trans from a subpopulation of TK-positive (TK+) viruses. To recapitulate this in vivo, mice were infected with mixtures of wild-type virus and a recombinant TK− mutant in various ratios. Following co-infection, the replication, number of latent viral genomes and reactivation efficiency of TK+ virus in trigeminal ganglia were reduced in a manner related to the amount of TK− virus in the inoculum. TK+ virus did not always complement the acute replication or increase the number of latent viral genomes of TK− mutant in mouse ganglia. Even so, TK+ virus could still confer the pathogenic phenotype to a TK− mutant, somehow providing sufficient TK activity in trans to permit a TK− mutant to reactivate from latently infected ganglia.
replication of TK⁺ virus in ganglia during acute infection. To understand how TK⁻ virus confers the pathogenic phenotype to a TK⁻ mutant, we investigated the interaction between TK⁺ and TK⁻ viruses during co-infection.

METHODS

Cells and viruses. Vero and TK⁻ human osteosarcoma (143) cells were propagated as described by Weller et al. (1983). Wild-type HSV-1 strain KOS and recombinant viruses, tkLTRZ1 (Davar et al., 1994) and KOS-GFP, were grown in Vero cells and titrated as described previously (Leib et al., 1991). KOS-GFP, kindly provided by Priscilla Schaffer (Harvard Medical School, Boston, MA, USA), was constructed by the insertion of a cytosine/green fluorescent immediate-early promoter–green fluorescent protein (GFP) gene cassette into the intergenic region between the UL26 and UL27 genes of KOS (Sainz & Haldow, 2002).

Assays of acute and latent infections in mice. All mouse experiment protocols were approved by the Laboratory Animal Committee at National Cheng Kung University. Seven-week-old male ICR mice (Charles River Laboratories) were maintained at our college laboratory animal centre. Mice were anaesthetized and then inoculated on scarified cornea with KOS, tkLTRZ1 or a mixture of both as described previously (Coen et al., 1989a; Leib et al., 1991). Virus titres at the site of inoculation and trigeminal ganglia were assayed by swabbing the eye at 1 day post-infection (p.i.) and by excising and homogenizing ganglia 3 days p.i. At 30 days p.i., mice were sacrificed and trigeminal ganglia were excised and tested for the presence of reactivatable virus following dissociation as described by Leib et al. (1991), except that cultures were screened for 10 days following explant and 5 days following replating.

Plaque autoradiography. Plaque autoradiography was performed as described previously (Chen et al., 1998; Horsburgh et al., 1998) with the following modifications. After 5 days at 37 °C, the methylcellulose overlay was removed and the cell monolayer was incubated with medium containing 2-6 μCi (96-2 KBg) [3H]thymidine (methyl-[^3H], 25 Ci mmol⁻¹; Amersham Biosciences) for 8 h at 37 °C. The cells were then stained, washed and air-dried to obtain images.

Quantitative PCR. Trigeminal ganglia were removed from latently infected mice, frozen in liquid nitrogen and homogenized in guanidine thiocyanate. One-tenth of each ganglion homogenate was assayed by quantitative PCR for viral DNA and cellular (adipsin) DNA as described by Kramer & Coen (1995) with the following modifications. The primer pair tk-12 (5′-GGCACACGTT-TATACAG) and tk-16 (5′-AACAATGGGATGCTTATGCC) was used for KOS, and the primer pair Lac1 (5′-AGCACAACACC-AAGCAGCA) and Lac2 (5′-AGCGACATCCAGGGAC) was used for tkLTRZ1. The reaction mixture for both KOS and tkLTRZ1 contained 1-5 mM Mg²⁺, the annealing temperature was 55 °C and the DNA was amplified for 30 cycles. PCR products were separated by electrophoresis on 1% agarose gels for KOS and 12% non-denaturating polyacrylamide gels for tkLTRZ1. Separated products were transferred to nylon membranes and probed with labelled oligonucleotide tk-10 (5′-TACCGTGCGGTATCTGCA) for KOS or with labelled oligonucleotide Lac3 (5′-CTGCACCTGAGTTGCGG) for tkLTRZ1. Primer pair tk-12 and tk-16 flanks the lacZ insertion in tkLTRZ1, so this primer pair will amplify a 541 bp PCR product for KOS and an approximately 4-6 kb PCR product for tkLTRZ1. Under our PCR conditions, tk-12 and tk-16 only amplified the expected 541 bp PCR product for KOS, but not the ~4-6 kb PCR product for tkLTRZ1. Primer pair Lac1 and Lac2 amplified the expected 163 bp PCR product specifically from the lacZ gene inserted in tkLTRZ1. Mouse cellular (adipsin) DNA was quantified as described by Katz et al. (1990). For each sample, the amount of viral DNA was normalized to cellular (adipsin) DNA and calculated on a per ganglion basis as described by Kramer & Coen (1995).

