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Detection of an Immediate Early Herpes Simplex Virus Type 1 Polypeptide in Trigeminal Ganglia from Latently Infected Animals

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In this study, trigeminal sensory ganglia from animals with acute herpes simplex virus, type 1 (HSV-1) infection were compared to those with a latent infection for the expression of HSV-specific antigens. By the indirect immunofluorescence assay, antisera to an immediate early polypeptide of molecular weight 175,000, designated VP175 or ICP4, and a hyperimmune antiserum to HSV-1 were used to determine whether early viral polypeptides were being expressed in neurons during the latent stage of infection. All 17 ganglia from animals with acute infection (sacrificed 3 to 12 days postinfection) exhibited positive staining when treated either with anti-HSV-1 or with anti-VP175. Forty of 42 ganglia from animals sacrificed during the latent stage of infection (22 to 200 days postinfection) exhibited immunofluorescent staining when treated with anti-VP175. The staining appeared to be similar to that observed in ganglia from acutely infected animals stained with anti-VP175, except that the number and distribution of stained cells were markedly reduced. No immunofluorescence was observed in ganglia from noninfected control animals when stained with anti-VP175 or anti-HSV-1, or when ganglia from latently infected animals were stained with anti-HSV-1 or preimmune serum.

Significant progress has been made towards the understanding of the natural history of diseases caused by herpes simplex virus (HSV); yet undefined are the physiological state of the latent virus and the mechanism by which latency is established and reversed. Although hypothesized by Goodpasture and Teague in 1929 (10), direct proof that HSV establishes a latent infection in the central nervous system, particularly in sensory ganglia, was not obtained until 1971 (33). After recovery following inoculation of HSV into rear footpads, mice were shown to harbor the virus (probably for life) in ipsilateral lumbosacral spinal ganglia. Virus was recovered only after "reactivation" triggered by explant and organ cocultivation. Virus could not be recovered from sciatic nerves, the neuraxis, or footpads from animals harboring virus in ganglia. The rate of centripetal progression of virus from foot to sciatic nerve to sacrosciatic ganglia to dorsal roots to spinal cord and brain suggested transport by retrograde intraaxonal transport (5). Application of similar techniques resulted in recovery of latent virus from trigeminal ganglia of both rabbits and mice after corneal inoculation, from lumbosacral ganglia of guinea pigs after rear footpad inoculation, and from human sensory ganglia at autopsy (1, 2, 12, 13, 18, 28, 29, 35, 37).

HSV was shown to be specifically associated with neurons during latent infection by in situ hybridization techniques (31, 32, 39). Application of autoradiographic techniques to latently infected ganglia reactivated in vitro showed initial deoxyribonucleic acid (DNA) synthesis in neurons with thymidine subsequently migrating to satellite cells (4). Knowledge concerning the natural history of recurrent disease is summarized by the neuronal hypothesis which assumes that, as a result of primary infection, virus invades superficial nerve endings and is subsequently transported intraaxonally to corresponding ganglia where latency is established. Upon "activation," virus or viral products travel centrifugally in axons to target organs and are released with or without production of lesions (20).

Although the state and activity of the viral genome during latency are not definitely known, much of the present experimental evidence supports conservation of the virus in a nonreplicating form. Virus-specific DNA has been detected

in latently infected ganglia of mice at the time of explant (21, 26); however, the detection of infectious virus or viral antigens has not been reported except in instances of suspected spontaneous reactivation (2, 27). Recent studies have reported the detection of HSV-specific ribonucleic acid in human sensory ganglia (9), suggesting that some transcription of the viral genome occurs in the ganglia. Nucleic acid hybridization studies indicate a dramatic increase in viral DNA genome equivalents and messenger ribonucleic acid in acutely infected and reactivated ganglia as opposed to latently infected ganglia (21, 31, 34). In attempts to establish model systems to study latency, investigators have utilized temperature-sensitive mutants to establish long-term persistent infections at the restrictive temperature in mice (18). Other studies have reported a restricted replication of HSV-1 in cells of neuronal origin (14, 17, 36).

