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Resistance of Peripheral Autonomic Neurons to In Vivo Productive Infection by Herpes Simplex Virus Mutants Deficient in Thymidine Kinase Activity

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We used a model involving acute and latent herpes simplex virus (HSV) infections of mouse superior cervical ganglia to assess in vivo neuronal infections with two thymidine kinase-deficient (TK^-) mutants of HSV type 1. Despite replication of the TK^- HSV strains at the site of inoculation in the eyes, little if any viral replication occurred in the superior cervical ganglia, as assessed by the viral titers of ganglion homogenates, viral antigens in tissue sections, and histopathological evidence of cytopathology or inflammation. Cyclophosphamide-induced immunosuppression and treatment with 6-hydroxydopamine, which enhanced productive infections of superior cervical ganglia with TK^+ HSV, did not induce TK^- HSV ganglionic infections. Latent TK^- HSV infections were not detected by cocultivation of ganglion explants. Efforts to infect ganglia in culture after they were removed from animals indicated that superior cervical ganglia, and particularly their neuronal elements, resisted productive TK^- HSV infection. These results supported the hypothesis that viral thymidine kinase facilitates acute and reactivated productive HSV infections of neurons.

The herpes simplex virus (HSV) genome codes for a thymidine kinase which is distinct from cellular thymidine kinase (7, 9, 18). This presumably confers a selective advantage to the virus, but at present the nature of this advantage is not well understood. One suggestion is that the presence of viral thymidine kinase is related to the central role of neurons in the "life cycle" of HSV. Neurons serve as reservoirs for latent virus, and viral reactivation leads to recurrent epithelial eruptions in the field of innervation of these infected neurons (1, 15). Because neurons are nondividing cells with presumed low levels of thymidine kinase activity, exogenously supplied enzyme may be needed to facilitate viral replication. In line with this hypothesis, it has been reported that mutant HSV strains lacking thymidine kinase activity (TK^-) replicate poorly and induce a low level of latent infection in sensory ganglia of experimental animals (16, 17). On the basis of such observations, it has been suggested that the viral TK gene may be involved not only in viral replication but also in the process of latency.

In this paper we describe studies of infections of superior cervical ganglia (SCG) of the autonomic nervous system in mice, in which we used two TK^- strains of HSV type 1. The SCG is infected after intraocular injection of virus, with the virus reaching the ganglia over postganglionic axons (13). This system provides a convenient model, allowing measurement of the replication of the input virus at the site of inoculation (i.e., the eye) in addition to assessment of the ganglion. In all aspects studied thus far, HSV infection of the SCG has resembled infection of sensory ganglia. In this study we supplemented the results of previous studies by testing whether measures which favored viral replication (in this case, cyclophosphamide-induced immunosuppression and treatment with the selective neurotoxin 6-hydroxydopamine [6-OHDA]) altered the course of ganglionic infection by TK^- HSV (11, 13). In addition, we also studied viral replication in ganglia which were first removed from the animals and then inoculated in culture. The following five strains of HSV type 1 were tested: a TK^- strain derived from strain KOS (KOS/TK^-) and its parental strain (KOS/TK^+); the TK^- strain B2006 and its TK^+ parent, strain C1 101; and strain F, a TK^+ strain which we used in our previous studies of this model. Strain KOS/TK^- was chosen for these experiments principally because it was used in the studies of

Tenser and colleagues (16, 17), whereas strain B2006 was used because it has been characterized extensively (2, 5, 18).

MATERIALS AND METHODS

Viruses. Viral stock pools were prepared on rabbit kidney cells as previously described (12). In the case of strain KOS/TK$^-$, a sample was obtained from R. Tenser and passed one time in the presence of 50 μg of 1-β-arabinofuranosylthymine (ara-T) per ml, and the inoculating stock was grown by a single passage in the absence of ara-T. Strain KOS/TK$^+$ was propagated in an identical manner, except that ara-T was omitted. Strains B2006 and C1 101 were obtained from D. R. Dubbs, and each of these strains was propagated by two passages in our laboratory in rabbit kidney cells. All of the viral stocks were diluted to the same titer for viral inoculation in comparative studies. Viral titrations were performed on rabbit kidney cells by using an antibody-containing pooled human serum overlay technique (4). Rabbit kidney cells were produced from New Zealand white rabbits and were used at the first or second passage. In almost all experiments, representative samples of recovered virus were tested for the emergence of TK$^+$ virus in TK$^-$-inoculated animals or ganglia by titration in the absence and presence of 50 μg of ara-T per ml (10); this provided a crude but simple and economical screening test for the emergence of TK$^+$ variants that were sensitive to drug inhibition (10). Samples were considered to be TK$^-$ when only a minor reduction in titer (usually 0.2 to 0.6 $\log_{10}$ plaque-forming units [PFU]) was observed in the presence of the inhibitor; in contrast, in TK$^+$ samples a reduction of at least 3 $\log_{10}$ PFU was characteristic. With the exceptions described below, in all of the samples tested the recovered virus was the same as the input virus with respect to growth in ara-T.

Animals. We used 4- to 6-week-old BALB/c female mice (Charles River Laboratories, Wilmington, Mass.). Intraocular inoculation was performed as described previously (12). The stock virus pools for all inoculations were diluted so that 4 μl of a pool containing 1.0×10^6 PFU/ml was injected into the anterior chamber of the right eye of each mouse inoculated.