Southern blot analysis. Viral DNA was extracted with phenol/chloroform, digested with BamHI, separated by electrophoresis and probed with a 506 bp BglII–SacI fragment from the pSVTK1 plasmid (Horsburgh et al., 1998).

Co-localization of TK⁺ and TK⁻ viruses in mouse ganglionic cells. Trigeminal ganglia were excised from 8-week-old ICR mice and digested into single-cell suspensions as described by Leib et al. (1991). These suspensions were infected with KOS-GFP or KOS-GFP plus tkLTRZ1. After 24 h, the suspensions were centrifuged, fixed with 2% paraformaldehyde for 20 min and washed with medium containing 0-1% saponin. The suspensions were incubated with anti-MAP2 antibody (Upstate) at room temperature for 1 h and then with secondary antibody conjugated with Alexa Fluor 550 (Molecular Probes) at room temperature for 1 h. After incubation, the suspensions were washed, resuspended with medium containing 300 μM chloroquine and incubated at 37 °C for 30 min to inactivate endogenous β-galactosidase activity. After incubation, the suspensions were washed, centrifuged, resuspended gently with medium containing 33 μM fluorogenic substrate of β-galactosidase (C₆₋RGI) (Molecular Probes) and incubated for 30 min at 37 °C. After incubation, the cultures were washed and observed under a fluorescence microscope.

Assay of TK activity. Trigeminal ganglia of mice mock-infected or infected with KOS, tkLTRZ1 or a mixture of both viruses were harvested at 3 days p.i.

Vero cells (2 × 10⁶ cells), mock-infected or infected with 1 × 10⁵ p.f.u. KOS, 1 × 10⁵ p.f.u. tkLTRZ1 or a mixture of both viruses, were harvested at 18 h p.i. Samples were lysed with 10 mM sodium phosphate buffer and then frozen at −80 °C for 2 h. The samples were thawed and assayed for viral TK activity as described by Jacobson et al. (1998) with the following modifications. The samples were spotted onto Whatman DE81 paper and each paper was washed and digested with 4 ml 75 mM sodium acetate (pH 7.5) containing 10 mg cellulase at 37 °C for 1 h. After digestion, the radioactivity was measured by scintillation counting. For each sample, the TK activity was normalized to the protein content, which was determined by Bradford assay.

RESULTS

Co-infection did not affect the replication of TK⁺ or TK⁻ virus during acute infection in the eyes of infected mice

Initially, we investigated how much TK⁺ virus is required during co-infection to permit a TK⁻ mutant virus to replicate in or reactivate from mouse ganglia. Mice were anaesthetized and scarified corneas were inoculated with 10⁵, 10⁶ or 10⁷ p.f.u. wild-type (wt) HSV-1 strain KOS mixed with 10⁶ p.f.u. virus tkLTRZ1. tkLTRZ1 is a recombinant virus derived from strain KOS that lacks TK activity due to an insertion within tk of the Escherichia coli lacZ downstream of the Moloney murine leukemia virus long terminal repeat (Davar et al., 1994). We have demonstrated previously that the insertion in tkLTRZ1 does not affect the expression of an adjacent gene, UL24, that regulates virus replication and reactivation in mouse trigeminal ganglia (Chen et al., 2004; Jacobson et al., 1998). This virus was chosen for this study because it does not replicate in or
reactivate from infected mouse trigeminal ganglia (Chen et al., 1998, 2004; Griffiths & Coen, 2003; Griffiths et al., 2003; Jacobson et al., 1998). In addition, tkLTRZ1 can be distinguished easily from KOS by plaque autoradiography assay, simple colorimetric assay (including X-Gal in the cell-culture medium) and its DNA pattern on a Southern blot.