In this study, trigeminal sensory ganglia from animals with acute HSV infection were compared to those with latent infection by indirect immunofluorescence, using monospecific antisera to VP175 as well as antisera to HSV-1 to determine whether the particular early viral polypeptide was expressed in neurons during the latent stage of infection. VP175 is a nonstructural alpha group polypeptide which is synthesized at highest rates 3 to 4 h postinfection (11). This polypeptide has been shown to be overproduced in cells infected both with certain temperature-sensitive mutants and with HSV-1 stocks containing a significant proportion of defective virus (6, 8, 19).

MATERIALS AND METHODS

Animals. New Zealand albino rabbits (3 kg each) were bilaterally infected via corneal scarification with 10^5 plaque-forming units of McKrae strain HSV-1. Beginning on day 3 postinfection, animals were monitored for shedding of infectious virus in the preocular tear film by ocular swab culture. Infected and uninfected control animals were sacrificed by intravenous administration of ketamine hydrochloride at appropriate times during both the acute and latent stages of disease. Animals sacrificed during the latent stage of disease had negative ocular cultures at the time of sacrifice. Trigeminal sensory ganglia were immediately removed, mounted in O.T.C. compound (Lab-Tek Products, Division of Miles Laboratories, Inc., Westmont, Ill.), and quick frozen at −30°C.

Preparation of antisera. The procedure used for preparation of antiserum to HSV-1-infected cells was described by Sim and Watson (30). Extracts of HSV-1-infected rabbit kidney cells (RK13) maintained in rabbit serum were used as the immunogen. The procedure used for preparation of the anti-VP175 and its characteristics were previously described (6). Briefly, the VP175 polypeptide was obtained from cells infected with an HSV-1 temperature-sensitive mutant designated *ts*B2 (provided by Priscilla A. Schaffer, Sidney Farber Cancer Institute, Boston, Mass.). The virus-infected cells were cultured at the nonpermissive temperature of 39°C. The VP175 polypeptide was purified by preparative polyacrylamide gel electrophoresis, and the purified polypeptide was used as the immunogen for inoculation of rabbits. The specificity of the immune serum for VP175 was demonstrated by immune precipitation tests with various HSV-specific polypeptides (data not shown). Although both antisera were used at dilutions of 1:2 or 1:8, the immunofluorescence endpoint titer for the anti-HSV-1 serum was approximately 20-fold higher than the titer of the anti-VP175 serum.

Indirect immunofluorescence test. The indirect immunofluorescence test described by Porter et al. (23) was employed with minor modifications. Serial sections (4 to 6 μm) were fixed in acetone at room temperature for 5 min and air dried. Before staining, the sections were rehydrated in phosphate-buffered saline for 5 min at room temperature. Sections were treated for 30 min at room temperature with either antiserum or preimmune rabbit serum diluted 1:2 or 1:8 in phosphate-buffered saline. Sections were then washed three times in phosphate-buffered saline and treated for 30 min with fluorescein-conjugated goat anti-rabbit gamma globulin (Hyland, Div. of Travenol Laboratories, Inc., Costa Mesa, Calif.). Sections were washed three times in phosphate-buffered saline, air dried, and mounted in 50% glycerol in phosphate-buffered saline. A minimum of 12 sections from each ganglion were stained and observed. To confirm initial results, several experiments were carried out in double-blind fashion. Test serum was sent under code, tissue sections were examined by several individuals, and results were recorded. The code was broken subsequently.

RESULTS

Acute infection. Thirty-one ganglia from 20 animals sacrificed during the acute stage of disease (3 to 12 days postinfection, positive ocular cultures at sacrifice) were treated with anti-VP175, stained, and observed. All ganglia exhibited typical patterns of specific nuclear fluorescence in more than 75% of the ganglion cell bodies present per section (Fig. 1a). However, upon focusing in more than one plane, a distinct granularity superimposed on the solid fluorescent background was observed. This granular staining pattern was pronounced on some sections (Fig. 1b). Positive immunofluorescence was observed throughout each ganglion, regardless of the level from which the stained section was taken.