Virus assay. Homogenates of right-sided ganglia from inoculated mice sacrificed during the acute phase of infection and homogenates of ganglia infected in vitro were assayed for virus similarly. Each ganglion was washed three times and homogenized in 1.2 ml of phosphate-buffered saline containing bovine serum albumin. After the homogenate was frozen and thawed three times, the titers of 10-fold dilutions were determined with rabbit kidney cells. The titers of preparations from eyes were determined similarly (12). Mean values were calculated from $\log_{10}$ viral titers, and comparisons were made by using the Student t test. Ganglia were assayed for latent infections by explantation and cocultivation, using human embryonic lung indicator cells (Biofluids, Rockville, Md.). These cultures were maintained and observed for the appearance of cytopathological effects for 3 weeks. The results of these experiments are presented below as the number of explants from which HSV was recovered per number of ganglia assayed; comparisons were made by using the chi-square contingency method.

Immunohistochemistry. Ganglia which were studied by the peroxidase-antiperoxidase method for the presence of viral antigens were fixed in Bouin solution and embedded in paraffin; sections were stained as previously described by using rabbit anti-HSV serum as the primary serum (6). Sections stained with hematoxylin and eosin were also prepared from each of these ganglia by using routine methods.

Infection of ganglia in vitro. Ganglia were inoculated with virus after they were placed in culture. These ganglia were removed from mice (in this study both right and left ganglia were used for separate determinations), washed three times in phosphate-buffered saline, and placed in 1 ml of maintenance medium (Eagle minimal essential medium supplemented with 2% fetal calf serum and 50 μg of gentamicin per ml), containing 1.0×10^5 PFU of the viral strain to be tested. After 2 h, the ganglia were washed three times, and then they were maintained for different intervals, until they were harvested for determinations of viral titers and for immunoperoxidase and histological evaluations.

RESULTS

Kinetics of viral replication in the SCG and eyes of immunocompetent mice inoculated with HSV strains KOS/TK$^+$ and KOS/TK$^-$. In our initial studies, we compared the replication of viral strains KOS/TK$^+$ and KOS/TK$^-$ in mouse SCG and in eyes (Fig. 1). In SCG, strain KOS/TK$^+$ was first detected in homogenates on day 2, and the virus titers rose to their highest values between days 3 and 5, after which the titers decreased; by day 8 infectious virus was no longer found in ganglion homogenates. This kinetic curve was similar to the curve found previously with HSV type 1 strain F, although the titers in this study were lower (13). In contrast, minimal titers of strain KOS/TK$^-$ were detected in single ganglia on days 1, 3, and 4. Using a sample of undiluted homogenate from a positive ganglion from an animal that was inoculated with strain KOS/TK$^-$ and sacrificed on day 3, we prepared a virus stock by a single passage in rabbit kidney cells. When the titer of this preparation was determined in the absence of ara-T, 5×10^6 PFU of virus per ml was measured, compared with $<1 \times 10^2$ PFU/ml in the presence of 50 μg of ara-T per ml. This pattern of ara-T sensitivity is characteristic of TK$^+$ viruses and suggested that a viral revertant emerged or was selected in the ganglion. The other two positive ganglia were not tested, but the above-described results suggested that even the small amounts of virus recovered from the TK$^-$ virus-inoculated mice may not have indicated ganglionic replication of TK$^-$ virus and that little, if any, replication of strain KOS/TK$^-$

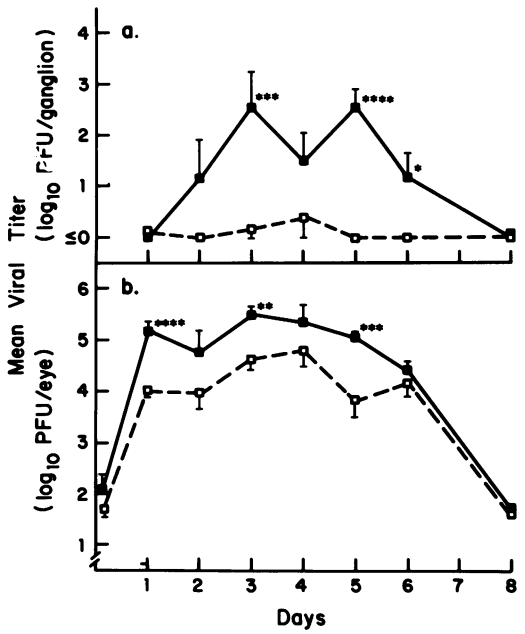

FIG. 1. *Kinetics of strain KOS/TK⁺ (■) and strain KOS/TK⁻ (□) replication in SCG and eyes. The mean viral titers in the SCG (a) and eyes (b) of mice inoculated with the two viral strains are shown. The ganglia and eyes of four or five mice were assayed at each interval after inoculation except on day 8, when only three ganglia and one eye of the TK⁺ virus-inoculated mice and five ganglia and five eyes of the TK⁻ virus-inoculated mice were assayed. One asterisk indicates P < 0.05, two asterisks indicate P < 0.02, three asterisks indicate P < 0.01, and four asterisks indicate P < 0.001 as determined by the Student t test.*

took place in the SCG. In contrast to these findings in the SCG, the TK⁻ strain replicated well in eyes, although the titers at all intervals tested were nearly 10-fold less than the titers in eyes inoculated with the TK⁺ parental strain.

