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Latent Infections in Spinal Ganglia with Thymidine Kinase-Deficient Herpes Simplex Virus

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A herpes simplex virus type 1 variant [C239(TK$^-$)] harboring a deletion in the thymidine kinase (TK) gene was assessed for capacity to establish latent infections. Outbred Swiss Webster mice were inoculated on both hind footpads, and numbers of neurons expressing latency-associated transcript and amounts of viral DNA in latently infected lumbosacral spinal ganglia were scored. C239(TK$^-$) established levels of latent infection that were only slightly lower than those found for either a TK rescued variant of this agent or the parent wild-type KOS. However, in contrast to the TK$^+$ viruses, C239(TK$^-$) could not be reactivated when spinal ganglia were cultured in vitro. The results presented show that expression of the viral TK gene plays no major role in establishment of the latent state but that it functions during reactivation of latent virus from explanted ganglia maintained in vitro.

The antiviral response raised in immunocompetent hosts eventually limits productive replication of herpes simplex virus (HSV) to levels beyond detectability. However, HSV escapes complete clearance from the host by establishment of a latent state in sensory neurons (12). HSV encodes a thymidine kinase (deoxypyrimidine kinase; TK) which is expressed during productive infection. Viruses deficient in TK expression (TK$^-$) replicate poorly in nervous tissue, exhibit reduced neurovirulence, and cannot be readily recovered when dorsal root ganglia are cultivated in vitro (2, 11, 15). These findings suggest that TK plays a major role either in establishment of the latent state or in reactivation. To determine which of these features is affected by the absence of viral TK, we studied the capacity of an HSV type 1 (HSV-1)-derived TK$^-$ virus, a TK rescued progeny of this agent, and the parental KOS virus to establish and be reactivated from latent infection in mice. Levels of virus latency were assessed by scoring for the latency-associated transcript (LAT) and by comparing the amounts of viral DNA in spinal ganglia.

The TK$^-$ virus C239(TK$^-$) was derived by cotransfection of unit-length KOS DNA with a plasmid termed pTKC. The latter was constructed from the TK gene cloned as *Bam*HI-P (0.51 to 0.55 map units [m.u.]) by deletion of a 560-base-pair *Kpn*I-to-*Bgl*II fragment (0.51 to 0.52 m.u.) containing the TK promoter regulatory region as well as the transcription start site. A chloramphenicol acetyltransferase (CAT) gene under the control of a simian virus 40 early promoter without the enhancer sequences from pSV1-CAT (3) was inserted in place of the deleted TK regulatory sequences. The resulting virus did not express detectable levels of TK mRNA as assessed by Northern (RNA) blot analysis using a 550-base-pair *Bgl*II-to-*Sst*I fragment as probe. This probe covers the region from +55 in the 5′-nontranslated region of the TK gene through the amino-terminal half of the peptide coding region. While no TK could be seen, CAT mRNA could be detected with a CAT-specific probe (data not shown). A TK$^+$ derivative virus of C239(TK$^-$) was produced by cotransfection of LM(TK$^-$) with unit-length C239(TK$^-$) and the KOS *Bam*HI P fragment (0.51 to 0.55 m.u.) by the $CaSO_4$ precipitation technique as modified for HSV (9, 10). The TK$^+$ phenotype was selected by intracerebral inoculation of 4- to 6-week-old outbred Swiss Webster mice. When the animals died, virus was isolated from the brain and plaque purified on rabbit skin cell monolayers. This kind of selection was possible since mice infected intracerebrally with 10^3 PFU of either KOS or the TK$^+$ derivative died 3 to 5 days later, while animals inoculated with the same or a 100-times-larger dose of C239(TK$^-$) survived. It should also be noted that neither C239(TK$^-$) nor the rescued progeny virus killed mice following peripheral infection with large doses ($\geq 10^6$ PFU per footpad). This is comparable to what was previously obtained for the parental KOS strain (16).

The expression of TK was assayed by a modification (6) of the test used by Jamieson and Subak-Sharpe (5). Levels of TK activity detected for the neurovirulent progeny virus of C239(TK$^-$) used in this study [C239R(TK$^+$)] ([2.4 ± 0.3] × 10^5 cpm) were comparable to those assessed for wild-type KOS ([2.8 ± 0.2] × 10^5 cpm). Those measured for C239(TK$^-$) ([0.3 ± 0.05] × 10^5) did not differ significantly from values determined for mock-infected control cells ([0.4 ± 0.05] × 10^5). To further analyze C239(TK$^-$) and C239R(TK$^+$), growth characteristics of the viruses were assessed on primary neuron cultures prepared in serum-free medium in 12-well Costar plates precoated with poly-L-lysine (1). Resulting cultures were ≥95% neuronal cells as judged by morphologic analysis. When compared with C239R(TK$^+$) or KOS, C239(TK$^-$) exhibited about 1,000-fold-restricted growth 48 h after infection, and even after 72 h only very little C239(TK$^-$) was present in neuron cultures (Fig. 1). Most of the neuronal cells (>90%) infected with C239(TK$^-$) were still alive 72 h after infection as detected by trypan blue staining, while the opposite was the case for cultures infected with C239R(TK$^+$) or KOS.