Although ganglion cell bodies treated with anti-HSV-1 exhibited similar staining, close inspection revealed differences. Some cell bodies manifested diffuse staining of both cytoplasm and nucleus (Fig. 1e). There was a general lack of granularity in all of the stained cells (Fig. 1d). All 17 ganglia from animals with acute infection

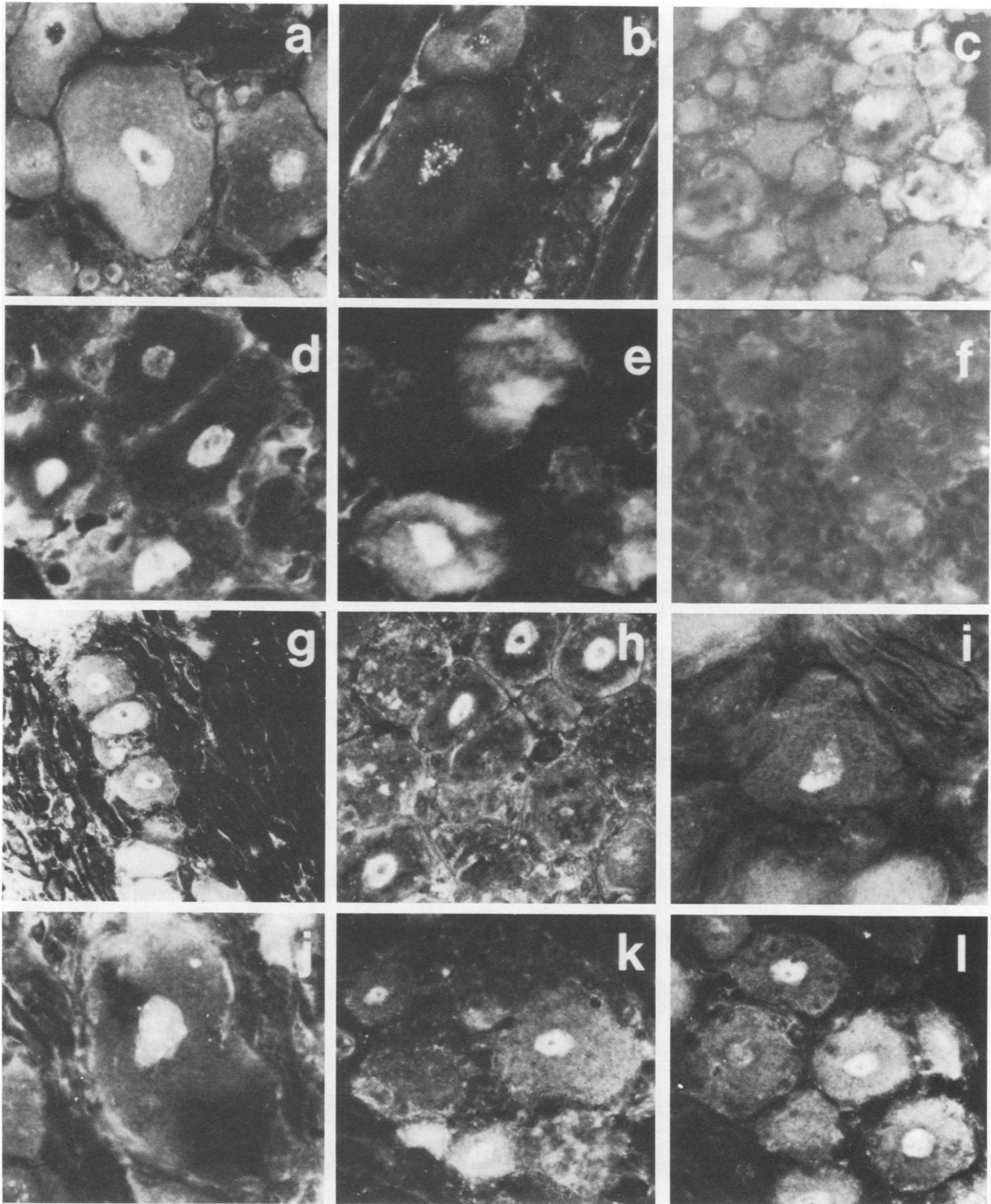

FIG. 1. *(a) Heavily stained nuclei of trigeminal ganglion (TG) cell bodies from an acutely infected rabbit sacrificed 3 days after ocular infection with 10^5 PFU of McKrae strain HSV-1. Section was stained with anti-VP175 at a dilution of 1:2 (×720). (b) Granular fluorescence in nuclei of TG cell bodies from an acutely infected rabbit sacrificed 7 days after ocular infection with HSV-1. Section was stained with anti-VP175, 1:2 (×760). (c) Lack of fluorescence in TG cell bodies from uninfected control animal after staining with anti-VP175, 1:2 (×400). (d) Heavily stained nuclei of TG cell bodies from an acutely infected rabbit sacrificed 4 days after ocular infection with HSV-1. Section stained with anti-HSV-1 1:2 (×350). (e) Heavily stained nuclei of TG cell bodies from an acutely infected rabbit sacrificed 4 days after ocular infection with HSV-1 (×1,000). (f) Lack of fluorescence in TG cell bodies from a latently infected animal sacrificed 43 days after ocular infection; section was stained with anti-HSV-1, 1:2 (×400). (g) Nuclear fluorescence after staining with anti-VP175, 1:2, in column of TG cell bodies from a latently infected rabbit sacrificed 30 days after ocular infection with HSV-1 (×200). (h) Nuclear fluorescence after staining with anti-VP175 (1:2); cell bodies from a latently infected rabbit sacrificed 30 days after ocular infection with HSV-1 (×800). (i) Nuclear fluorescence after staining with anti-VP175, 1:8, of TG cell body from a latently infected animal sacrificed 80 days after ocular infection with HSV-1 (×1,100). (j) Nuclear fluorescence similar to that in (b) in TG cell bodies from a latently infected rabbit sacrificed 150 days after ocular infection with HSV-1; stained with anti-VP175 (×970). (k and l) Nuclear fluorescence after staining with anti-VP175 (1:2) of TG cell bodies from a latently infected rabbit sacrificed 200 days after ocular infection with HSV-1 (×900).*