The results of immunoperoxidase and histological evaluations of SCG sections paralleled the viral titers. Mice were inoculated with all five viral strains, and three to seven animals were sacrificed on days 3, 4, 5, and 7. In the case of the TK⁺ strains (KOS/TK⁺, C1 101, and F), antigen-positive neurons and supporting cells were clearly detected on day 3 and were present on days 4 and 5 (Fig. 2a and b), but by day 7 only traces of antigen were detected. In the histological examinations, abnormal neurons with intranuclear inclusions admixed with inflammation were observed on days 3 through 5 (Fig. 3a and b), and by day 7 the inflammatory response obscured or replaced the abnormal neurons. In contrast to these findings with the TK⁺ strains, after inoculation with both strain KOS/TK⁻ and strain B2006 ganglia appeared completely normal; neither viral antigens nor abnormal neurons were found, and no evidence of inflammation was detected in SCG sections (data not shown).

Latent infection. Next, animals infected with the different viral strains were evaluated for the presence of latent infections. Mice were sacrificed between days 21 and 28 after inoculation, and the SCG were explanted. Table 1 shows the combined results of four experiments. Each of the three TK⁺ strains (KOS/TK⁺, C1 101, and F) induced detectable latent infections, whereas virus was not recovered from any of the ganglia from mice inoculated with the two TK⁻ strains (KOS/TK⁻ and B2006).

Attempts to potentiate SCG infection by immunosuppression and 6-OHDA administration. Next, we evaluated infections of the SCG with the different viral strains in mice subjected to two experimental manipulations which were designed to increase the magnitude of acute SCG infection, namely, immunosuppression with cyclophosphamide and administration of 6-OHDA.

Immunosuppression by cyclophosphamide was used to test the hypothesis that TK⁻ virus may be at a selective disadvantage because the virus or virus-infected cells or both are eliminated more readily by immune defenses. Such an increased susceptibility to immune defenses might mask the capacity of TK⁻ virus to replicate in neurons. Figure 4 shows the results of two experiments in which mice received 250 mg of cyclophosphamide per kg intraperitoneally 1 day after viral inoculation. In the first experiment, we compared SCG and eye infections in cyclophosphamide-treated mice inoculated with either strain KOS/TK⁺ or strain KOS/TK⁻, and in the second we made a similar comparison in mice inoculated with strains C1 101, B2006, and F. The animals infected with strains KOS/TK⁺ and KOS/TK⁻ were sacrificed on days 4, 6, 7, and 8 after infection, and, as in previous studies, cyclophosphamide administration allowed infection of the SCG to persist through day 8 in mice infected with the TK⁺ strain (13). In contrast, virus was not detected in the ganglia in any of the strain KOS/TK⁻-inoculated mice. This difference between the parental and mutant strains occurred despite similar viral titers on days 4 and 6 in eye homogenates. Experiments with strains C1 101 and B2006 assayed on days 5, 6, and 7 after inoculation produced similar results. None of the strain B2006-inoculated mice exhibited virus in SCG homogenates, whereas virus was detected readily in the ganglia of strain

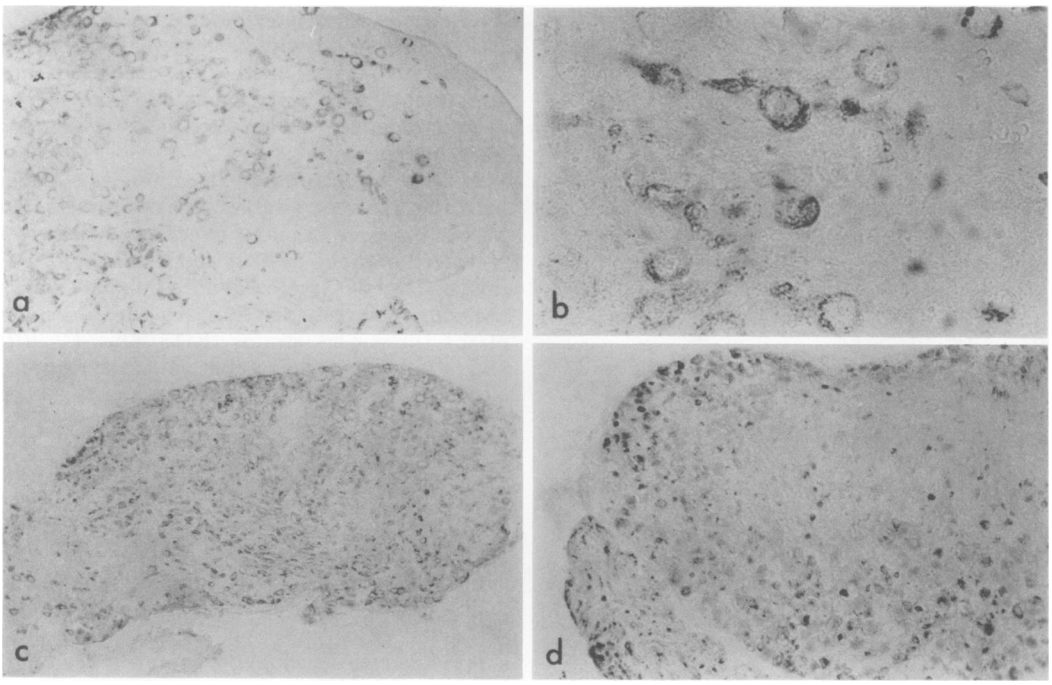

FIG. 2. *Viral antigens in TK⁺ HSV-infected ganglia. (a) Immunoperoxidase staining of HSV antigen located in many neurons of an SCG section from a mouse sacrificed 5 days after inoculation with strain KOS/TK⁺. ×40. (b) Greater magnification of the same section as in (a), illustrating the neuronal localization of the peroxidase-antiperoxidase staining of viral antigens. ×160. (c) Immunoperoxidase staining of SCG from a mouse treated with cyclophosphamide and sacrificed 5 days after inoculation with strain KOS/TK⁺. Almost all neurons are stained. ×40. (d) Immunoperoxidase staining of SCG from a mouse treated with 6-OHDA and sacrificed 6 days after inoculation with strain KOS/TK⁺. Widespread neuronal staining and peripheral nerve staining (lower left) are present. ×40.*

C1 101-inoculated mice (the latter group could only be assayed through day 6 because the remaining mice died before day 7). In these groups, the titers of the eye homogenates differed on days 5 and 6; the strain C1 101 titers were higher, but, interestingly, the strain B2006 mean ocular titers were higher than the titers in strain KOS/TK⁺-inoculated mice and on day 7 were as high as the titers in animals inoculated with strain F.