Virus titres at the site of inoculation were monitored by assaying virus in the tear film at 1 day p.i. (Chen et al., 2004). In agreement with our previous study (Chen et al., 2004), the titre of KOS in the eye increased concomitantly with inoculation (Table 1). The titre in the eyes of mice infected with $10^6$ p.f.u. tkLTRZ1 was almost identical to that of mice infected with $10^5$ p.f.u. KOS, perhaps suggesting that, at these inocula, a maximal level of replication in the eye had been reached. Consistent with this idea, virus titres in the eyes co-infected with both viruses ($10^8$ p.f.u. tkLTRZ1 plus $10^5$ p.f.u. KOS) were not higher than when these inocula were used individually. To determine the TK phenotype of the viruses replicating in the eyes of co-infected mice, we employed plaque autoradiography. It was found that, in each of the co-infected groups, >99% of the virus replicating in the eyes was TK$^{-}$ (Table 1). Taken together, these results indicate that co-infection had no discernible effect on the replication of wt and TK$^{-}$ viruses in the eyes of infected mice at 1 day p.i.

**Acute replication of TK$^{+}$ virus was reduced at all three co-infection ratios, whilst acute replication of TK$^{-}$ mutant was only observed with a high ratio of TK$^{+}$:TK$^{-}$ virus in the inoculum**

The ability of the virus to replicate in trigeminal ganglia was assessed by titration of the virus in homogenized ganglia harvested at 3 days p.i. In agreement with our previous observations (Chen et al., 2004), virus could not be detected in ganglia from mice infected with $10^8$ p.f.u. tkLTRZ1 (Table 1). The titre of KOS in ganglia increased concomitantly with inoculum. Despite robust replication in the ganglia of mice co-infected with $10^5$ p.f.u. KOS, the mean titre ($10^{12.2}$ p.f.u.) was significantly lower than that ($10^{14.4}$ p.f.u.) when inoculum contained only $10^5$ p.f.u. KOS ($P<0.01$, Student’s $t$-test). Plaque autoradiography revealed that TK$^{-}$ virus was present in all five ganglia tested (Table 1) and more than two-thirds (69%) of the viruses in these samples were TK$^{-}$. Therefore, a minimum ratio of 1:1000 of TK$^{+}$:TK$^{-}$ virus in the inoculum was required to permit the replication of TK$^{-}$ mutant in mouse ganglia. In contrast, in the mice infected with tkLTRZ1 and $10^5$ p.f.u. KOS, no virus could be detected in ganglia. Therefore, co-infection of mice with TK$^{-}$ mutant reduced the ganglionic replication of TK$^{+}$ virus significantly in all three co-infected groups compared with infection with KOS alone. Notably, ganglionic replication of KOS in animals infected with $10^5$ p.f.u. KOS and $10^8$ p.f.u. tkLTRZ1 was reduced by more than 2000-fold (Table 1).

**In latently infected ganglia, co-infection reduced the number of genomes of TK$^{+}$ virus in all three co-infected groups, but increased the number of genomes of TK$^{-}$ mutant in two groups with high ratios of TK$^{+}$:TK$^{-}$ virus in the inoculum**

We next investigated the effect of co-infection on the efficiency with which TK$^{+}$ and TK$^{-}$ viruses establish latency

<table>
<thead>
<tr>
<th>Table 1. Acute infections of mice</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Virus phenotype (inoculum, p.f.u.)</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>TK$^{+}$ ($10^6$)</td>
</tr>
<tr>
<td>TK$^{+}$ ($10^5$)</td>
</tr>
<tr>
<td>TK$^{+}$ ($10^4$)</td>
</tr>
<tr>
<td>TK$^{+}$ ($10^3$)</td>
</tr>
<tr>
<td>TK$^{+}$ ($10^3$) + TK$^{-}$ ($10^6$)</td>
</tr>
<tr>
<td>TK$^{+}$ ($10^3$) + TK$^{-}$ ($10^5$)</td>
</tr>
<tr>
<td>TK$^{+}$ ($10^3$) + TK$^{-}$ ($10^4$)</td>
</tr>
</tbody>
</table>

*Calculated by averaging the logarithms of the titres.
†Determined by plaque autoradiography.
§ND, Not done.
$P<0.01$ compared with TK$^{+}$ ($10^5$) group, Student’s $t$-test.
$P<0.01$ compared with TK$^{+}$ ($10^4$) group, Student’s $t$-test.
$P<0.01$ compared with TK$^{+}$ ($10^3$) group, Student’s $t$-test.
by quantifying their viral genomes in latently infected ganglia. Ganglia were harvested 30 days p.i. and the numbers of KOS and tkLTRZ1 genomes were determined by quantitative PCR. Table 2 shows that, compared with infection with tkLTRZ1 alone, the presence of $10^7$ p.f.u. KOS in the inoculum increased the number of tkLTRZ1 genomes in latently infected ganglia significantly (3-2-fold).