(sacrificed 3 to 12 days postinfection) exhibited positive staining when treated either with anti-HSV-1 or with anti-VP175 (Table 1). No immunofluorescence was observed in 20 ganglia from uninfected control animals when stained with anti-VP175 (Fig. 1c) or anti-HSV-1 (Fig. 1f) or when ganglia from latently infected animals were stained with anti-HSV-1 or preimmune serum (not shown).

Latent infection. Forty-two ganglia from 22 animals sacrificed during the latent stage of infection (22 to 200 days postinfection, negative ocular cultures at sacrifice) were treated with anti-VP175, stained, and observed (Table 2). Forty of 42 ganglia exhibited immunofluorescent staining which appeared to be synonomous with that observed in ganglia from acutely infected animals stained with anti-VP175 (Fig. 1, g through l). Both distribution within the ganglion and the number of positively stained cells per section decreased with time after acute infection.

TABLE 1. *Immunofluorescent staining of trigeminal nerve ganglia from animals sacrificed during acute infection with HSV-1*

Days postinfection	Immunofluorescence[a]	
	Anti-VP175	Anti-HSV-1
3	+ (7)	+ (2)
4	+ (7)	+ (1)
5	+ (7)	+ (6)
6	+ (2)	+ (2)
8	+ (2)	+ (2)
10	+ (2)	+ (2)
12	+ (2)	+ (2)

[a] Parentheses indicate number of ganglia sectioned and stained for indicated experiment.

TABLE 2. *Immunofluorescent staining of trigeminal nerve ganglia from animals sacrificed during latent infection with HSV-1*

Days postinfection	Immunofluorescence[a]	
	Anti-VP175	Anti-HSV-1
22	+ (2)	ND
25	+ (2)	ND
30	+ (1)	ND
40	+ (10)	ND
47	+ (2)	ND
58	+ (6)	− (6)
70	+ (2)	ND
80	+ (2)	ND
90	+ (3)	− (1)
120	+ (1)	ND
150	+ (7); − (1)	− (1)
200	+ (2); − (1)	ND
Uninfected	− (20)	− (10)

[a] Parentheses indicate the number of ganglia sectioned and stained. ND, Not done.