Previously, we reported that administration of the neurotoxin 6-OHDA potentiated acute infections of SCG with HSV and decreased subsequent latency (11). We hypothesized that this effect resulted from a direct action of the drug on nerve terminals, which altered the metabolic state of the neurons such that productive lytic infection was favored over latency. This effect was observed most easily in animals that were passively immunized with antiviral antibody; the background of viral replication was reduced in untreated controls, yet high viral titers were still observed in the ganglia of drug-treated mice. In this experiment, 250 mg of 6-OHDA per kg was administered intraperitoneally to animals 1 day after viral inoculation, and mice were sacrificed 5 days after inoculation for ganglion homogenization and assessment of viral titers; because the purpose of this study was not to test the 6-OHDA effect but to optimize viral replication, no antibody was given. In addition to animals treated with 6-OHDA, groups of control mice received an ascorbate-containing drug vehicle 1 day after viral inoculation. Table 2 shows the results of this study. Virus was detected in an SCG homogenate in only one TK⁻ virus-inoculated animal (an animal not receiving 6-OHDA). In this mouse, which was inoculated with strain B2006, only 3 PFU of virus was found; because no virus was recovered from a second sample of this ganglion homogenate, a further analysis of the recovered virus was not performed.

Immunoperoxidase staining for HSV antigens and histological evaluations were also performed on ganglion sections from mice inoculated with the five viral strains and subjected to cyclophosphamide or 6-OHDA treatment. The results of these experiments paralleled those of the viral titer experiments. In the case of the three TK⁺

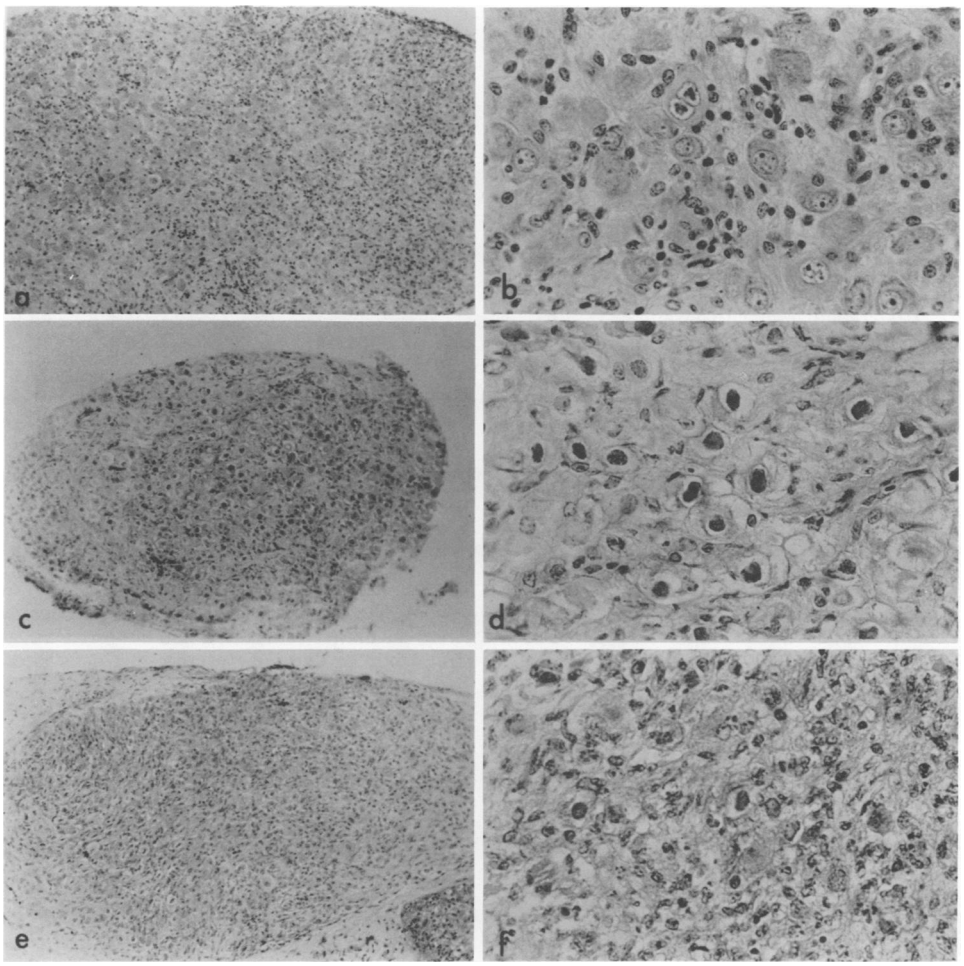

FIG. 3. *Light microscopy of TK⁺ HSV-infected ganglia. (a) SCG of an immunocompetent mouse sacrificed 5 days after inoculation with strain F. Inflammation is admixed with normal and abnormal neurons. ×40. (b) Greater magnification of the same section as in (a). A rare binucleate, inclusion body-bearing neuron is present. ×160. (c) SCG of a cyclophosphamide-treated mouse sacrificed 6 days after inoculation with strain C1 101. Virtually every neuron contains an intranuclear inclusion, although inflammation is absent. ×40. (d) Greater magnification of the same ganglion as in (c). ×160. (e) SCG of a mouse treated with 6-OHDA and inoculated with strain KOS/TK⁺ 6 days before sacrifice. All neurons are abnormal, and mononuclear inflammation is evident. ×40. (f) Greater magnification of the same section as in (e). All sections were stained with hematoxylin and eosin.*

