JOURNAL OF VIROLOGY, Nov. 1994, p. 7212–7220
0022-538X/94/$04.00+0
Copyright © 1994, American Society for Microbiology
Vol. 68, No. 11

# Secondary Herpes Simplex Virus Latent Infection in Transplanted Ganglia

RICHARD B. TENSER,[1,2]* WADE A. EDRIS,[2] ANDREW GAYDOS,[1] AND KATHLEEN A. HAY[1]

*Departments of Medicine (Neurology)[1] and Microbiology and Immunology,[2]*
*Pennsylvania State University College of Medicine,*
*Hershey, Pennsylvania 17033*

Received 27 December 1993/Accepted 26 July 1994

**Sensory ganglia latently infected with herpes simplex virus (HSV) were transplanted beneath the renal capsule of syngeneic recipients, and the latent infection remaining was investigated. HSV latency-associated transcript (LAT) expression and reactivation of HSV after explant of transplanted dorsal root ganglia were monitored as markers of latency. Two to four weeks after transplantation, both indicated evidence of HSV latency in transplants. At those times, infectious virus was not detected in direct ganglion homogenates. In addition, viral antigen and infected cell polypeptide 4 RNA were not detected. Taken together, the results suggested that HSV latent infection rather than persistent infection was present in transplants. From these results, two explanations seemed possible: latency was maintained in transplanted neurons, or alternatively, latency developed after transplantation, in neurons not previously latently infected. The latter was considered putative secondary latency and was investigated in three ways. First, evidence of reactivation which might serve as a source for secondary latency was evaluated. Reactivation of HSV in transplants was evident from HSV antigen expression (52% of transplants) and the presence of cell-free virus (38% of transplants) 3 to 5 days after transplantation. Second, putative secondary latency was investigated in recipients immunized with HSV prior to receiving latently infected ganglia. Reactivation was not detected 3 to 5 days after transplantation in immunized recipients, and LAT expression was rare in these recipients after 3 to 4 weeks. Lastly, the possibility of secondary latency was investigated by comparing results obtained with standard HSV and with reactivation-defective thymidine kinase-negative ($TK^-$) HSV. Defective reactivation of $TK^-$ HSV was demonstrated by immunohistochemistry and by the inability to isolate infectious virus. Donor dorsal root ganglia latently infected with $TK^+$ HSV showed many LAT-positive neurons 2 or more weeks after transplantation (average, 26 per transplant). However, LAT expression was undetectable or minimal >2 weeks after transplantation in donor ganglia latently infected with $TK^-$ HSV (average, 0.2 per transplant). Impaired reactivation of $TK^-$ HSV-infected donor ganglia after transplantation, therefore, was correlated with subsequent limited LAT expression. From these results, the occurrence of secondary latency was concluded for ganglia latently infected with $TK^+$ HSV and transplanted beneath the kidney capsule. In vivo reactivation in this transplant model may provide a more useful means to investigate HSV reactivation than in usual in vitro explant models and may complement other in vivo reactivation models. The occurrence of secondary latency was unique. The inhibition of secondary latency by the immune system may provide an avenue to evaluate immunological control of HSV latency.**

Herpes simplex virus (HSV) is a common neurotropic virus which in humans and experimental animals readily establishes latent infection of sensory ganglion neurons (8, 30). During latency, limited viral transcription occurs and latency-associated transcripts (LAT) are detected in neuronal nuclei (5, 16, 17, 34, 36, 42). Viral antigens are not detected during latency, and infectious virus is not present. However, virus is easily isolated after reactivation of HSV from explant cultures of latently infected ganglia (10, 32). Viral factors important for HSV reactivation have been investigated by the infection of experimental animals with HSV mutants and the subsequent reactivation or lack of reactivation of HSV from ganglion explants (4, 9, 18, 19, 31, 37, 39). Investigation of host factors important for reactivation, including immunological factors, have been less amenable to study. This has been in part because of the difficulty in inducing reactivation in vivo in mice and also because explant culture to induce reactivation removes potentially important host factors. Destruction of ganglion explants occurs after HSV reactivation in explants in vitro. This is very different from usual in vivo reactivation in humans, in which case destruction of the latently infected ganglion does not occur and latency is maintained after reactivation. The immune system probably plays a role in this. However, while immunological control has been well established as being of importance in the general control of systemic HSV infection, a role for the immune system in the establishment and maintenance of latency is less clear. Investigators have used immunosuppression to induce or enhance in vivo HSV reactivation (21, 25, 30), but no evidence has been provided for an effect of the immune system on reactivation at the cellular or molecular level or on the maintenance of HSV latency.

We report here results of a ganglion transplantation model which permitted HSV reactivation under somewhat more physiological conditions than are usually used for reactivation in ganglion explants. In addition, latency was maintained in transplants after reactivation. In this model, ganglia latently infected with HSV were transplanted beneath the renal capsule of syngeneic mice. Reactivation was detected by evidence of HSV antigen and cell-free virus several days after transplantation. Latency in transplants was detected by the presence of

* Corresponding author. Mailing address: Pennsylvania State University College of Medicine, 500 University Dr., Division of Neurology, Room C6840, Hershey, PA 17033. Phone: (717) 531-8692. Fax: (717) 531-4694. Electronic mail address: rtenser@med.hmc.psu.edu.

LAT and also by HSV reactivation in explants. HSV was not detected in direct homogenates. In addition, HSV RNAs other than LAT and HSV antigens were not detected in transplants, further supporting the conclusion that HSV latent infection was present. The effect of the immune system on control of reactivation and latency was determined by comparison of results between naive and previously HSV-immunized transplant recipients. Lastly, reactivation and latency in transplanted ganglia previously latently infected with thymidine kinase-negative ($TK^-$) HSV were investigated. From these studies, we concluded that secondary latent infection occurred in transplants, in which neurons not previously latently infected became latently infected. In summary, we observed (i) reactivation of HSV and then probable secondary latency in transplants, (ii) inhibition of secondary latency in previously immunized recipients, and (iii) secondary latency in $TK^+$ but only rarely in $TK^-$ HSV-infected transplants. The occurrence of secondary latency and its inhibition by immunization may relate to mechanisms for the maintenance of HSV latency in vivo.

## MATERIALS AND METHODS

**Establishment of HSV latent infection.** Latent HSV infection of the fourth lumbar (L-4) and L-5 dorsal root ganglia (DRG) was established after bilateral rear footpad inoculation (50 μl) of 6- to 8-week-old male and female C57BL/6 mice. $TK^+$ HSV-1 (strain KOS; $10^8$ PFU/ml) and deletion mutant $TK^-$ HSV-1 (KOS 3B; $5 \times 10^7$ PFU/ml) were grown in Vero cells (36). TK activity of $TK^-$ HSV was <1% of the parental level (36). Mouse inoculation (under general anesthesia [methoxyfluorane]) was performed as previously described (36). By this method, HSV latent infection is detected in 90 to 95% of DRG (unpublished observation). During latency (>21 days after footpad inoculation), HSV can be isolated from DRG after explant culture of ganglia but not from direct ganglion homogenates.