In addition, the pattern of nuclear staining became more distinctly granular (Fig. 1j). Positively stained ganglion cell bodies were more consistently found in the anterior medial dorsal aspect of the ganglion. Positive staining of all sections throughout a given ganglion was seldom observed. Clusters of three to five positively stained cells were commonly observed; isolated cell bodies with positive staining patterns were rarely observed. Of eight ganglia treated with anti-VP175, anti-HSV-1, and preimmune serum, all exhibited specific nuclear staining with anti-VP175; no staining was observed with anti-HSV-1 or preimmune serum. Increased numbers of heavily staining cells were observed in ganglia from animals with multiple documented episodes of virus shedding in preocular tear film. These recurrent episodes decreased in frequency with increasing time after initial infection.

DISCUSSION

In this paper, "latent infection" is used in the sense of a long-term infection of the host during which infectious virus is not detectable by culture of cell-free homogenates, although the potential for reactivation with production of infectious virus is retained (as demonstrated by whole-cell or organ cocultivation). Ninety-five percent of ganglia from animals in the latent stage of infection showed positive nuclear fluorescence at explant using the monospecific antiserum to VP175. Although spontaneous reactivation cannot be ruled out, the frequency of our findings in ganglia from randomly selected latently infected animals would appear to be far too great to be explained on this basis. An additional point against spontaneous reactivation is the fact that all ocular cultures were negative for several days before, as well as at the time of sacrifice. Ultrastructural studies using horseradish peroxidase-labeled anti-VP175 might aid in resolution of this question.

The fact that positive nuclear fluorescence was obtained only with anti-VP175 during the late acute stage and continuing throughout the latent stage of disease suggests that infection in certain ganglion cells is somehow converted from a productive to a nonproductive or latent stage. Whether these findings represent VP175 retained in infected ganglia or VP175 newly synthesized during the latency period cannot be determined. Our observation of a decrease in the number of positively labeled cells as the acute stage of infection is modulated toward the latent stage is consistent with the findings of Openshaw et al., who compared viral DNA and ribonucleic acid equivalents in acute and latently infected ganglia from mice (21). Although they detected

no viral ribonucleic acid during the latent stage, the study did not exclude the possibility of transcription of a small portion of the viral genome.

The characteristics of VP175 or ICP4 have been studied by a number of investigators. After synthesis, the majority of the VP175 is associated with the nucleus (3, 6, 22). The polypeptide can be detected in three forms which differ in electrophoretic mobility (22). The protein has DNA-binding properties (24) and is phosphorylated (22), and recent studies have suggested that the bound phosphate may cycle on and off (38). The HSV gene, which is thought to code for the synthesis of VP175, has been identified as acting early in the HSV replicative cycle and controls the transition from immediate early to early and late protein synthesis (15, 16, 25). It has also been suggested that VP175 may be required continuously to maintain early protein synthesis and may act to inhibit immediate early protein synthesis (7). In view of the regulatory functions associated with VP175 within the infected cell, it is tempting to speculate on the functional role this protein may play in the initiation or maintenance of the latent state within the neuron. However, definitive studies will be difficult to approach until a cell culture latency model can be established.

