The Role of Herpes Simplex Virus Type 1 Thymidine Kinase in Pathogenesis

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SUMMARY

A genetically engineered herpes simplex virus type 1 (HSV-1) thymidine kinase (TK) deletion mutant has been constructed and used to investigate the role of this gene in pathogenesis. Inoculation of mice with the HSV TK deletion mutant resulted in the establishment of latent ganglionic infection as demonstrated by superinfection of explanted ganglia with wild-type (wt) virus but not by routine explant culture suggesting that the virus-encoded TK is not essential for the establishment of latent infection but may be necessary for either reactivation or virus replication following reactivation. In addition, Southern blot hybridization has been used to demonstrate in vivo complementation of this mutant by wt virus in both peripheral and central nervous system tissues of mice during acute infection and to show that such complementation can result in the establishment and reactivation of latent TK- infection.

INTRODUCTION

Several studies have suggested that mutants of herpes simplex virus type 1 (HSV-1) with a defective thymidine kinase gene (TK-) have a reduced ability to establish latent infections in vivo, and that this may be related to a decrease in the ability of such mutants to replicate in tissues of the nervous system (Field & Wildy, 1978; Tenser & Dunstan, 1979; Field & Darby, 1980). More recent studies using genetically engineered TK- deletion mutants have shown that an HSV-2 mutant cannot establish either acute or latent ganglionic infection in mice (McDermott et al., 1984; Tenser & Edris, 1987). Similarly, an HSV-1 mutant was shown to be latency-negative in mice but to establish latent infection in at least a proportion of infected rabbits, suggesting that the host may also be a determinant in the establishment of latent infection (Meignier et al., 1988). Whether failure of TK- virus to cause latent infection is due to a block in establishment, maintenance or reactivation remains unclear since failure to reactivate virus by routine explant culture does not define the stage at which such a block occurs. In this study we have investigated the ability of an HSV-1 (SC16) TK deletion mutant to establish latent infection in mice and have used Southern blot hybridization to follow in vivo complementation of this mutant by wild-type (wt) virus in both peripheral (PNS) and central nervous system (CNS) tissues. We show by the reactivation of latent virus following superinfection of explanted ganglia with wt virus that the TK- deletion mutant is able to establish latency ganglionic infection. However, infection with the mutant was quantitatively different from that caused by wt virus in that virus DNA could not be detected in nervous system tissue during either acute or latent infection.

METHODS

Viruses. The virus used for both the construction of the TK- deletion mutant SC16 TKDM21 and for the animal experiments was the HSV-1 strain SC16 (Hill et al., 1975), an isolate with low passage history. Virus infectivity was measured by plaque titration using BHK-21 C13 cells grown in Eagle's medium containing 10% (v/v) tryptose phosphate broth and 10% (v/v) calf serum (ETC).
Fig. 1. (a) Summary of the construction of an 816 bp deletion within the TK-coding region. The insert containing the deleted version of the TK gene was purified following AluI digestion and used to transfect cells with HSV-1 DNA to generate the TK deletion mutant SC16 TKDM21. (b) Southern blot hybridization of BamHI-digested wt SC16 (lane 1) and TKDM21 DNA (lane 2) with pTKdelA showing the presence of the 2.7 kb deleted BamHI Q fragment in TKDM21 DNA.

Plasmids. The 3-5 kb BamHI Q fragment of HSV-1 (SC16) cloned into pBR322 has been described (Darby et al., 1986). A 2043 bp PvuII fragment containing the entire TK-coding sequence was introduced into the Smal site of M13mp8 and this clone was designated pM13TK1. pM13TK1 was digested with the restriction enzymes NruI and Smal to remove 816 bp of TK-coding sequence. The resultant clone which contained a deleted version of the TK gene was designated pTKdelA (Fig. 1 a).

Recombination between pTKdelA and HSV-1 DNA. pTKdelA was digested with AluI and a 1-3 kb AluI fragment containing the deleted TK gene was gel-purified and cotransfected into BHK cells with high M, HSV-1 (SC16)-infected cell DNA by the calcium phosphate precipitation method (Graham & van der Eb, 1973) and dimethyl sulphoxide boost (Stow & Wilkie, 1976). Monolayers were left for 4 days and then harvested. Sonicated infected cell suspensions were then assayed on BHK cells maintained in Glasgow modified Eagle's medium and 10% newborn calf serum containing 1 μg/ml acyclovir (ACV) to select TK- variants. One of 10 virus clones analysed with the restriction enzyme BamHI had the expected 816 bp deletion within the HSV-1 (SC16) BamHI fragment. This virus stock was re-cloned and shown by Southern blot hybridization to have the expected deletion (Fig. 1 b). Virus clone TKDM21 was used in subsequent experiments.