Three kinds of experiments were performed to assess the capacity of C239(TK$^-$) and its TK$^+$ progeny to establish latent infection in nervous tissue. First, spinal ganglia were explanted and cocultivated on rabbit skin cells (12) 21 days after outbred Swiss Webster mice (five mice for each virus) had been inoculated on both hind footpads with 10^6 PFU of

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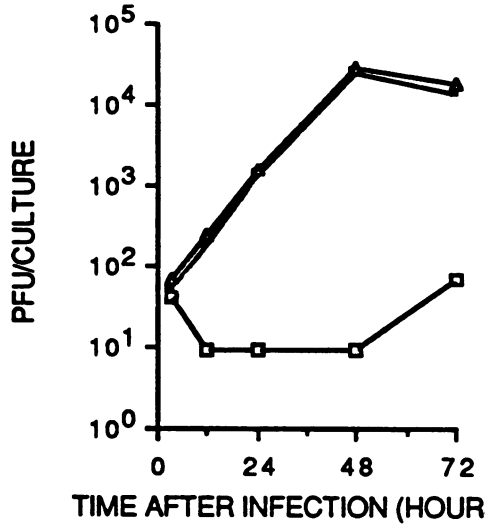

FIG. 1. Replication cycles of HSV-1 variants C239(TK$^-$) (□), C239R(TK$^+$), (■), and KOS (△) in primary cultures of brain neurons. Neuron cultures prepared as previously described (1) were infected at multiplicities of 0.05 of the variants and incubated at 37°C for various times. Cells and supernatants were harvested and frozen at −70°C for later titration. Values represent averages of three cultures titrated individually; the level of sensitivity was 10 PFU per culture.

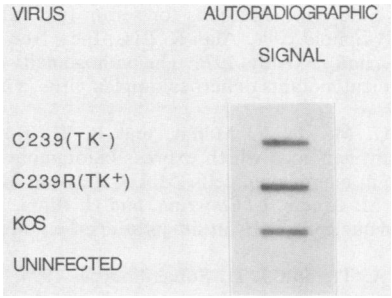

FIG. 2. Viral DNA present in murine spinal ganglia latently infected with HSV-1 variants C239(TK$^-$), C239R(TK$^+$), and KOS. Pooled latently infected lumbar spinal ganglion numbers 4 and 5 from five mice previously inoculated on both hind feet with C239(TK$^-$), C239R(TK$^+$), or KOS were homogenized in 10 mM Tris–150 mM EDTA–250 mM NaCl (pH 8). They were then digested with proteinase K (1.5 mg/ml) after addition of sodium dodecyl sulfate to a final concentration of 1% at 56°C for 14 h, extracted with phenol-chloroform, and precipitated with ethanol (8). The samples were suspended in 10 mM Tris–10 mM EDTA (pH 8), digested with RNase (2 h; 37°C), treated again with proteinase K (2 h; 37°C), extracted with phenol-chloroform, and precipitated with ethanol. DNA concentrations of the samples suspended in 10 mM Tris–1 mM EDTA (pH 8) were estimated from the optical density at 280 nm and were adjusted to 4.5 μg of total DNA per 15 μl. DNA samples bound to a nylon membrane (ICN Biomedicals, Irvine, Calif.) were hybridized with a ^{32}P-labeled nick-translated *Hin*dIII N (0.87 to 0.90 m.u.; 10^8 cpm/ml) fragment of HSV-1 (7).

KOS, C239R(TK$^+$), or C239(TK$^-$). After 6 to 7 days in culture, reactivation of virus from ganglia of all the mice originally infected with KOS or C239R(TK$^+$) could be observed, while no virus could be detected in cultures of ganglia from C239(TK$^-$)-infected mice even after 21 days of cocultivation. In the second series of experiments, the number of neurons harboring latent virus was assessed by scoring for expression of the LAT (4) in sections of latently infected spinal ganglia. Here, in situ hybridization methods specific for the LAT were used (13). Numbers of neurons in sections of lumbar spinal ganglion numbers 4 and 5 were found to be very similar for the three viruses (Table 1). In the third group of experiments, amounts of C239(TK$^-$), C239R(TK$^+$), and KOS DNA in latent ganglia were compared by slot blot hybridization of DNA extracted from ganglia (7, 8). Here, a ^{32}P-labeled nick-translated *Hin*dIII N fragment (0.87 to 0.90 m.u.; 10^8 cpm/μg) was utilized as probe. Amounts of HSV DNA, as estimated by optical density, did not vary more than two- to threefold between C239(TK$^-$), C239R(TK$^+$), and KOS (Fig. 2).