**Transplantation of latently infected ganglia.** During HSV latency, DRG were surgically removed under general anesthesia and under sterile conditions. After being briefly washed in sterile balanced salt solution, DRG were transplanted beneath the renal capsule of syngeneic recipients. For this procedure, recipients were anesthetized and the fur on the left posterior flank was removed. The left kidney was exposed through a posterior incision under sterile conditions. The kidney capsule was nicked with a scalpel, and transplants were gently inserted beneath the capsule. Peritoneal and cutaneous tissues were closed with sutures or clips. Two DRG (one L-4 and one L-5 from the same donor) were transplanted together. In all discussion below, each pair of ganglia will be referred to as a transplant. Some recipients were immunized with HSV prior to receiving latently infected DRG (see Table 3 for methods of immunization). At several time points after transplantation, mice were sacrificed, and transplanted ganglia were assayed for HSV reactivation (by the detection of HSV antigen and infectious cell-free HSV) and for latency (by the detection of LAT and the isolation of HSV from transplant explants but not direct homogenates).

**HSV reactivation in transplants. (i) Infectious HSV in transplanted DRG homogenates.** Kidneys bearing transplants were removed from anesthetized and exsanguinated mice, and transplants were dissected free from surrounding kidney. Transplanted ganglia were frozen at −70°C in 0.5 ml of medium containing 2% serum. After homogenization and centrifugation, supernatants and debris were cultured on Vero cell monolayers to isolate infectious HSV. The isolation of HSV in direct homogenates indicates cell-free HSV and the occurrence of HSV reactivation. Transplants were scored as positive or negative for cell-free HSV.

**(ii) HSV antigen.** Transplants were removed and fixed with 4% paraformaldehyde, and paraffin sections were prepared. By using peroxidase-antiperoxidase methodology, HSV antigen was detected with polyclonal HSV antibody (1:500; Dako, Santa Barbara, Calif.), polyclonal glycoprotein C (gC) antiserum (1:500) (6; provided by R. Courtney), or monoclonal antibody to HSV infected cell polypeptide 4 (ICP4; 1:200; ATCC HB 8183. designation 58-S). Serial sections (10 μm) were examined, and transplants were scored as positive or negative for HSV antigen. In brief, after rehydration, tissue sections were treated with 3% $H_2O_2$ in methanol and then 5% dimethyl sulfoxide in buffered saline. After blocking with 5% powdered milk in buffered saline, primary antibody was added and sections were incubated overnight at 4°C. After washing, secondary antibody (1:50) was added for 60 min at room temperature. Peroxidase-antiperoxidase (1:50) and then diaminobenzidine were added to detect HSV antigen. HSV antigen can be detected during acute infection and reactivation but not during latency. HSV antigen present in transplants was considered to indicate reactivation.

**HSV latent infection in transplants. (i) Isolation of HSV in transplanted DRG explants but not in direct homogenates.** Transplants were removed after 21 to 28 days and tested for infectious virus in direct homogenates as described above or tested for HSV reactivation in explants. For the latter, transplants were dissected free from surrounding kidney and explanted at 37°C by usual methods for 6 days. After that period, ganglia were freeze-thawed, homogenized, and assayed for infectious virus on monolayer cells. Detection of infectious virus after explantation of transplants but not in direct DRG transplants, which were homogenized without being explanted, was considered to indicate latent infection. The isolation of cell-associated HSV in explants but not cell-free HSV (in direct homogenates) is a typical marker of HSV latency. Transplants were scored as positive or negative for latency.

**(ii) HSV antigen in transplanted DRG.** The lack of HSV antigen 10 or more days after transplantation, at a time when LAT was present and HSV could be isolated from explant culture of transplants, was considered to indicate HSV latent infection.

**(iii) HSV LAT in transplanted DRG.** HSV-1 LAT was assayed by in situ hybridization on serial sections (10 μm). LAT was detected by hybridization with a strand-specific oligonucleotide, using methods as described previously (36). In brief, a synthetic oligonucleotide corresponding to nucleotides 611 to 640 of the HSV-1 sequence published by Wagner et al. (41), which is antisense to LAT, was 3′ end-labeled ([$^{35}$S] dCTP; 1,000 Ci/mmol; Amersham). A control oligonucleotide probe used was specific for HSV-1 ICP4 RNA. The ICP4 RNA oligonucleotide corresponds to nucleotides 1722 to 1751 of the published sequence (22). Labeling was as for the LAT oligonucleotide. It was expected that the control probe would detect HSV RNA during acute but not latent infection. When testing the ICP4 RNA probe on transplants, we included acutely infected trigeminal ganglion tissue (3 to 4 days after corneal inoculation) as a positive control. Specific activities of probes were $1 \times 10^8$ to $2 \times 10^8$ cpm/μg, and tissue sections were hybridized with 1 to 3 ng (approximately $10^5$ cpm) for 72 h (10% dextran sulfate, 45% formamide, 2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]). Individual LAT-positive neurons were counted. Hybridization detection of LAT in the typical neuronal nucleus distribution and the

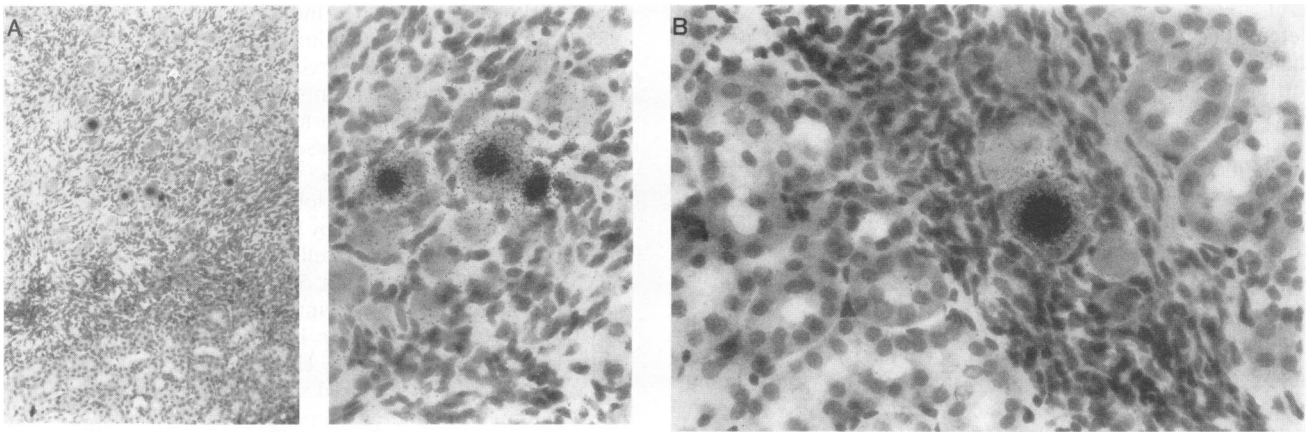


FIG. 1. HSV LAT in DRG neurons 24 days after transplantation of ganglion latently infected with $TK^+$ HSV (in situ hybridization, hematoxylin-eosin counterstain). (A) Low-power (left; magnification, ×48) and higher-power (right; magnification, ×192) fields showing LAT-positive neurons. An area of adjacent kidney tubules is seen on the bottom left. (B) High-power (magnification, ×192) field showing LAT-positive neuron and adjacent kidney tubules. Typical neuronal nuclear localization of LAT is seen in both panels.

absence of hybridization detection of HSV ICP4 RNA were considered to be evidence of latency.