Mice. Four to 5-week-old female BALB/c mice were obtained from Tucks U.K. Ltd and were infected at 5 to 6 weeks of age.

Inoculation of mice. Twenty μl of virus suspension containing 5 × 10⁶ p.f.u. was inoculated subcutaneously into the left ears of anaesthetized mice (Hill et al., 1975).

Measurement of virus titres in mouse tissues. Tissue samples were stored at −70 °C. Samples were later homogenized in 0.2 ml of ETC in small glass grinders. The presence of infectious virus was determined by assay on BHK cells.

Reactivation of latent virus from dorsal root ganglia. Pooled CII, CIII and CIV cervical dorsal root ganglia were explanted directly into penny bottles containing 0-5 ml of Glasgow modified Eagle's medium supplemented with 1% calf serum. Cultures were then incubated for 5 days at 37 °C in a 5% CO₂ humidified atmosphere before homogenization and assay for infectious virus. In cases where superinfection of ganglia was used for the reactivation of mutant virus, explanted ganglia were cultured in medium containing 10⁵ p.f.u. of wt HSV-1 (SC16) for 5 days before homogenization and assay.

DNA extraction. High M, DNA was extracted from mouse tissues and BHK cells as described previously (Efstathiou et al., 1986).

Blot hybridization. Restriction endonuclease-digested DNA was electrophoresed in 0.8% agarose gels containing 1 μg/ml ethidium bromide and transferred to nitrocellulose (Schleicher & Schuell) by the technique of Southern (1975). DNA fragments to be used as hybridization probes were labelled with [α-32P]dCTP (3000 Ci/mmol; Amersham) either by nick translation (Rigby et al., 1977) or by extension of a random primer (Feinberg & Vogelstein, 1983) to a specific activity of 2 × 10⁶ to 4 × 10⁷ c.p.m./μg. Prehybridization and hybridization of filters were performed as described previously (Efstathiou et al., 1986). Autoradiography was carried out at −70 °C using preflashed Kodak X-Omat 5 film and intensifying screens for exposures of 1 to 10 days.

One-step growth curves. BHK cell monolayers in 50 mm tissue culture dishes were infected at a multiplicity of 5 p.f.u./cell. After an adsorption period of 1 h the cells were washed three times with ETC and incubated at 37 °C. Cells were harvested in the medium at various times after infection and stored at −70 °C before sonication and assay on BHK-21 cells.
Pathogenicity studies

Five- to 6-week old female BALB/c mice (120) were divided into three experimental groups and inoculated in the left ear pinna with either $5 \times 10^6$ p.f.u. of wt HSV-1 (SC16), $5 \times 10^6$ p.f.u. of the deletion mutant TKDM21 or for in vivo complementation studies with a 1:1 mixture.
comprising $2.5 \times 10^6$ p.f.u. of wt HSV-1 (SC16) and $2.5 \times 10^6$ p.f.u. of TKDM21. The relatively high doses resulted in a 10% mortality in animals inoculated with wt HSV-1 (SC16), a 5% mortality in animals inoculated with a mixture of wt and mutant virus and no deaths in those animals inoculated with the TK deletion mutant TKDM21. Infectious virus assays were performed on homogenates of ear, left cervical ganglia (CII, CIII and CIV), spinal cord (0.5 cm$^3$ from the cervical region) and brainstem (pons medulla) from mice killed at 1, 3, 5 and 10 days post-infection (Fig. 4).