Interestingly, $10^4$ p.f.u. KOS also increased the number of tkLTRZ1 genomes slightly, despite this inoculum of KOS not supporting measurable ganglionic replication of tkLTRZ1 at acute times. Therefore, as few as 1 p.f.u. TK$^+$ per 10 000 p.f.u. TK$^-$ virus in the inoculum was sufficient to increase the number of tkLTRZ1 genomes in latently infected ganglia, even in the absence of detectable replication at acute times. In contrast, co-infection with $10^7$ p.f.u. KOS did not cause an increase in the number of tkLTRZ1 genomes.

Consistent with the observation that co-infection with TK$^-$ mutant reduced the replication of wt virus in ganglia significantly during acute infection, co-infection with a TK$^-$ mutant reduced the number of TK$^+$ virus genomes in latently infected ganglia in all three co-infected groups and by as much as 5-fold (Table 2). Notably, KOS genomes were detected in three of six ganglia (50 %) from mice infected with $10^7$ p.f.u. KOS alone, but co-infection with tkLTRZ1 decreased the detection rate significantly to one of eight ganglia (12 %).

**Co-infection permitted the reactivation of TK$^-$ mutant, but reduced the reactivation efficiency of TK$^+$ virus, in latently infected mouse ganglia**

To determine the effect of co-infection on the reactivation of TK$^+$ and TK$^-$ viruses, latently infected ganglia were harvested 30 days p.i. and reactivation was assessed.

Consistent with previous studies (Chen et al., 1998, 2004), no reactivation was observed in 38 ganglia infected latently with tkLTRZ1 alone, and the reactivation efficiency of KOS-infected ganglia increased concomitantly with inoculum (Table 2). Reactivation was observed in all three groups of ganglia co-infected with both viruses, and the reactivation efficiency correlated with the amount of KOS in the inoculum. These data demonstrated that only 1 p.f.u. TK$^+$ virus per 100 000 p.f.u. TK$^-$ virus in the inoculum was sufficient to permit reactivation of the TK$^-$ mutant. It is worth mentioning that this mixed inoculum did not permit detectable ganglionic replication of tkLTRZ1 at acute times and resulted in a ratio of only 1 KOS genome for every 10 000 tkLTRZ1 genomes in latently infected ganglia. Levels of reactivation from ganglia infected latently with $10^4$ or $10^5$ p.f.u. KOS alone were comparable to those of the co-infected samples. However, co-infection with tkLTRZ1 reduced reactivation from ganglia latently infected with $10^3$ p.f.u. KOS significantly, from 64 to 19 % (Table 2).

Plaque autoradiography revealed that TK$^-$ virus was present in the viruses that reactivated from every ganglion in all three co-infected groups. The percentage of reactivated TK$^-$ virus in the co-infected groups (21, 30 and 37 %) appeared to correlate with the amount of KOS in the inoculum.

To examine the viruses that reactivated, samples from the three co-infected groups were grown in medium containing X-Gal (5-bromo-4-choro-3-indoyl-β-D-galactopyranoside), and ‘blue’ and ‘white’ plaques were picked and then plaque-purified twice more in medium containing X-Gal, as described previously (Griffiths et al., 1998). These isolates were subjected to plaque autoradiography and Southern blot analyses. Representative results of three white and three blue isolates are shown in Fig. 1. We found that the blue plaques purified from reactivated viruses lacked TK activity and retained the lacZ gene insertion in the tk gene.