strains, cyclophosphamide and 6-OHDA markedly increased both the distribution of viral antigen within ganglia and the histopathological alterations of ganglion cells. Immunosuppression by cyclophosphamide markedly depleted the inflammatory response and resulted in infection of almost 100% of the ganglionic neurons, as detected by the presence of viral antigens (Fig. 2c) and by marked alterations of these cells, which had prominent intranuclear inclusions and cytoplasmic pallor (Fig. 3c and d). Although 6-OHDA did not depress the inflammatory response, it also induced a more extensive distribution of viral antigen and neuronal cytopathology (Fig. 2d and 3e and f). Despite this marked potentiation of infection in the TK⁺ virus-inoculated animals, viral antigens or histopathological changes were not detected in SCG sections in any of the mice that were inoculated with either strain KOS/TK⁻ or strain B2006 and treated with the two drugs (data not shown).

Infection of ganglia in vitro. Next, we studied replication of the TK⁻ strains in the SCG when the ganglia were inoculated with virus

TABLE 1. *Latent SCG infections: comparison of ganglion explants from mice inoculated with TK⁺ and TK⁻ strains*

HSV strain	No. of ganglia positive/no. of ganglia tested	
KOS/TK⁺	10/15	(67)[a]
KOS/TK⁻	0/23[b]	(0)
C1 101	4/8	(50)
B2006	0/15[c]	(0)
F	3/12	(25)

[a] Numbers in parentheses are percentages.
[b] $P < 0.001$ compared with strain KOS/TK⁺, as determined by chi-square contingency.
[c] $P < 0.01$ compared with strain C1 101, as determined by chi-square contingency.

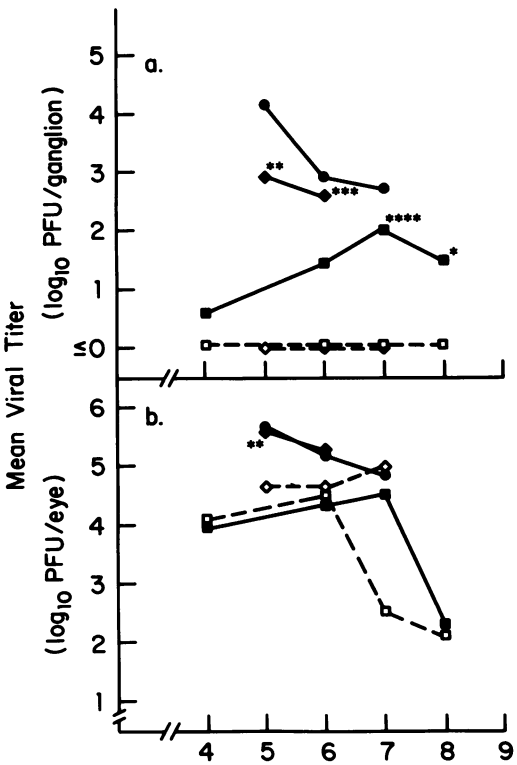

FIG. 4. *Comparison of mean viral titers in homogenates of SCG and eyes of cyclophosphamide-treated mice inoculated with TK⁺ and TK⁻ viral strains. The results of two experiments are combined, as explained in the text. Each point represents the mean titer of SCG or eye homogenates from four or five animals. One asterisk indicates $P < 0.05$, two asterisks indicate $P < 0.02$; three asterisks indicate $P < 0.01$, and four asterisks indicate $P < 0.001$, as determined by the Student t test comparing corresponding strains (i.e., KOS/TK⁻ versus KOS/TK⁺ and B2006 versus C1 101). All other such comparisons were not significant. Symbols: ■, strain KOS/TK⁺; □, strain KOS/TK⁻; ◆, strain C1 101; ◇, strain B2006; ●, strain F.*

after they were removed from the animals and placed in culture. This procedure was designed to eliminate both host immune defenses and the influence of peripheral replication at the site of inoculation. Homogenates of these ganglia were assessed for viral titers on days 3 through 6 after inoculation. As Fig. 5 shows, the three TK⁺ strains replicated to high titers by days 5 and 6. Strain KOS/TK⁻ also replicated but yielded lower titers, whereas minimal amounts of virus

TABLE 2. *Effect of 6-OHDA treatment on SCG infections after inoculation with TK⁺ and TK⁻ strains*

HSV strain	Viral titers (log_{10} PFU)[a]	
	Control group	6-OHDA-treated group
KOS/TK⁺	2.89 ± 0.26	3.40 ± 0.47
KOS/TK⁻	0[b]	0[c]
C1 101	3.14 ± 0.09	2.90 ± 0.33
B2006	0.06 ± 0.04[d]	0[d]
F	3.50 ± 0.25	4.64 ± 0.21[e]

[a] Mean ± standard error of the mean. Mean values were calculated from results of homogenates of ganglia from four or five mice sacrificed 5 days after viral inoculation.
[b] $P < 0.05$ compared with strain KOS/TK⁺.
[c] $P < 0.001$ compared with strain KOS/TK⁺.
[d] $P < 0.001$ compared with strain C1 101.
[e] $P < 0.05$ compared with strain F control group.
The differences between the control groups and the 6-OHDA-treated groups for the strains other than F were not significant, as determined by the Student *t* test.