## RESULTS

**Latent infection after transplantation of donor ganglia previously latently infected with HSV.** In initial studies, it was evident that HSV LAT was present in transplanted ganglia for at least several weeks after transplantation. Following this observation, studies were performed to determine if the presence of LAT indicated the presence of latency. This was thought to be the case. Finally, therefore, studies were performed to investigate mechanisms of latency in transplants.

**(i) LAT expression in donor ganglia after transplantation.** Ganglion morphology was well maintained after transplantation, and HSV LAT was readily detected in DRG which had been transplanted beneath the renal capsule (Fig. 1). When transplanted ganglia were examined 2 to 4 weeks after transplantation, LAT was evident in the typical neuronal nuclear distribution. This LAT pattern, which has been well described in latently infected ganglia in other studies (5, 16, 17, 34, 37), suggested the possibility of latent infection in transplants. Hybridization with an oligonucleotide antisense to ICP4 RNA was not detected 4 weeks after transplantation (Fig. 2B). Hybridization was detected, however, with the ICP4 probe in control, acutely infected ganglia (Fig. 2A). The latter result helps to technically validate the lack of hybridization with the ICP4 probe 4 weeks after transplantation. Hybridization with only the LAT probe supported the conclusion that latency, rather than a persistent infection, was present. This conclusion was supported by virus isolation and HSV antigen results (see below).

The number of neurons expressing LAT was decreased from the time before transplantation (average of 108 on day 0) (Table 1) to the period after transplantation (average of 16 on day 1). In part, the decrease probably reflected the death of some previously LAT-positive transplanted neurons. The number of positive neurons showed minimal change in the period of 1 to 27 days posttransplantation (Table 1). LAT-positive neurons were evident in transplants for over 5 weeks, the

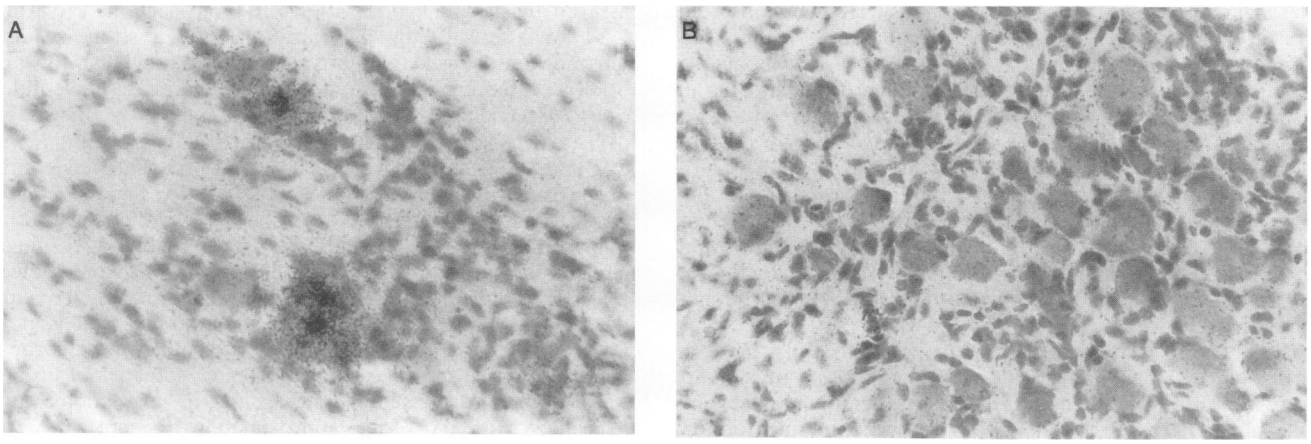


FIG. 2. HSV ICP4 RNA in control trigeminal ganglion acutely infected with $TK^+$ HSV (4 days after corneal inoculation) (A) but not in ganglion 20 days after transplantation (B) (magnification, ×196). Tissue processing and hybridization were as for LAT except that time under emulsion was 10 rather than 5 days. The lack of detectable ICP4 RNA in the transplanted ganglion suggests that persistent HSV infection was not present.

TABLE 1. Residual latent infection after transplantation of latently infected ganglia

| Virus used to establish latency in donor DRG | Average no. of LAT-positive neurons ± SD in transplants (no. tested) at indicated day(s) after transplantation | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0[a] | 1[b] | 3–4 | 5–7 | 10–12 | 15–17 | 20–27 |
| $TK^+$ HSV | 108 ± 43 (4) | 16 ± 8 (4) | 15 ± 12 (5) | 14 ± 8 (6) | 13 ± 3 (3) | 29 ± 24 (6) | 23 ± 21 (7) |
| $TK^-$ HSV | 94 ± 21 (4) | 26 ± 12 (5) | 23 ± 11 (4) | ND[c] | 11 ± 4 (8) | 0.2 ± 0.4[d] (7) | 0.2 ± 0.4[e] (12) |

[a] Ganglia examined without transplantation. The number of LAT-positive neurons shown is for two DRG, to allow comparison with other time periods during which ganglion pairs were examined. The number of ganglia tested (four) refers to four pairs of DRG.
[b] For all transplantation studies, pairs of DRG (the ipsilateral L-4 and L-5 DRG) were transplanted together. Results shown are for pairs of ganglia.
[c] ND, not done.
[d] Difference between average number of LAT-positive neurons in comparison with the $TK^+$ HSV group is significant at the $P < 0.005$ level ($t = 3.168$, 11 df).
[e] $P < 0.005$ ($t = 3.908$, 17 df).

longest period studied (see Table 4). Ganglion preservation was generally good, and the juxtaposition of LAT-positive neurons and adjacent renal tubules was striking (Fig. 1B). Neurons were less well preserved after liver and spleen capsule transplantation (data not shown). The presence of LAT several weeks after transplantation suggested HSV latency. This was further investigated by the determination of HSV isolation after explantation and in direct homogenates.

**(ii) Isolation of infectious HSV from transplants after explant culture but not from direct homogenates.** After 21 to 28 days, transplanted ganglia were dissected free of surrounding kidney tissue and explanted. Following explantation for 6 days, tissues were homogenized and assayed for infectious virus. Infectious HSV was isolated from 8 of 12 (67%) of transplants. Although, the frequency of HSV-positive explants was less than that usually noted for latently infected ganglia tested by explant culture (without prior transplantation, approximately 90 to 95% positive; unpublished observation), considering the nonspecific loss of neurons as part of the transplant procedure, the percent positive was thought acceptable. Removal of transplants followed by immediate freeze-thawing and direct homogenization did not result in HSV isolation in any of six samples. Therefore, these results were similar to standard testing of ganglia for latency; the isolation of HSV from explants but not from direct homogenates is a usual operational definition of HSV latency.