**Table 2. Latent infections in the ganglia of mice**

<table>
<thead>
<tr>
<th>Virus phenotype (inoculum, p.f.u.)</th>
<th>Viral genome/ganglion [log(mean ± SEM)]*</th>
<th>Reactivation</th>
<th>Avg. TK$^-$ virus (%)†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TK$^-$ mutant (no. positive samples/total)</td>
<td>wt virus (no. positive samples/total)</td>
<td>Reactivating ganglia/total ganglia (%)</td>
</tr>
<tr>
<td>TK$^-$ ($10^8$)</td>
<td>4·5 ± 0·3 (8/8)</td>
<td>ND</td>
<td>0/38 (0)</td>
</tr>
<tr>
<td>TK$^+$ ($10^4$)</td>
<td>ND†‡</td>
<td>1·0 ± 0·4 (3/6)</td>
<td>18/28 (64)</td>
</tr>
<tr>
<td>TK$^+$ ($10^5$)</td>
<td>ND†‡</td>
<td>3·3 ± 0·1 (6/6)</td>
<td>22/28 (79)</td>
</tr>
<tr>
<td>TK$^+$ ($10^5$)</td>
<td>ND†‡</td>
<td>4·3 ± 0·2 (7/7)</td>
<td>33/36 (92)</td>
</tr>
<tr>
<td>TK$^+$ ($10^5$) + TK$^-$ ($10^4$)</td>
<td>4·4 ± 0·2 (8/8)</td>
<td>0·5 ± 0·5 (1/8)§</td>
<td>3/16 (19)§</td>
</tr>
<tr>
<td>TK$^+$ ($10^5$) + TK$^-$ ($10^4$)</td>
<td>4·8 ± 0·4 (8/8)</td>
<td>2·7 ± 0·3 (8/8)§</td>
<td>12/14 (86)</td>
</tr>
<tr>
<td>TK$^+$ ($10^5$) + TK$^-$ ($10^4$)</td>
<td>5·2 ± 0·3II (6/6)</td>
<td>3·6 ± 0·2 (6/6)§</td>
<td>16/16 (100)</td>
</tr>
</tbody>
</table>

*Calculated by averaging the logarithms of viral genomes.
†Determined by plaque autoradiography.
‡ND, Not done.
§$P<0·01$ compared with TK$^+$ ($10^5$) group, Fisher’s exact test.
‖$P<0·03$ compared with TK$^-$ ($10^8$) group, Student’s t-test.
¶$P<0·01$ compared with TK$^+$ ($10^5$) group, Student’s t-test.
indicating that the reactivated TK\(^-\) virus retained the expected TK phenotype and genomic structure.

**The TK\(^-\) mutant dually infected ganglionic cells with TK\(^+\) virus and reduced the TK activity of TK\(^+\) virus during co-infection**

Our study found that co-infection with a TK\(^-\) mutant reduced the replication of TK\(^+\) virus during the acute phase. It is thought that TK is functional as a homodimer, and the high-resolution crystal structure of the dimer has been solved. The insertion of lac\(Z\) into tkLTRZ1 should result in the generation of a polypeptide that has the first two of the six amino acids that are thought to make important dimer–dimer contacts (reviewed by Evans *et al.*, 1998). Therefore, it is possible that, when the ganglionic cell is infected dually with both KOS and tkLTRZ1, the truncated polypeptide from tkLTRZ1 forms a non-functional heterodimer with the wt TK from KOS, reducing the TK activity and thus limiting the ability of KOS to replicate.

To investigate the possibility that the ganglionic cell can be infected dually with TK\(^+\) and TK\(^-\) viruses, trigeminal ganglia were excised from uninfected mice and digested into single-cell suspensions as described by Leib *et al.* (1991). These suspensions (with approximately 3 \(\times\) 10\(^6\) cells per ganglion) were infected with 2 \(\times\) 10\(^6\) p.f.u. KOS-GFP or KOS-GFP plus tkLTRZ1 (4 \(\times\) 10\(^6\) p.f.u. in total). KOS-GFP is a TK\(^+\), recombinant virus derived from KOS with the expression of GFP (Sainz & Halford, 2002). After 24 h, the cultures were stained with microtubule-associated protein MAP2, a marker specific for neurons (Di Stefano *et al.*, 2001). The cultures that were infected with tkLTRZ1 were incubated with a fluorogenic substrate (C\(_{12}\)RG) that forms a red fluorescent product following cleavage by \(\beta\)-galactosidase. All of the cultures were then observed under a fluorescence microscope. In the ganglion culture infected with KOS-GFP, most cells emitting green fluorescence also emitted blue fluorescence (Fig. 2). This demonstrates that most KOS-GFP-infected cells were neurons. In the ganglion culture co-infected with KOS-GFP and tkLTRZ1, about 355 \(\pm\) 15 cells emitted green fluorescence, 213 \(\pm\) 13 cells emitted red fluorescence and 35 \(\pm\) 3 cells emitted both green and red fluorescence. This suggests the presence of ganglionic cells that were dually infected with both KOS-GFP and tkLTRZ1 (Fig. 3). Most cells that emitted green, red or both green and red fluorescence were neurons.