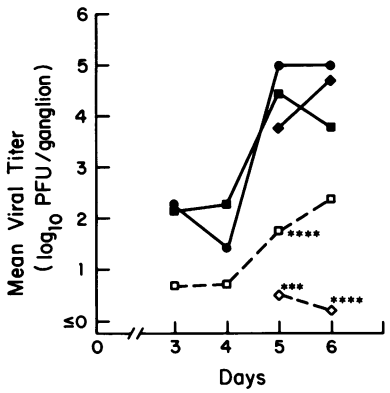

FIG. 5. *Mean viral titers of homogenates of SCG inoculated in culture. Each point represents the mean of the viral titers of five ganglia. Three asterisks indicate $P < 0.01$, and four asterisks indicate $P < 0.001$, as determined by the Student t test comparing corresponding strains (i.e., KOS/TK⁻ versus KOS/TK⁺ and B2006 versus C1 101). All other such comparisons were not significant. Symbols: ■, strain KOS/TK⁺; □, strain KOS/TK⁻; ◆, strain C1 101; ◇, strain B2006; ●, strain F.*

were detected in ganglia inoculated with strain B2006.

To assess the cell type in which replication occurred, immunoperoxidase staining for viral antigen was assessed in four or five ganglia that were infected with each viral strain and harvested after 5 and 6 days of incubation in vitro. Extensive infections of ganglia with the three TK^+ strains were observed. Antigen staining was greatest at the periphery but penetrated well into the substance of each ganglion; only the central region was not stained (Fig. 6a). Both nonneuronal elements and neurons were stained in abundance (Fig. 6b). In contrast, more limited infections with strains KOS/TK^- and B2006 were noted. In ganglia inoculated with these two TK^- strains, little antigen was found, and even in the ganglia that were maximally involved only a superficial peripheral margin of antigen-positive cells was found, with the exception of the nerve exiting or entering the ganglion, where more extensive antigen staining occurred (Fig. 6c through f). Almost all of the staining was localized to nonneuronal elements, and in some cases antigen-positive satellite cells surrounded negative neurons (Fig. 6c and d). Neurons were lightly stained only very rarely (Fig. 6f). Histological examinations confirmed the results of immunoperoxidase staining. In ganglia inoculated with all five viral strains, central areas of necrosis were found frequently, and these areas could probably be attributed to death in vitro of these portions of the ganglia due to insufficient diffusion of nutrients or metabolites. However, in other respects the ganglia inoculated with the TK^+ and TK^- viruses differed. In almost all ganglia inoculated with strain KOS/TK^+, C1 101, or F, we observed marked neuronal abnormalities and widespread intranuclear inclusions (Fig. 6g). In contrast, we found no evidence of neuronal inclusions in the ganglia inoculated with strains KOS/TK^- and B2006 (Fig. 6h), although occasionally abnormal nonneuronal cells were observed peripherally in a distribution similar to the distribution of immunoperoxidase staining.

DISCUSSION

In this study little, if any, replication of the two TK^- strains was detected in the SCG during the acute phase of infection. This was true not only in otherwise untreated immunocompetent mice, but also in mice immunosuppressed with cyclophosphamide or treated with 6-OHDA. Likewise, viral antigens, light microscopic cytopathology, and evidence of inflammation were not detected in the ganglia of untreated or treated animals inoculated with the TK^- viruses. In addition, latent TK^- virus was not rescued from ganglion explants. Furthermore, a marked resistance of ganglia, particularly neuronal elements, to TK^- HSV infection in vitro was found.

The rationale for treating mice with cyclophosphamide and with 6-OHDA was that both of these drugs potentiate productive ganglionic infections (11, 13). This effect was confirmed clearly in animals treated with cyclophosphamide and infected with the TK^+ viruses, in which elevated viral titers were sustained in ganglion homogenates until the animals died on day 7 or 8 after inoculation. Similarly, immunoperoxidase and histopathological evaluations of these ganglia revealed widespread viral antigen and morphological alterations of neurons without appreciable inflammation. The fact that such changes were not observed in mice inoculated with TK^- viruses indicates that the selective advantage against the TK^- strains did not result from merely a greater susceptibility to elimination of TK^- viruses and virus-infected cells by immune defenses. Similarly, 6-OHDA failed to potentiate ganglionic infections in TK^- virus-inoculated animals; no productive infection, viral antigen, or histopathological evidence of inflammation was found in these mice. As previously reported, titers of ganglia from mice inoculated with strain F were higher in 6-OHDA-treated animals than in control mice (11). However, in the case of the other two TK^+ strains, higher titers were not observed in animals that received 6-OHDA. Whether this failure to detect higher titers in drug-treated mice inoculated with strains KOS/TK^+ and C1 101 resulted from the small sample size, from the fact that an effect of 6-OHDA was difficult to recognize in nonimmunized mice, or from a more fundamental biological difference in the infections with these two strains was not pursued. The question posed in this study was whether treatment with the drug could uncover or potentiate TK^- viral replication. Our results showed that with 6-OHDA and with cyclophosphamide there was no evidence of acute infection of the ganglia, which emphasized that the SCG are resistant to productive infection by TK^- HSV in vivo.