**(iii) Lack of HSV antigen in transplants.** Transplants were tested with polyclonal HSV and gC antibody and with monoclonal antibody to ICP4. HSV antigen was not detected in any of five transplants during the period of putative secondary latency (data not shown), at a time when LAT was readily detected. As shown below, HSV antigen was detected only acutely after transplantation (Table 2).

In summary, hybridization detection of LAT but not ICP4 RNA, the isolation of infectious HSV from explants but not from direct ganglion homogenates, and the lack of HSV antigen supported the concept that HSV latency was present. The mechanism of latency was then investigated to determine whether latency was residual latency in donor ganglia or whether it was newly established subsequent to transplantation, i.e., putative secondary latency.

**Mechanisms of secondary latency. (i) Detection of HSV antigen and cell-free infectious virus 3 to 7 days after transplantation.** The possible basis of LAT expression which was present several weeks after transplantation of latently infected ganglia was first evaluated by study of HSV reactivation in ganglia shortly after transplantation. It was thought that secondary latency would be more feasible if reactivation were demonstrated. HSV antigen was detected in 71 and 57% of transplanted ganglia 4 and 5 days after transplantation, respectively, and to a lesser degree before and after those times (Table 2). HSV antigen was not detected 7 or more days after transplantation. HSV antigen detected after transplantation of latently infected ganglia is shown in Fig. 3.

Additional evidence of HSV reactivation was provided by direct homogenization of transplants and the detection of infectious HSV in cell-free suspension. Cell-free HSV was evident 3 to 5 days after transplantation; 25 to 60% of transplants were positive (Fig. 4). HSV reactivation in transplants suggested a mechanism by which secondary latency might occur.

**(ii) Inhibition of reactivation by prior immunization of transplant recipients.** To further investigate the possibility that LAT expression several weeks after transplantation was re-

TABLE 2. Reactivation of HSV in transplants: HSV antigen expression in transplanted ganglia

| Virus used to establish latency in donor DRG | % of transplanted ganglia positive for HSV antigen (no. positive/total tested)[a] at indicated day(s) after transplantation | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 1–2 | 3 | 4 | 5 | 6–7 |
| $TK^+$ HSV | 0 (0/4) | 0 (0/4) | 28 (2/7) | 71 (5/7) | 57 (4/7) | 17 (1/6) |
| $TK^-$ HSV | ND[b] | ND | 0 (0/4) | 20 (1/5) | 28 (2/7) | 0 (0/5) |

[a] Reactivation of $TK^+$ HSV showed 1 to 2 antigen-positive cells in antigen-positive ganglia on days 3 and 6 to 7, while there were 5 to >100 antigen-positive cells in positive ganglia on days 4 to 5. For $TK^-$ HSV-infected ganglia, one to three antigen-positive cells were seen. Results were obtained with polyclonal HSV antiserum.
[b] ND, not done.

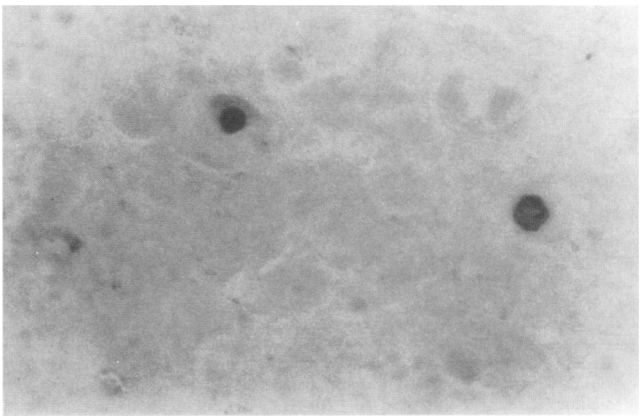

FIG. 3. HSV antigen 4 days after transplantation of DRG previously latently infected with $TK^+$ HSV (polyclonal antiserum; magnification, ×192). HSV reactivation is evident from the presence of viral antigen.

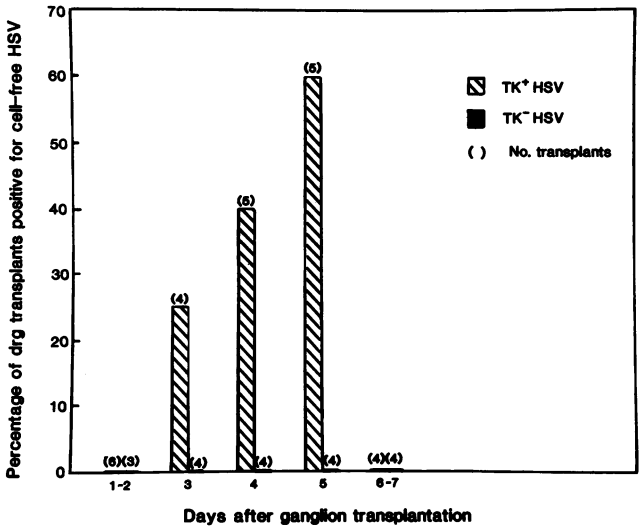


FIG. 4. Cell-free HSV in direct homogenates of transplanted ganglia. The presence of cell-free HSV in ganglion homogenates 3 to 5 days after transplantation suggests reactivation of $TK^+$ HSV but not $TK^-$ HSV. The number of determinations is given in parentheses.

lated to reactivation after transplantation, mice receiving transplants were immunized prior to transplantation. It was hypothesized that if reactivation produced secondary latency and reactivation were inhibited by prior immunization, secondary latency would also be decreased. Reactivation of HSV several days after transplantation was inhibited, as indicated by the inability to detect HSV antigen in any of five samples. In addition, cell-free HSV was not detected in direct ganglion homogenates, in contrast to ready detection in two unimmunized control groups (Table 3). Overall, cell-free HSV was detected in 11 of 23 control transplant recipients and none of 9 immunized recipients. The median neutralizing antibody titer in immunized mice was 1:64 (range, 1:32 to 1:128).

**(iii) Inhibition of secondary latency by prior immunization of transplant recipients.** Immunization of transplant recipients also markedly decreased the number of LAT-positive ganglia several weeks after transplantation of $TK^+$ HSV-infected ganglia. In control unimmunized mice tested on days 20 to 27 and 35 to 38, there were averages of 26 and 19 LAT-positive neurons, respectively, in transplants (Table 4). After immunization of mice and subsequent transplantation, there were averages of one and two LAT-positive neurons, respectively (Table 4).