We attempted to recapitulate these data in vivo. Mice were infected with 10\(^5\)–10\(^6\) p.f.u. KOS-GFP. The viral titre in ganglia harvested at day 3 p.i. was comparable to that from mice infected with KOS. However, when ganglia were dissociated into single-cell suspensions and observed under a fluorescence microscope, the green fluorescence emitted by cells was too weak to be detected and not significantly different from that of mock-infected control (data not shown).

To investigate the possibility that co-infection with a TK\(^-\) mutant reduces the TK activity of wt virus, mice were infected with 10\(^5\) p.f.u. KOS, 10\(^8\) p.f.u. tkLTRZ1 or a mixture of both. Trigeminal ganglia harvested at day 3 p.i. were processed and assayed for TK activity. Fig. 3(a) shows that co-infection with tkLTRZ1 reduced the TK activity of KOS in mouse ganglia significantly (\(P<0.01\), Student’s *t*-test). We also tested this in Vero cells and found similar results (Fig. 3b).
Levels of TK\(^{-}\) mutant reactivated from latently infected mouse ganglia correlated with the amount of TK\(^{+}\) mutant in the inoculum

Our results showed that the relative amount of TK\(^{-}\) mutant in reactivated viruses correlated with the amount of wt virus in the inoculum. Lastly, we asked whether the amount of TK\(^{-}\) mutant in the inoculum would affect its own reactivation from latently co-infected ganglia. Mice were infected with 10\(^{4}\), 10\(^{6}\) and 10\(^{8}\) p.f.u. tkLTRZ1 mixed with 10\(^{5}\) p.f.u. KOS, and reactivation was assayed as before. Virus reactivated from every ganglion tested in all three infected groups (Table 3). As determined by plaque autoradiography, approximately 5% of reactivated viruses were TK\(^{-}\) in the group containing 10\(^{4}\) p.f.u. tkLTRZ1. This is approximately 9-fold less than in the group containing 10\(^{6}\) p.f.u. KOS, and reactivation was assayed as before. Virus reactivated from every ganglion tested in all three infected groups (Table 3). As determined by plaque autoradiography, approximately 5% of reactivated viruses were TK\(^{-}\) in the group containing 10\(^{4}\) p.f.u. tkLTRZ1. This is approximately 9-fold less than in the group containing 10\(^{6}\) p.f.u. tkLTRZ1, and this difference is statistically significant (\(P<0.03\), Student’s \(t\)-test). In groups infected with 10\(^{6}\) and 10\(^{8}\) p.f.u. tkLTRZ1, the percentages were not statistically significantly different (\(P=0.63\), Student’s \(t\)-test), suggesting that a plateau had been reached at 10\(^{6}\) p.f.u.

DISCUSSION

In this study, we have demonstrated that co-infection with TK\(^{-}\) HSV mutant reduced the replication, numbers of latent viral genomes and efficiency of reactivation of TK\(^{+}\) virus in mouse ganglia by decreasing TK activity. We present quantitative data showing that very little TK\(^{+}\) virus in the inoculum (1 in 100 000 p.f.u.) was needed to support reactivation of the TK\(^{-}\) mutant. However, 10-fold more TK\(^{+}\) virus was required to see a measurable increase in the numbers of TK\(^{-}\) genomes that established latency, and a further 10-fold to permit detectable levels of replication in ganglia at acute times. These results also provide the first evidence that the level of TK activity required at each step of ganglionic infection may be different. Only the highest dose of TK\(^{+}\) virus (10\(^{5}\) p.f.u. per mouse) permitted the acute replication of the TK\(^{-}\) mutant, so TK\(^{+}\) virus did not permit...
the acute replication of the TK\(^-\) mutant under all conditions. Even without permitting the acute replication and increasing latent TK\(^-\) mutant viral genomes in ganglia, TK\(^+\) virus still could confer the pathogenic phenotype to a TK\(^-\) mutant by complementing it to reactivate from latently infected ganglia. In addition, our data showed that enzymic compensation by wt virus, but not gene recombination, allows the TK\(^-\) mutant to behave more like a TK\(^+\) virus during co-infection.