The results of our experiments are open to two broad hypothetical interpretations, depending largely upon whether TK^- viruses reach the SCG after eye inoculation. Unfortunately, this issue was not tested directly in these experiments and had to be assessed indirectly. If TK^- viruses failed to reach the ganglia, then interpretation of our results is straightforward; failure of the viruses to reach the ganglia will clearly result in the absence of detectable viral replication,

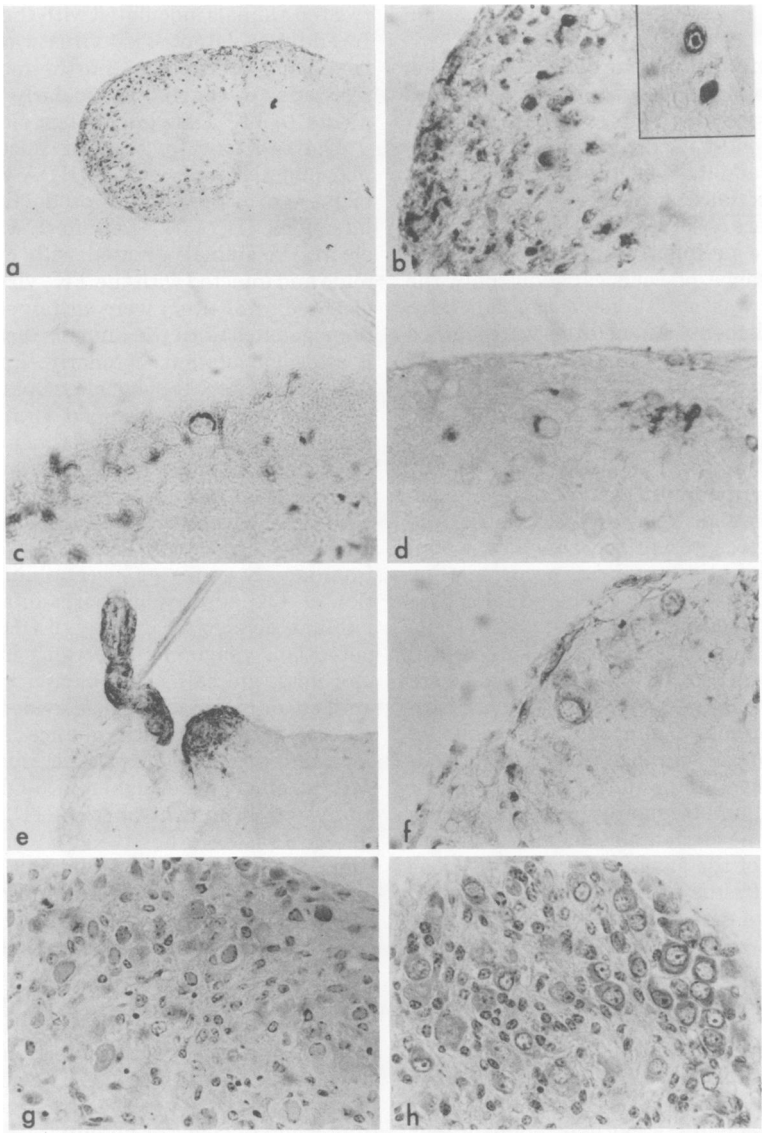

FIG. 6. *Immunoperoxidase staining and light microscopy of SCG inoculated in culture. (a) Immunoperoxidase staining of SCG inoculated with strain KOS/TK$^+$ 5 days earlier. ×40. (b) Greater magnification of the same section as in (a), showing antigen localization in neurons and nonneuronal elements. The insert shows well-defined neuronal staining. ×160. (c) Immunoperoxidase staining of SCG inoculated with strain KOS/TK$^-$ 5 days earlier. Staining of nonneuronal elements with sparing of neurons is present. ×160.(d) Immunoperoxidase staining of SCG inoculated with strain KOS/TK$^-$ 6 days earlier. Staining of nonneuronal elements is present. ×160. (e) Immunoperoxidase staining of SCG inoculated with strain KOS/TK$^+$ 5 days earlier. Strong staining of the nonneuronal cells in the nerve is present, with complete sparing of ganglionic neurons. ×40. (f) Immunoperoxidase staining of SCG inoculated with strain B2006 6 days earlier. Light neuronal staining and nonneuronal staining are present. ×160. (g) Section of SCG inoculated with strain C1 101 5 days earlier and stained with hematoxylin and eosin. Marked neuronal cytopathology and intranuclear inclusions are evident. ×160. (h) Section of SCG inoculated with strain B2006 5 days earlier and stained with hematoxylin and eosin. Neurons show normal morphology. ×160.*

viral antigen expression, and latency. However, why should TK$^-$ virus fail to reach the ganglia? Two possible reasons are (i) that TK$^-$ virus cannot be taken up and transported by postganglionic SCG axons and (ii) that the amounts of virus available at the nerve terminals in the eyes were too small for such transport to occur. The first of these possibilities seems unlikely. It is

difficult to understand why a TK⁻ virus would be at a selective disadvantage in relation to axonal uptake and transport. These phenomena presumably depend upon viral surface molecules that are recognized by cellular receptors and perhaps also upon other structural molecules that are required to interact with the axonal transport system. A defect in the synthesis or function of viral thymidine kinase should not alter these properties of infectious virions. On the other hand, less available virus at nerve terminals is likely to diminish ganglionic infection. However, it is questionable whether the magnitude of the decrease in eye infections observed in these experiments could, by itself, account for the virtually complete absence of SCG infections. Thus, within individual experiments, at times higher titers were observed in the eyes of TK⁻ virus-inoculated mice in which ganglia were negative for virus than in the eyes of TK⁺ virus-inoculated mice in which positive ganglia were found. Also, in the cyclophosphamide-treated animals, in which higher viral titers were found in the eyes of TK⁻ virus-inoculated mice than in the eyes of their TK⁺ virus-inoculated counterparts, the SCG were negative. In fact, in these experiments the mean viral titers in the eyes of mice inoculated with strain B2006 were higher than the titers in the eyes of mice inoculated with strain KOS/TK⁺, yet no strain B2006 virus was detected in ganglia. These results suggest that under our experimental conditions, TK⁻ virus should have been able to reach the ganglia.