Peak virus titres in cervical ganglia, brainstem and spinal cord tissue homogenates of animals inoculated with wt HSV-1 (SC16) were observed 5 days after infection. A similar pattern of virus replication in the nervous system of mice inoculated with a mixture of wt and mutant virus was observed, with peak virus titres detected in cervical ganglia 3 days after infection and peak titres in brainstem and spinal cord tissue homogenates occurring 5 days after infection. In contrast, infectious virus could not be detected in cervical ganglia, brainstem or spinal cord tissue homogenates assayed from animals inoculated with TKDM21 at any time after infection. Comparison of virus titres in the inoculated ears of animals from each experimental group (Fig. 4a) showed lower titres in those animals inoculated with TKDM21 alone as compared to
animals inoculated with either wt virus or a mixture of wt and mutant virus and is likely to reflect poor replication of the mutant at the site of inoculation. The inability to detect virus in nervous system tissue of animals inoculated with TKDM21 was not related solely to the observed reduction in virus titres at the periphery since an additional group of animals inoculated with $10^8$ p.f.u. of TKDM21 in the left ear pinna had peripheral virus titres equivalent to wt virus-inoculated animals but infectious virus remained undetectable in nervous system tissue assayed 5 days after infection (Fig. 4). These results show that the TK deletion mutant TKDM21 is significantly less neurovirulent than wt virus. Since this block did not appear to be related to peripheral virus titres during acute infection, the inability to detect virus in the nervous system is more likely to reflect a true neuropathogenic attenuation than merely a block in the ability of virus to enter the nervous system.

Southern blot hybridization was used next to determine whether the inability to detect infectious virus in the nervous system of mice inoculated with TKDM21 was reflected in the amount of virus DNA present in such tissues and to assess complementation of TKDM21 following infection of animals with a mixture of wt and mutant virus.

**Detection of HSV DNA in mouse neural tissue during acute infection**

DNA was extracted from brainstems, spinal cords and cervical ganglia of five mice from each experimental group at 5 days post-infection. The DNA was digested with BamHI and analysed by Southern hybridization using the pTKdelA fragment as probe. Fig. 5 shows the detection of the 3-5 kb HSV-1 (SC16) BamHI Q fragment in brainstem, cervical ganglia and spinal cord DNA of animals inoculated with wt SC16. The amount of HSV DNA detected in these samples varied from 0-1 to five copies/cell in individual mice assessed by comparison with the one
Fig. 6. Detection of the wt 3.5 kb SC16 BamHI Q fragment or the 2.7 kb deleted TKDM21 BamHI fragment in individual mouse brainstems (lanes 1 to 5) or pooled cervical ganglia (lane 6) from wt-, wt/TKDM21- or TKDM21-inoculated animals sampled 16 weeks post-infection. Samples (10 µg) of DNA were digested in each case with BamHI, transferred to nitrocellulose and hybridized to pTKdelA. U is uninfected whole mouse brain DNA. Re is one copy/cell reconstruction of either wt SC16 or TKDM21 DNA. One brainstem DNA sample contained a high Mr fragment (●), the nature of which is not known but could be due to either a partial digestion product or an unrelated contaminating DNA species.

Detection of HSV DNA in mouse neural tissue during latent infection

Sixteen weeks after infection when latent infections had been established five animals from each experimental group were killed and DNA was extracted from brainstems of individual mice and from pooled cervical ganglia (CII, CIII and CIV). Ten µg of DNA from each sample was digested with BamHI and was analysed by Southern hybridization using the pTKdelA fragment as probe. The 3.5 kb SC16 BamHI Q fragment was detected in all brainstem and cervical ganglia DNA samples from animals inoculated with wt virus. The amounts corresponded to approximately one copy/cell assessed by comparison with the reconstruction, with little mouse-to-mouse variation in copy number (Fig. 6). Confirmation of the latent status of the virus DNA was made by re-probing filters with the BamHI K junction fragment which detected endless DNA characteristic of the latent state (Rock & Fraser, 1983; Efstathiou et al., 1986) (data not shown). Southern blot analysis of DNA extracted from brainstem and pooled cervical ganglia from animals inoculated with a mixture of wt and mutant virus revealed the presence of both the wt 3.5 kb BamHI Q fragment and the deleted 2.7 kb restriction fragment indicating that wt helper function had enabled mutant TKDM21 to establish latent infection. Virus DNA could not be detected in DNA extracted from individual brainstems, or pooled cervical ganglia from five mice inoculated with the mutant TKDM21, a result consistent with the inability to detect virus DNA in the nervous system of these mice during the acute disease.

Explant culture of cervical ganglia (CII, CIII, CIV) of five mice from each experimental group resulted in virus reactivation from all five mice inoculated with wt virus, four of five mice inoculated with a mixture of wt and mutant virus and none of the 10 mice inoculated with either
Pathogenicity of an HSV TK mutant

5 × 10⁶ p.f.u. or 10⁸ p.f.u. of mutant TKDM21. DNA extracted from monolayers infected with reactivated virus was analysed by Southern blot hybridization (Fig. 7). The wt 3.5 kb BamHI Q fragment was detected in monolayers infected with reactivated virus from five mice infected with wt SC16. Both the wt 3.5 kb BamHI Q fragment and deleted 2.7 kb fragment were detected in virus reactivated from four of five mice infected with a mixture of wt and mutant virus although a greater amplification of wt virus was reflected by the relative amounts of wt and mutant virus DNA, indicating considerable selection for wt virus during the reactivation procedure.