**How does a TK\(^-\) mutant reduce the ganglionic infection of a TK\(^+\) virus?**

Our study found that co-infection with a TK\(^-\) mutant reduced the replication, latency establishment and reactivation efficiency of TK\(^+\) virus in mouse trigeminal ganglia. This was particularly evident when the ratio of TK\(^+\) : TK\(^-\) virus was low. Besides the ratios that we tested, Tenser & Edris (1987) showed that co-infection of mice with equal amounts of wt and mutant viruses reduced the replication of wt virus in mouse ganglia by 20-fold. A plausible explanation for these results is provided upon consideration of the structure of active TK, which was not available at the time of previous co-infection reports (Efstathiou et al., 1989; Ellis et al., 1989; Field, 1982; Field & Lay, 1984; Tenser & Edris, 1987; Tenser et al., 1981). It is possible that the truncated TK polypeptide from \(t\)kLTRZ1 forms a non-functional heterodimer with the normal TK polypeptide from KOS in co-infected cells, causing a reduction in the TK activity that would be observed in the cells infected with KOS alone. Our results showing that ganglionic cells were infected dually with both KOS-GFP and \(t\)kLTRZ1 and that co-infection with \(t\)kLTRZ1 indeed reduced the TK activity of KOS both in vitro and in vivo support this possibility.

**How does wt virus complement the ganglionic infection of a TK\(^-\) mutant?**

We found that not only the step of ganglionic infection, but also the degree of complementation of TK\(^-\) mutant by TK\(^+\) virus, varied in a dose-dependent manner. For example, with the same inoculum of TK\(^-\) virus, the TK\(^+\) : TK\(^-\) virus ratio in reactivated samples did not remain constant as the amount of TK\(^+\) virus in the inoculum increased, and there was a positive relationship between the amount of TK\(^+\) virus in the inoculum and TK\(^-\) virus that reactivated.

Co-infection with \(10^5\) p.f.u. wt virus resulted in more TK\(^-\) genomes in latently infected ganglia than did \(10^4\) p.f.u. wt virus. The increase in the number of latent \(t\)kLTRZ1 genomes in ganglia of mice co-infected with TK\(^-\) virus plus \(10^4\) p.f.u. KOS (Table 2) was unexpected, as very little virus was observed at acute times (Table 1). Previously, we have demonstrated that the titres of wt and TK\(^-\) viruses in the infected eye were comparable 24 h p.i., but titres of TK\(^-\) mutant declined more rapidly over the subsequent 2 days (Chen et al., 1998; Horsburgh et al., 1998). Co-infection may prolong the replication of TK\(^-\) mutant in the eye, perhaps by providing TK activity in trans, although this increase may not always be detectable. The concept of TK activity being provided in trans has been suggested previously (Coen et al., 1989b; Tenser et al., 1996), and our finding that ganglionic cells were infected dually with both TK\(^-\) and TK\(^+\) viruses addresses this possibility.

Given that many acyclovir-resistant clinical isolates comprise viruses with multiple phenotypes, including TK\(^+\) and TK\(^-\), the data presented in this paper provide an insight into how these mixed populations are pathogenic. It has previously been proposed that isolates that limit their ribosomal expression of TK, for example via frameshifting, synthesize sufficient TK to activate acyclovir, but not enough TK for pathogenesis. It is possible that an isolate that contains TK\(^+\) and TK\(^-\) viruses is behaving similarly, except that rather than having 1% of wt TK activity, one TK\(^+\) virus among 100,000 TK\(^-\) viruses is sufficient to support reactivation from latently infected ganglia.

**ACKNOWLEDGEMENTS**

We thank Donald Coen for critical review of the manuscript, Angela Pearson for providing UL24 information and H.-Y. Lei, Ai-Li Shiao and K.-S. Hsu for helpful discussion. This research was supported by grant 92-2320-B-006-109 from the National Science Council in Taiwan (S.-H. C.).

**REFERENCES**