However, if TK⁻ virus did indeed reach the ganglia, how can our results be fully explained? The following two possible reasons for the presence of negative ganglion homogenates despite delivery of HSV to the ganglia during the acute stages of infection warrant consideration: (i) ganglionic infection was aborted at the stage of viral deoxyribonucleic acid synthesis because the neurons were nonpermissive for TK⁻ virus; and (ii) virus became latent without any initial productive infection. Either of these mechanisms could explain adequately the failure to detect infectious viruses in ganglion homogenates. However, if infection was aborted only because of insufficient thymidine kinase activity, we would expect that viral antigens might still expressed in such abortively infected cells. Because HSV thymidine kinase is a member of the so-called β group of viral polypeptides (14), both α and β polypeptides should still be synthesized in abortively infected cells and should be detected by immunoperoxidase staining. However, viral antigens were not detected in any of the ganglia from TK-virus-inoculated animals, including those treated with cyclophosphamide and 6-OHDA. This suggests that upon reaching a ganglion, TK⁻ virus became latent, without detectable antigen expression. In this way TK⁻ virus infection may not differ from TK⁺ virus latency, in which viral antigen is found neither during the latent stage nor during the acute stage of infection in immunized mice (unpublished data).

However, if the two TK⁻ viruses became latent, why could they not be recovered from ganglion explants? An explanation for this may be provided by the results of the experiments in which ganglion explants were inoculated with virus in cell culture. These studies showed that ganglia were resistant to infection by TK⁻ virus, although they readily supported TK⁻ HSV infections. Moreover, the neurons within the ganglia appeared to be particularly resistant to TK⁻ virus infection; when present, the majority of viral antigen was located in nonneuronal cells. In contrast, in TK⁺ virus-inoculated ganglia most viable neurons exhibited viral antigen. If ganglionic neurons are resistant to TK⁻ HSV replication even under explantation conditions (i.e., the conditions used for viral rescue), we would anticipate that latent virus may not be recovered. Detection of latency depends on reactivation of virus, presumably in neurons, with productive infection followed by amplification as a result of spread and further replication in neighboring cells. This process may well be interrupted in the case of the TK⁻ strains.

Using this line of reasoning, we favor the hypothesis that the TK⁻ viruses reached the ganglia and established latent infections, which went undetected by the usual explantation rescue techniques because the TK⁻ viruses replicated poorly in ganglionic neurons. This hypothesis predicts that ganglionic latency is established readily by TK⁻ viruses and that when additional methods (such as nucleic acid hybridization and complementation rescue) are refined and applied to this model, the presence of the latent genome will be detected. Indeed, because under a variety of experimental conditions the magnitude of an acute productive infection and the subsequent prevalence of latency appear to be inversely related (13), we predict that ganglionic latent infections are established regularly by TK⁻ viruses. These formulations also imply that establishing latency is mechanistically unrelated to the presence of thymidine kinase activity, but rather that the following two independent constraints operated to alter HSV replication in our experiments: thymidine kinase activity and a cell-determined mechanism which controlled whether productive or latent infection occurred. This cell-determined mechanism is not well understood, but in the case of neurons it is probably best demonstrated by experimental

neurectomy, which potentiates replication during acute infections and reactivation of latent virus (12, 13, 19). This interpretation allows reconciliation of our results with the data of Field and Wildy (3), who found that strain B2006 induced explantation-detectable latent infections in cervical sensory ganglia. The ability of these investigators to detect latent strain B2006 virus may reflect differences in the experimental methods used, perhaps in the strain or age of mice used. The evolutionary significance of HSV thymidine kinase remains uncertain, but our results suggest that if conservation of thymidine kinase is related to neuronal infection, then it is related primarily to reactivation rather than to establishment of latent infection.

The mechanism of resistance of neurons to TK^- HSV infection is also not entirely clear. Neurons are postmitotic cells that undergo minimal (repair-related) deoxyribonucleic acid synthesis. To support productive HSV infection, thymidine kinase is probably needed to expand the pool of phosphorylated thymidine derivatives rapidly for incorporation into deoxyribonucleic acid and perhaps for regulation of other synthetic pathways involved in deoxyribonucleic acid synthesis (8). Without thymidine kinase supplied by the virus, neurons behave like serum-starved cells in culture (7) and provide a poor milieu for viral replication. However, this is apparently not an obligatory character of neurons. We have found that isolated neurons in culture support TK^- HSV replication in a manner indistinguishable from replication of TK^+ parent strains (unpublished data). It may be that neurons in vivo, even when subsequently explanted, differ from neurons maintained in isolation within cell cultures with respect to the ability to support replication of TK^- HSV. Whether this difference results from neuronal maturation, from the trophic influence of satellite cells or innervated target organs, or from other epigenetic influences operating in vivo remains to be determined.

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