TABLE 1. Expression of HSV-1 LAT in sensory neurons latently infected with HSV-1 variants C239(TK$^-$), C239R(TK$^+$), and KOS[a]

Virus	No. of LAT-positive neurons/ganglion (avg)
C239(TK$^-$)	1–4 (2.2)
C239R(TK$^+$)	0–6(2.7)
KOS	1–5(2.9)

[a] Four- to six-week-old outbred Swiss Webster mice (five per group) were infected on both scarified hind feet with 10^6 PFU of virus in 30 μl and sacrificed 21 days later. Lumbar spinal ganglion numbers 4 and 5 from mice in each group were pooled, and in situ hybridization was carried out on cryocut sections by using a ^{35}S-labeled DNA probe (ATD-19; 0.78 to 0.783 m.u. [13]) specific for LAT. Positive neurons were scored in 6 to 10 sections per ganglion. Numbers represent the ranges and averages of neurons expressing LAT in a representative ganglion section. Averages were calculated from individual values determined from sections of 10 to 14 ganglia.

The results presented here indicate that a TK$^-$ HSV-1 strain is able to establish levels of latent infection in sensory neurons of mice which are comparable to those of TK$^+$ control agents with respect to both number of neuronal sites expressing LAT and amount of viral DNA assessed in ganglia (Table 1; Fig. 2). The poor replication of TK$^-$ viruses in nervous tissue of mice reported by others (2, 11) and the inability of C239(TK$^-$) to substantially replicate in primary neuron cultures (Fig. 1) indicate that extensive replication of virus within neuronal tissue is not a prerequisite for the efficient establishment of latent infection. However, the lack of expression of TK activity had a pronounced negative effect on reactivation of virus from nervous tissue of mice when ganglia were cocultivated in vitro. From this we conclude that TK has no detectable influence on the establishment of latent infections in these systems but that it facilitates the reactivation induced by in vitro cultivation. It is now of interest to determine whether reactivation in vivo is also affected by the expression of the viral TK.

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ADDENDUM

After the manuscript had been submitted for publication, a report by Tenser et al. was published (14). Similar latency characteristics for TK$^-$ viruses were described.

LITERATURE CITED

1. **Bodenstein, J.** 1984. Culture methods for growth of neuronal cell lines in defined media, p. 3–13. *In* W. Barnes, D. A. Sirbasku,

and G. H. Sato (ed.), Methods for serum free culture of neuronal and lymphoid cells. Alan R. Liss, Inc., New York.
2. **Field, H. J., and P. Wildy.** 1978. The pathogenicity of thymidine kinase deficient mutants of herpes simplex virus in mice. J. Hyg. **81**:267–277.
3. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
4. **Haase, A., M. Brodie, L. Stowring, and H. Blum.** 1984. Detection of viral nucleic acid by in situ hybridization. Methods Virol. **7**:189–226.
5. **Jamieson, A. T., and J. H. Subak-Sharpe.** 1974. Biochemical studies on the herpes simplex virus-specified deoxypyrimidine kinase activity. J. Gen. Virol. **24**:481–492.
6. **Javier, R. T., K. M. Izumi, and J. G. Stevens.** 1988. Localization of a herpes simplex virus neurovirulence gene dissociated from high-titer virus replication in the brain. J. Virol. **62**:1381–1387.
7. **Kafatos, F. C., C. Weldon-Jones, and A. Efstradiadis.** 1979. Determination of nucleic acid sequence homologies and relative concentrations by dot blot hybridization procedure. Nucleic Acids Res. **7**:1541–1552.
8. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
9. **Miller, G., P. Wertheim, G. Wilson, J. Robinson, J. L. M. C. Geelan, J. Van Der NoorDaa, and A. J. Van Der Eb.** 1979. Transfection of human lymphoblastoma cells with herpes simplex viral DNA. Proc. Natl. Acad. Sci. USA **76**:949–953.
10. **Post, L. E., S. Mackem, and B. Roizman.** 1981. Regulation of alpha genes of herpes simplex virus: expression of chimeric genes produced by fusion of thymidine kinase with alpha gene promoters. Cell **24**:555–565.
11. **Price, R., and A. Kahn.** 1981. Resistance of peripheral autonomic neurons to in vivo productive infection by herpes simplex virus mutants deficient in thymidine kinase activity. Infect. Immun. **34**:571–580.
12. **Stevens, J. G., and M. L. Cook.** 1971. Latent herpes simplex in spinal ganglia of mice. Science **173**:843–845.
13. **Stevens, J. G., E. K. Wagner, G. B. Devi-Rao, M. L. Cook, and L. T. Feldman.** 1987. RNA complementary to a herpesvirus alpha gene is prominent in latently infected neurons. Science **235**:1056–1059.
14. **Tenser, R. B., K. A. Hay, and W. A. Edris.** 1989. Latency-associated transcript but not reactivatable virus is present in sensory ganglion neurons after inoculation of thymidine kinase-negative mutants of herpes simplex virus type 1. J. Virol. **63**:2861–2865.
15. **Tenser, R. B., R. L. Miller, and F. Rapp.** 1979. Trigeminal ganglion infection by thymidine kinase-negative mutants of herpes simplex virus. Science **205**:915–917.
16. **Thompson, R. L., M. L. Cook, G. B. Devi-Rao, E. K. Wagner, and J. G. Stevens.** 1986. Functional and molecular analysis of avirulent wild-type herpes simplex virus type 1 strain KOS. J. Virol. **58**:203–211.