TABLE 3. HSV reactivation after transplantation of latently infected ganglia into HSV-immunized recipients: detection of cell-free virus[a]

| Immunization treatment of recipients[b] | Transplanted ganglia positive for cell-free HSV (no. positive/total tested) at indicated day after ganglion transplantation | |
|---|---|---|
| | 4 | 5 |
| None | 38 (3/8) | 38 (3/8) |
| FCA + medium | 67 (2/3) | 75 (3/4) |
| FCA + HSV | 0 (0/5) | 0 (0/4) |

[a] Latent infection was established in donor DRG with $TK^+$ HSV.
[b] Transplant recipients were immunized as follows: none, no treatment; FCA + medium, Freund's complete adjuvant (FCA) plus medium subcutaneously (s.c.) and intraperitoneally (i.p.), after 2 weeks medium alone s.c. and after 2 weeks medium alone s.c. (mice were used for transplants 1 week after the last treatment); FCA + HSV, FCA plus HSV s.c. and i.p., after 2 weeks HSV alone, and after 2 weeks HSV alone s.c. (mice were used for transplants 1 week after the last treatment).

TABLE 4. Decreased latent infection after transplantation of latently infected ganglia into HSV-immunized recipients[a]

| Immunization treatment of recipients[b] | Avg no. of LAT-positive neurons ± SD (no. of ganglia tested) at indicated days after transplantation | |
|---|---|---|
| | 20–27 | 35–38 |
| None | 26 ± 24 (7) | 19 ± 27 (7) |
| FCA + medium | 23 ± 29 (6) | 28 ± 28 (6) |
| FCA + HSV | 1 ± 1[c] (8) | 2 ± 3[d] (8) |

[a] Latent infection was established in donor DRG with $TK^+$ HSV.
[b] See in Table 3, footnote *b*.
[c] Difference between average number of LAT-positive neurons in comparison with the no-treatment group is significant at the $P < 0.01$ level ($t = 2.909$, 13 df).
[d] $P < 0.05$ ($t = 1.835$, 13 df).

**Latent infection and reactivation in transplanted ganglia latently infected with $TK^-$ HSV. (i) LAT expression after transplantation of ganglia latently infected with $TK^-$ HSV.** It was hypothesized that if secondary latency resulted from reactivation and subsequent latent infection of neurons not previously latently infected, secondary latency would be unlikely in ganglia latently infected with reactivation defective $TK^-$ HSV. Although ganglia latently infected with $TK^-$ HSV contained similar numbers of LAT-positive neurons as $TK^+$ HSV-infected ganglia on day 0, and in the days shortly after transplantation (Table 1), LAT-positive neurons were rare subsequently in $TK^-$ HSV transplants (Table 1). Therefore, unlike after transplantation of ganglia latently infected with $TK^+$ HSV, latency (i.e., LAT expression) was not maintained in ganglia infected with $TK^-$ HSV.

**(ii) Detection of HSV antigen and infectious virus 3 to 7 days after transplantation.** To determine if $TK^-$ HSV was defective for reactivation in this model, as has been reported for reactivation in explants, isolation of HSV was investigated after transplantation. Cell-free infectious virus was not detected in direct homogenates (Fig. 4), nor was HSV isolated from explants (data not shown).

HSV antigen-positive cells were occasionally seen with polyclonal HSV antibody but were uncommon, particularly in comparison with ganglia infected with $TK^+$ HSV (Table 2). It was thought that antigen expressed was likely that of immediate-early proteins, since $TK^-$ HSV has been shown to be defective for reactivation in other studies (4, 19, 37) and since infectious virus was not detected (Fig. 4). This conclusion was investigated through the use of antibodies specific for HSV ICP4 and HSV gC. ICP4 antibody detected antigen in occasional neurons in $TK^-$ HSV-infected transplants (Fig. 5A), while gC antibody was negative (Fig. 5C). As would be expected, both showed evidence of HSV antigen in control acutely infected ganglia (Fig. 5B and D). The presence of immediate early HSV antigen and absence of late antigen in $TK^-$ HSV-infected transplants was consistent with abortive reactivation.

The correlation of the limited evidence of reactivation after transplantation of $TK^-$ HSV-infected ganglia and the very limited LAT expression >2 weeks after transplantation supported the hypothesis that the maintained presence of LAT-positive neurons in $TK^+$ HSV transplants was related to reactivation.