**Reactivation of mutant TKDM21 following superinfection of latently infected ganglia**

The inability to detect the TK⁻ deletion mutant TKDM21 in nervous system tissue either by direct homogenization and assay during acute infection or by Southern blot hybridization during both acute and latent infection and the failure to reactivate virus from cervical ganglia by explant culture suggested either that this virus could not establish a latent infection or that the presence of small amounts of virus during both acute and latent infection failed to be detected.

Superinfection of human ganglia with temperature-sensitive (ts) mutants has been used in the detection of non-reactivable or defective HSV DNA (Brown et al., 1979; Lewis et al., 1984) presumably by a process of complementation and/or recombination. Using a similar approach, superinfection of explanted ganglia with wt SC16 was used in an attempt to reanimate virus from dorsal root ganglia of TKDM21-infected animals. Explanted cervical ganglia (CII, CIII, CIV) from five mice inoculated 16 weeks previously with mutant TKDM21 were placed in medium containing 10⁵ p.f.u. of wt SC16. Following incubation at 37°C for 5 days tissue homogenates were used to infect BHK cell monolayers. DNA extracted from infected
Fig. 8. (a) Southern blot analysis of virus from the ganglia of TKDM21-inoculated animals removed during the latent stage of infection and superinfected with wt SC16. Superinfected cervical ganglia from five mice (lanes 1 to 5) were homogenized and assayed on BHK cells. BHK cell DNA (10 μg) was in each case digested with BamHI and analysed by Southern hybridization using pTKdelA as probe. Rc is either wt SC16 or TKDM21 DNA. (b) Virus obtained from the superinfected ganglia from one mouse (lane 5) was further analysed by Southern hybridization following one passage in medium containing 1 μg/ml ACV to reduce the amount of wt SC16 superinfecting virus (b, lane 3). Lane 1, TKDM21; lane 2, SC16.

monolayers was analysed by Southern blot hybridization using pTKdelA as probe (Fig. 8). Although a faint band corresponding in size to the 2.7 kb deleted BamHI Q fragment was detected in each set of ganglia from five mice, the weak intensity of these bands made the unambiguous identification of TKDM21 virus DNA difficult. For this reason ACV enrichment of one reactivant (SIJ; Fig. 8a, lane 5) was carried out. Fig. 8 clearly shows the presence of the mutant virus 2.7 kb deleted BamHI Q fragment with a concomitant decrease in the wt virus 3.5 kb BamHI Q indicating the successful reactivation of mutant virus TKDM21. The ability to reactivate the TK deletion mutant TKDM21 following superinfection with wt virus indicates that this mutant is able to establish and maintain latency in the absence of detectable virus replication during acute infection. This observation is in agreement with previous studies which have shown that HSV ts mutants unable to replicate at the body temperature of the mouse are able to establish latent infection (McLennan & Darby, 1980; Watson et al., 1980).

Although the inability to reactivate virus following routine explant culture suggests a block in reactivation, it is possible that superinfection is a more sensitive method by which to detect reactivation from low levels of latent virus.

**DISCUSSION**

This paper describes studies on the pathogenesis of an HSV-1 (SC16) TK deletion mutant, TKDM21. As expected, this engineered deletion mutant, lacking 816 bp of TK-coding sequence, expresses no TK activity, grows normally in tissue culture and has the same non-syncytial plaque morphology as the parental SC16 virus. It is of interest that two HSV-1 (strain 17) TK deletion mutants previously described by Sanders et al. (1982) had an observed change in plaque morphology from non-syncytial to a small syncytial phenotype and grew poorly in tissue culture suggesting that a second gene function was affected in these mutants. This is most likely due to the fact that these mutants had large deletions covering much of the TK gene and its promoter which would have disrupted the overlapping reading frame UL24 (McGeoch et al., 1988) the product of which has been shown to be important but not essential for virus growth in tissue culture (D. M. Coen, personal communication). Mutant TKDM21 was constructed such
Pathogenicity of an HSV TK mutant

that 292 bp downstream of the initiating ATG of the HSV TK gene was retained. Of the two in vitro and in vivo initiation transcription sites that could potentially encode UL24 (Read et al., 1984) the transcript initiating proximal to the first initiating methionine of UL24 is maintained in mutant TKDM21 and is therefore most likely to be involved in the expression of this gene product.