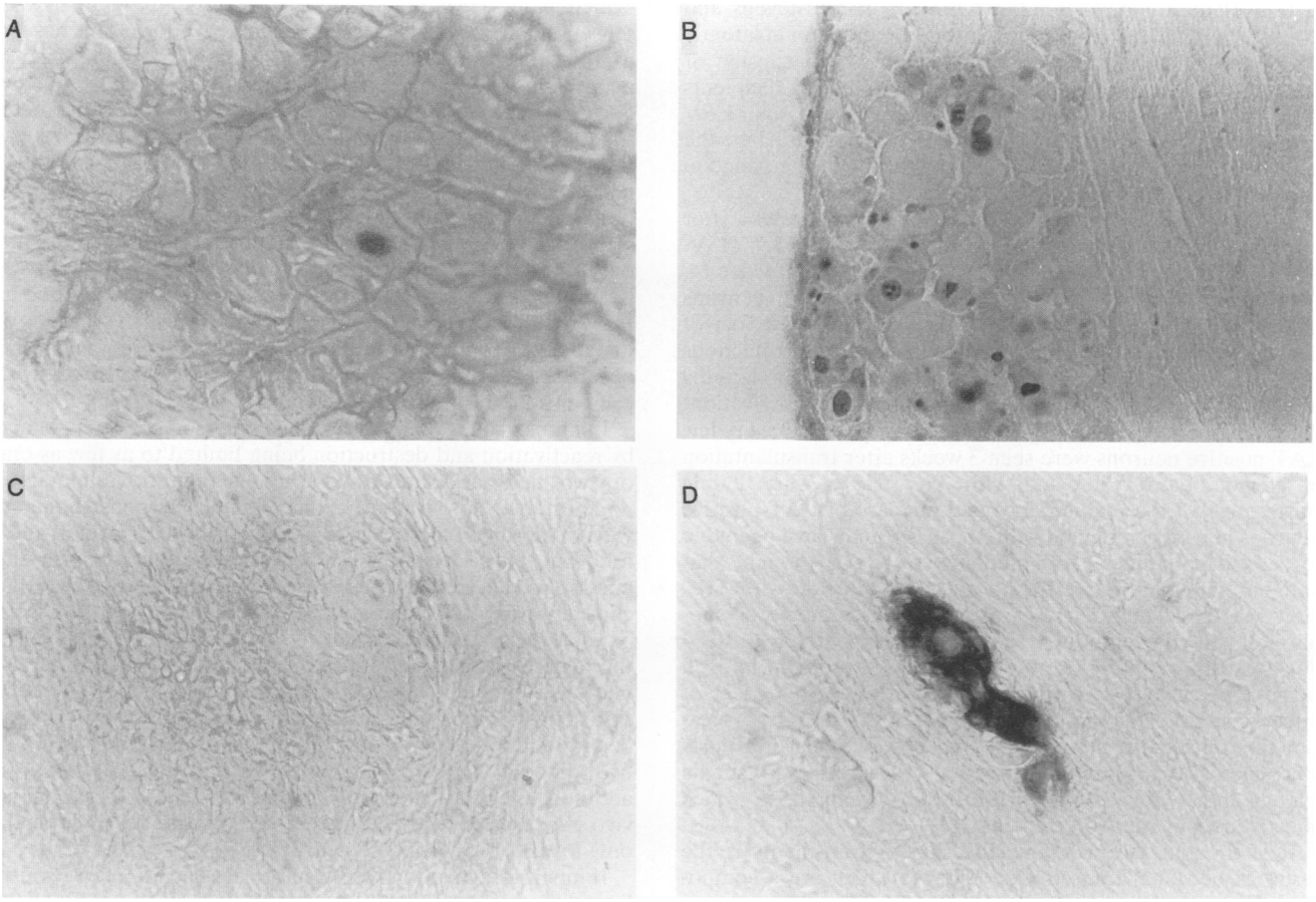


FIG. 5. HSV antigen in transplanted ganglion showing abortive reactivation of $TK^-$ HSV. DRG were infected by footpad inoculation of $TK^-$ HSV. After 28 days, ganglia were transplanted, and 4 days after transplantation, they were tested for HSV antigens. (A) HSV antigen detected in the nucleus of a neuron tested with ICP4 monoclonal antibody. (B) Control trigeminal ganglion acutely infected with $TK^+$ HSV (3 days after corneal inoculation) showing neurons with typical nuclear localization of ICP4 antigen. (C) Lack of HSV antigen in $TK^-$ HSV transplant tested with gC antibody. (D) Control trigeminal ganglion acutely infected with $TK^+$ HSV (3 days after corneal inoculation) showing extensive gC antigen. Magnification, ×196.

## DISCUSSION

The presence of LAT-positive neurons in transplants several weeks after transplantation suggested two possible explanations: (i) LAT-positive neurons were previously latently infected neurons which maintained LAT expression, or (ii) LAT-positive neurons represented neurons infected after transplantation, presumably following reactivation of HSV in transplants. This occurrence is referred to as secondary latency. Decrease of LAT as part of the axon reaction in which expression of many RNA species is modified (20, 35, 40, 43), and the subsequent restoration of LAT was thought a possible explanation for the decrease and then continued presence of LAT-positive neurons. Temporary decrease of neuronal RNAs and proteins has been well described after neurectomy (35, 40, 43). The second explanation was thought possible if it were shown that reactivation occurred after transplantation of ganglia. Three types of studies were performed to investigate putative secondary latency.

The first means to investigate possible secondary latency after transplantation of latently infected ganglia was to determine the presence of HSV antigen and to determine cell-free HSV in direct homogenates of transplanted ganglia. These are measures of HSV reactivation, which was thought necessary if secondary latency in previously uninfected neurons were to occur. HSV antigen was readily detected in transplanted DRG (Fig. 3 and Table 2), and cell-free HSV could be isolated when transplanted DRG were removed and directly homogenized (Fig. 4). The observations of HSV antigen and cell-free virus in transplants indicated the occurrence of HSV reactivation and a means by which secondary latency could be established.

In studies of HSV reactivation in vivo in transplanted ganglia, it was striking that ganglia were not destroyed, as uniformly happens following reactivation of HSV in vitro in explanted ganglia. This finding suggested the possibility of host mechanisms which curtailed the reactivated infection and which permitted the establishment of secondary latency.

The second means to investigate the putative occurrence of secondary latency was to compare latency in transplanted ganglia in hosts which were or were not previously immunized with HSV. It was thought that if secondary latency resulted from the infection of neurons after HSV reactivation, this would be inhibited if the transplant recipient could clear reactivated virus and viral infected cells by a brisk immune response. Inhibition of reactivation (Table 3) and secondary latency (Table 4) were in fact observed in immunized recipients. The observation of inhibition of HSV reactivation in vivo

in transplanted ganglia builds on the report by Stevens and Cook, who noted decreased HSV antigen expression in latently infected ganglia transplanted beneath the spleen capsule or into the peritoneal cavity in previously immunized recipients (33). In the present study, inhibition of reactivating HSV antigen was also noted in immunized recipients, but the emphasis was on the preservation of neurons in the ganglion and on the concept of secondary latency.

Lastly, we determined if LAT expression was present after transplantation of ganglia latently infected with $TK^-$ HSV. Since $TK^-$ HSV mutants are largely reactivation defective (4, 7, 19, 37), it was thought that while LAT-positive neurons might be maintained, it would be unlikely that new foci of latency subsequent to reactivation would result. Infectious HSV was not detected after transplant of ganglia latently infected with $TK^-$ HSV (Fig. 4), supporting the reactivation-defective phenotype of $TK^-$ HSV. As seen in Table 1, very few LAT-positive neurons were seen 3 weeks after transplantation of ganglia latently infected with $TK^-$ HSV. This finding suggested that the maintenance and increase of LAT-positive neurons in ganglia infected with $TK^+$ HSV may have been due to secondary latency. As noted above, it was thought that antigen expressed in transplanted ganglia infected with $TK^-$ HSV was HSV immediate-early protein. This view was supported by the detection of HSV antigen with antibody to ICP4 but not with antibody to gC. Lack of gC expression antigen suggests abortive reactivation and is consistent with the lack of infectious HSV. Limited and sparse $TK^-$ HSV antigen expression in transplants is similar to the observations of Knosz-Vnenchak et al. of limited expression of transcripts in ganglia acutely infected with $TK^-$ HSV (12) or ganglia in which abortive reactivation is occurring (13).

It is thought that HSV reactivation in transplants is the result of neuronal transcription changes which are a component of the axon reaction (20, 35, 40, 43). Following neurectomy, marked changes in neuronal transcription and protein expression occur, and given the very proximal neurectomy (and rhizotomy) performed as part of the DRG transplant procedure, significant changes in neuronal transcription are likely. It is suggested that for $TK^+$ HSV, LAT expression was decreased and reactivation occurred. In the case of $TK^-$ HSV, it is hypothesized that LAT expression was similarly decreased, but since reactivation and therefore the establishment of secondary latency were precluded, the latent infection was eliminated. In keeping with this suggested mechanism, decreased DRG LAT and HSV DNA were noted in latently infected mice after sciatic neurectomy (36). Secondary latency was not apparent in that study, possibly because all mice were presumably immunized against HSV as part of the initial footpad infection procedure. The mechanism suggested might involve antisense inhibition of LAT (41). It is understood that discussion of antisense inhibition requires consideration of mechanisms of latency of $LAT^-$ HSV. Without extensive discussion at this time, it is noted that reactivation of $LAT^-$ HSV in vivo may differ from that in explant culture (1, 9, 39). In addition, it is probable that factors other than decrease of LAT are required for reactivation to occur. The sequence of events may be similar to those reported for bovine herpesvirus 1, in which case LAT was reported to be decreased in many neurons but reactivation occurred in few (27).

Although only antibody titer was measured in immunized mice, the results obtained in this study support a role for the immune system in the maintenance of HSV latency after reactivation in this animal model. Mechanisms for the maintenance of latency after in vivo reactivation in humans are unclear. In humans, areas of loss of sensation are not usually evident after HSV reactivation (unlike the frequent loss of sensation after varicella-zoster virus reactivation). This has led to the conclusion that HSV reactivation does not destroy neurons in humans. In addition, HSV reactivation in humans does not typically eliminate the latent infection. It has been suggested that latency is maintained in the setting of reactivation by round-trip axoplasmic transport of HSV or maintenance of the latent HSV genome in neurons despite reactivation (15, 32). These explanations seek to explain reactivation of infectious HSV and subsequent maintenance of latency without destruction of the neurons in which reactivation occurred. Neuron-sparing reactivation of a generally lytic virus such as HSV requires speculation of mechanisms by which synthesis of infectious HSV spares neurons in which reactivation has occurred (32). Possibly, however, neurons are destroyed with each episode of in vivo reactivation.

In humans, lack of apparent sensory loss might be explained by reactivation and destruction being limited to as few as one or two neurons. Sensory loss might then be minimal or undetectable. Sprouting of axons of intact neurons to denervated skin may then reinervate the surface. Reinervation following each episode of reactivation would diminish the possibility that sensory loss would accumulate and become clinically evident. Reinervation of denervated skin by sprouting of adjacent nerve fibers has been reported (8, 11). Support for the hypothesized reactivation of HSV and destruction of few neurons is provided by experimental studies. In mice, evidence of reactivation and neuronal destruction after reactivation has been found in as few as one to three neurons in a ganglion in two different in vivo models of reactivation (24, 28). In addition, the lack of evident increase of HSV DNA after in vivo reactivation was considered to indicate reactivation in only a small proportion of neurons (1).

If in vivo reactivation results in the destruction of previously latently infected neurons, and latency is maintained by latent infection of previously uninfected adjacent neurons, the present model of secondary latency might apply. A potential difficulty in applying this model to humans would be the observation that despite people being HSV seropositive, human latency is not eliminated (26, 29); in the present transplant model, latency was decreased or eliminated. However, the differences between species, the markedly altered neuronal function after neurectomy and transplantation, and the milieu of the transplant site are among the factors that may result in immune control that is more effective in the mouse model. HSV immunization prior to infection with HSV, including treatment with antibody (14, 23, 35) and with immune cells (2, 3, 44), has been shown to decrease primary latency. Similar methods will be useful for further study of the mouse transplant model of secondary latency.

## ACKNOWLEDGMENTS

These studies were supported by National Institutes of Health grant NS20684 (R.B.T.).

Helpful discussions with Satvir Tevethia and the secretarial assistance of Tricia Spitler and Kathy Conner are gratefully acknowledged.

## REFERENCES

1. **Bloom, D. C., G. B. Devi-Rao, J. M. Hill, J. G. Stevens, and E. K. Wagner.** 1994. Molecular analysis of herpes simplex virus type 1 during epinephrine-induced reactivation of latently infected rabbits in vivo. J. Virol. **68**:1283–1292.
2. **Bonneau, R. H., and S. R. Jennings.** 1989. Modulation of acute and latent herpes simplex virus infection in C57Bl/6 mice by