Mutant TKDM21 replicated less efficiently in the ears of infected mice and could not be detected in either sensory ganglia or brainstem tissue homogenates by conventional virus assay techniques, a result consistent with previous studies of TK− mutants (Field & Wildy, 1978; Tenser & Dunstan, 1979; Klein et al., 1981; Price & Khan, 1981). These had suggested that virus-encoded TK is required for replication in non-dividing neuronal cells and for efficient replication in epidermal cells involved early in the infection. Southern blot hybridization analysis failed to detect TKDM21 virus DNA during either acute or latent infection and attempts to reactivate virus from sensory ganglia by routine explant culture proved unsuccessful, an observation that has been made for both HSV-1 and HSV-2 TK deletion mutants following experimental infection of mice (McDermott et al., 1984; Tenser & Edris, 1987; Meignier et al., 1988). Recovery of TKDM21 from explanted ganglia following superinfection with wt virus indicated that the TK deletion mutant used in this study had established a latent infection and supports the view that virus-encoded TK is not an essential requirement for the establishment of latent infection. Whether the virus-encoded TK plays an essential role in the reactivation process is more difficult to determine and further work is needed to show whether this function is required for the reactivation process or for virus replication following reactivation.

In vivo complementation of TKDM21 was followed through the PNS and CNS by Southern blot hybridization following infection of mice with a 1:1 mixture of wt TK+ virus. The deleted 2.7 kb BamHI Q fragment present in the TKDM21 genome provided a marker which allowed the separation of the TK+ and TK− components necessary for the evaluation of TK complementation. The presence of TK− virus during mixed infection had little effect on virus titres at the periphery or in the PNS or CNS of mice during the acute stage of disease and Southern blot analysis of brainstem, cervical ganglia and spinal cords of animals revealed the presence of both wt TK+ and TK− virus DNA in all tissues (Fig. 5). Similarly, during latent infection both wt TK+ and TK− virus DNA could be detected. However, by comparison to reconstructions it appears that the ratio of mutant to wt virus DNA was greater in acute than in latent infection (compare Fig. 5a to Fig. 6) suggesting that complementation between TK+ and TK− virus may be less efficient during the establishment of latent infection.

Successful reactivation of TKDM21 from ganglia containing both wt TK+ and TK− virus was possible although the small amounts of TKDM21 virus DNA detected following reactivation suggested a reduced efficiency of complementation during this process. One possibility which may explain this observation is that efficient complementation is multiplicity-dependent in vivo in that initial infection involving high doses of both wt TK+ and TK− results in efficient complementation at the site of inoculation but that the lower titres detected during acute infection of both the PNS and CNS could influence the numbers of cells co-infected with wt TK+ and TK− virus and thus the number of cells destined to become latently infected of which only a proportion may be co-infected with these viruses. It is at present impossible to know the proportion of latently infected cells containing both wt TK+ and TK− virus although it seems likely that latent infection of neurons with both TK+ and TK− viruses does occur. A number of previous studies demonstrating in vitro complementation/recombination of either TK+/TK− virus mixtures or between avirulent viruses have been published (Tenser et al., 1981; Field & Lay, 1984; Javier et al., 1986; Tenser & Edris, 1987; Sedarati et al., 1988) however, the results presented here are the first to follow complementation of a deletion mutant within the CNS and PNS during both acute and latent infection by Southern blot hybridization. It is of interest that studies on the sensitivity of clinical isolates to ACV from early clinical trials with this compound found a number of the isolates to contain mixtures of resistant and sensitive viruses (Dekker et
al., 1983) and that similar observations have been made in experimental infections of mice (Field & Lay, 1984). These results suggest that in vivo complementation between TK+ and TK− viruses may be a mechanism by which TK− drug-resistant virus could be maintained in the population.

In conclusion we have demonstrated by superinfection of explanted ganglia with wt virus that a TK− deletion mutant is able to establish latent ganglionic infection suggesting that the virus-encoded TK is not essential for the establishment of latent infection. However, it may be necessary for either reactivation or virus replication following reactivation. In addition we have followed the in vivo complementation of a TK− deletion mutant by Southern hybridization and have confirmed previous reports (Tenser et al., 1985; Tenser & Edris, 1987) which had shown that such complementation can result in the establishment and reactivation of latent TK− virus infection.

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Pathogenicity of an HSV TK mutant


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