adoptive transfer of immune lymphocytes with cytolytic activity. J. Virol. **63:**1480–1484.
3. **Bonneau, R. H., and S. R. Jennings.** 1990. Herpes simplex virus-specific cytolytic T lymphocyte restricted to a normally low responder H-2 allele are protective in vivo. Virology **174:**599–604.
4. **Coen, D. M., M. Kosz-Vnenchak, J. G. Jacobson, D. A. Leib, C. L. Bogard, P. A. Schaffer, K. L. Tyler, and D. M. Knipe.** 1989. Thymidine kinase-negative herpes simplex virus mutants establish latency in trigeminal ganglia but do not reactivate. Proc. Natl. Acad. Sci. USA **86:**4736–4740.
5. **Deatly, A. M., J. G. Spivack, E. Lavi, D. R. O'Boyle, and N. W. Fraser.** 1988. Latent herpes simplex virus type 1 transcripts in peripheral and central nervous system tissues of mice map to similar regions of the virus genome. J. Virol. **62:**749–756.
6. **Eberle, R., and R. J. Courtney.** 1980. Preparation and characterization of specific antisera to individual glycoprotein antigens comprising the major glycoprotein region of herpes simplex virus type 1. J. Virol. **35:**902–917.
7. **Efstathiou, S., S. Kemp, G. Darby, and A. C. Minson.** 1989. The role of herpes simplex virus type 1 thymidine kinase in pathogenesis. J. Gen. Virol. **70:**869.
8. **Greenfield, Z., and M. Devor.** 1981. Collatoral reinervation of rat hindlimb skin does not depend on repeated sensory testing. Neurosci. Lett. **25:**305–309.
9. **Hill, J. M., F. Sedarati, R. T. Javier, E. K. Wagner, and J. G. Stevens.** 1990. Herpes simplex virus latent phase transcription facilitates in vivo reactivation. Virology **174:**117–125.
10. **Ho, D. Y.** 1992. Herpes simplex virus latency: molecular aspects. Prog. Med. Virol. **39:**76–115.
11. **Jackson, P. C., and J. Diamond.** 1984. Temporal and spatial constraints on the collatoral sprouting of low-threshold mechanosensory nerves in the skin of rats. J. Comp. Neurol. **226:**336–345.
12. **Kosz-Vnenchak, M., D. M. Coen, and D. M. Knipe.** 1990. Restricted expression of herpes simplex virus lytic genes during establishment of latent infection by thymidine kinase-negative mutant viruses. J. Virol. **64:**5396–5402.
13. **Kosz-Vnenchak, M., J. Jacobson, D. M. Coen, and D. M. Knipe.** 1993. Evidence for a novel regulatory pathway for herpes simplex virus gene expression in trigeminal ganglion neurons. J. Virol. **67:**5385–5393.
14. **Klein, R. J.** 1980. Effect of immune serum on the establishment of herpes simplex virus infection in trigeminal ganglia of hairless mice. J. Gen. Virol. **49:**401–405.
15. **Klein, R. J.** 1985. Initiation and maintenance of latent herpes simplex virus infections: the paradox of perpetual immobility and continuous movement. Rev. Infect. Dis. **7:**21–30.
16. **Krause, P. R., K. D. Croen, S. E. Straus, J. M. Ostrove.** 1988. Detection and preliminary characterization of herpes simplex virus type 1 transcripts in latently infected human trigeminal ganglia. J. Virol. **62:**4819–4823.
17. **Leib, D. A., C. L. Bogard, M. Kosz-Vnenchak, K. A. Hicks, D. M. Coen, D. M. Knipe, and P. A. Schaffer.** 1989. A deletion mutant of the latency-associated transcript of herpes simplex virus type 1 reactivates from the latent state with reduced frequency. J. Virol. **63:**2893–2900.
18. **Leib, D. A., D. M. Coen, C. L. Bogard, K. A. Hicks, D. R. Yager, D. M. Knipe, K. L. Tyler, and P. A. Schaffer.** 1989. Immediate-early regulatory gene mutants define different stages in the establishment and reactivation of herpes simplex virus latency. J. Virol. **63:**759–768.
19. **Leist, T. P., R. M. Sandri-Goldin, and J. G. Stevens.** 1989. Latent infections in spinal ganglia with thymidine kinase-deficient herpes simplex virus. J. Virol. **63:**4976–4978.
20. **Lieberman, A. R.** 1971. The axon reaction: a review of the principal features of perikaryal responses to axon injury. Int. Rev. Neurobiol. **14:**49–124.
21. **Martin, J. R., and S. Suzuki.** 1987. Inflammatory sensory polyradiculopathy and reactivated peripheral nervous system infection in a genital herpes model. J. Neurol. Sci. **79:**155–171.
22. **McGeoch, D. J., A. Dolan, S. Donald, and D. H. K. Brauer.** 1986. Complete DNA sequence of the short repeat region in the genome of herpes simplex virus type 1. Nucleic Acids Res. **14:**1727–1745.
23. **McKendall, R. R.** 1985. IgG-mediated viral clearance in experimental infection with herpes simplex virus type 1: role for neutralization and Fc-dependent functions but not C′ cytolysis and C5 chemotaxis. J. Infect. Dis. **151:**464–470.
24. **McLennon, J. L., and G. Darby.** 1980. Herpes simplex virus latency: the cellular location of virus in dorsal root ganglia and the fate of the infected cell following virus activation. J. Gen. Virol. **51:**233–243.
25. **Openshaw, H., L. V. Shavrina Asher, C. Wohlenberg, T. Sekizawa, and A. L. Notkins.** 1979. Acute and latent infection of sensory ganglia with herpes simplex virus immune control and reactivation. J. Gen. Virol. **44:**205–215.
26. **Reeves, W. C., L. Corey, H. G. Adams, L. A. Vontver, and K. K. Holmes.** 1981. Risk of recurrence after first episodes of genital herpes. N. Engl. J. Med. **305:**315–319.
27. **Rock, D., J. Lokensgard, T. Lewis, and G. Kutish.** 1992. Characterization of dexamethasone-induced reactivation of latent bovine herpesvirus 1. J. Virol. **66:**2484–2490.
28. **Sawtell, N. M., and R. L. Thompson.** 1992. Rapid in vivo reactivation of herpes simplex virus in latently infected murine ganglionic neurons after transient hypothermia. J. Virol. **66:**2150–2156.
29. **Shillitoe, E. J., J. M. A. Wilton, and T. Lehner.** 1977. Sequential changes in cell-mediated immune responses to herpes simplex virus after recurrent herpetic infection in humans. Infect. Immun. **18:**130–137.
30. **Shimeld, C., T. J. Hill, W. A. Blyth, and D. L. Easty.** 1990. Reactivation of latent infection and induction of recurrent herpetic eye disease in mice. J. Gen. Virol. **71:**397–404.
31. **Steiner, I., J. G. Spivack, S. L. Deshmane, C. I. Ace, C. M. Preston, and N. W. Fraser.** 1990. A herpes simplex virus type 1 mutant containing a nontransinducing Vmw65 protein establishes latent infection in vivo in the absence of viral replication and reactivates efficiently from explanted trigeminal ganglia. J. Virol. **64:**1630–1638.
32. **Stevens, J. G.** 1989. Human herpesviruses: a consideration of the latent state. Microbiol. Rev. **53:**318–332.
33. **Stevens, J. G., and M. L. Cook.** 1974. Maintenance of latent herpetic infection: an apparent role for anti-viral IgG. J. Immunol. **113:**1685–1693.
34. **Stevens, J. G., E. K. Wagner, G. B. Devi-Rao, M. L. Cook, and L. T. Feldman.** 1987. RNA complementary to a herpes virus alpha gene mRNA is prominent in latently infected neurons. Science **235:**1056–1059.
35. **Tenser, R. B.** 1985. Sequential changes of sensory neuron (fluoride-resistant) acid phosphatase in dorsal root neurons following neurectomy and rhizotomy. Brain Res. **332:**386–389.
36. **Tenser, R. B., W. A. Edris, and K. A. Hay.** 1993. Neuronal control of herpes simplex virus latency. Virology **195:**337–347.
37. **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 with thymidine kinase-negative mutants of herpes simplex virus type 1. J. Virol. **63:**2861–2865.
38. **Tenser, R. B., and G. D. Hsiung.** 1977. Pathogenesis of latent herpes simplex virus infection of the trigeminal ganglion in guinea pigs: effects of age, passive immunization, and hydrocortisone. Infect. Immun. **16:**69–74.
39. **Trousdale, M. D., I. Steiner, J. G. Spivak, S. L. Deshmane, S. M. Brown, A. R. MacLean, J. H. Subak-Sharpe, and N. W. Fraser.** 1991. In vivo and in vitro reactivation impairment of a herpes simplex virus type 1 latency-associated transcript variant in a rabbit eye model. J. Virol. **65:**6989–6993.
40. **Villar, M., R. Cortes, E. Theodorsson, Z. Wiesenfeld-Hallin, M. Schalling, J. Fahrenkrug, P. C. Emson, and T. Hokfelt.** 1989. Neuropeptide expression in rat dorsal root ganglion cells and spinal cord after peripheral nerve injury with special reference to galanin. Neuroscience **33:**587–604.
41. **Wagner, E. K., G. Devi-Rao, L. T. Feldman, A. T. Dobson, Y. F. Zhang, W. M. Flanagan, and J. G. Stevens.** 1988. Physical

characterization of the herpes simplex virus latency-associated transcript in neurons. J. Virol. **62:**1194–1202.

42. **Wechsler, S. L., A. B. Nesburn, R. Watson, S. M. Slanina, and H. Ghiasi.** 1988. Fine mapping of the latency-related gene of herpes simplex virus type 1: alternative splicing produces distinct latency-related RNAs containing open reading frames. J. Virol. **62:**4051–4058.
43. **Wong, J., and M. M. Oblinger.** 1991. NGF rescues substance P expression but not neurofilament or tubulin gene expression in axotomized sensory neurons. J. Neurosci. **11:**543–552.
44. **Zarling, J. M., P. A. Moran, L. Brewer, R. Ashley, and L. Corey.** 1988. Herpes simplex virus (HSV)-specific proliferation and cytotoxic T-cell responses in humans immunized with an HSV type 2 glycoprotein subunit vaccine. J. Virol. **62:**4481–4485.