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LITERATURE CITED

1. **Baringer, J. R., and P. Swoveland.** 1973. Recovery of herpes-simplex virus from human trigeminal ganglions. N. Engl. J. Med. **228**:648-650.
2. **Baringer, J. R., and P. Swoveland.** 1974. Persistent herpes simplex virus infection in rabbit trigeminal ganglia. Lab. Invest. **30**:230-240.
3. **Cabral, G. H., R. J. Courtney, P. A. Schaffer, and F. Marciano-Cabral.** 1980. Ultrastructural characterization of an early, nonstructural polypeptide of herpes simplex virus type 1. J. Virol. **33**:1192-1198.
4. **Cook, M. L., V. B. Bastone, and J. G. Stevens.** 1974. Evidence that neurons harbor latent herpes simplex virus. Infect. Immun. **9**:946-951.
5. **Cook, M. L., and J. G. Stevens.** 1973. Pathogenesis of herpetic neuritis and ganglionitis in mice: evidence of intraaxonal transport of infection. Infect. Immun. **7**: 272-288.
6. **Courtney, R. J., and M. Benyesh-Melnick.** 1974. Isolation and characterization of a large molecular-weight polypeptide of herpes simplex virus type 1. Virology **62**: 539-551.
7. **Dixon, R. A. F., and P. A. Schaffer.** 1980. Fine-structure mapping and functional analysis of temperature-sensitive mutants in the gene encoding the herpes simplex virus type 1 immediate early protein VP175. J. Virol. **36**:189-203.
8. **Frenkel, N. R., J. Jacob, R. W. Honess, G. S. Hayward, H. Locker, and B. Roizman.** 1975. Anatomy of herpes simplex virus DNA. III. Characterization of defective DNA molecular and biological properties of virus populations containing them. J. Virol. **16**:153-167.
9. **Galloway, D. A., C. Fenoglio, M. Shevchuk, and J. K. McDougall.** 1979. Detection of herpes simplex RNA in human sensory ganglia. Virology **95**:265-268.
10. **Goodpasture, E. W., and O. Teague.** 1923. Transmission of the virus of herpes febrillis along nerves in experimentally infected rabbits. J. Med. Res. **44**:139-184.
11. **Honess, R. W., and B. Roizman.** 1974. Regulation of herpes virus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. J. Virol. **14**:8-19.
12. **Knotts, F. B., M. L. Cook, and J. G. Stevens.** 1973. Latent herpes simplex virus in the central nervous system of rabbits and mice. J. Exp. Med. **138**:740-744.
13. **Knotts, F. B., M. L. Cook, and J. G. Stevens.** 1974. Pathogenesis of herpetic encephalitis in mice after ophthalmic inoculation. J. Infect. Dis. **130**:16-27.
14. **Lancz, G. J., and T. L. Zottlemoyer.** 1976. Restricted replication of herpes simplex virus in neural cells (39384). Proc. Soc. Exp. Biol. Med. **152**:302-306.
15. **Leiden, J. M., R. Buttyan, and P. G. Spear.** 1976. Herpes simplex virus gene expression in transformed cells. I. Regulation of the viral thymidine kinase gene in transformed L cells by products of superinfecting virus. J. Virol. **20**:413-424.
16. **Leung, W. C.** 1978. Evidence for a herpes simplex virus-specific factor controlling the transcription of deoxypyrimidine kinase. J. Virol. **27**:269-274.
17. **Levine, M., A. L. Goldin, and J. C. Glorioso.** 1980. Persistence of herpes simplex virus genes in cells of neuronal origin. J. Virol. **35**:203-210.
18. **Lofgren, K. W., J. G. Stevens, H. S. Marsden, and J. H. Subak-Sharpe.** 1977. Temperature-sensitive mutants of herpes simplex virus differ in the capacity to establish latent infections in mice. Virology **76**:440-443.
19. **Murray, B. K., N. Biswal, J. B. Bookout, R. E. Lanford, R. J. Courtney, and J. L. Melnick.** 1975. Cyclic appearance of defective interfering particles of herpes simplex virus and the concomitant accumulation of early polypeptide VP175. Intervirology **5**:173-184.
20. **Nesburn, A. B., and M. T. Green.** 1976. Recurrence in ocular herpes simplex infection. Invest. Opthalmol. **15**: 515-518.
21. **Openshaw, H., A. Puga, and A. L. Notkins.** 1979. Herpes simplex virus infection in sensory ganglia; immune control, latency and reactivation. Fed. Proc. **38**: 2660-2664.
22. **Pereira, L., M. H. Wolf, M. Fenwick, and B. Roizman.** 1977. Regulation of herpes virus macromolecular synthesis. V. Properties of α-polypeptides made in HSV-1 and HSV-2 infected cells. Virology **77**:733-749.
23. **Porter, D. D., I. Wimberly, and M. Benyesh-Melnick.** 1969. Prevalence of antibodies to EB virus and other herpes viruses. J. Am. Med. Assoc. **208**:1675-1679.
24. **Powell K. L., and D. J. M. Purifoy.** 1976. DNA-binding proteins of cells infected by herpes simplex virus type 1 and type 2. Intervirology **7**:225-239.
25. **Preston, C. M.** 1979. Control of herpes simplex virus type 1 mRNA synthesis in cells infected with wild-type virus or the temperature-sensitive mutant *tsk*. J. Virol. **29**: 275-284.
26. **Puga, A., J. D. Rosenthal, H. Openshaw, and A. L. Notkins.** 1978. Herpes simplex virus DNA and mRNA sequences in acutely and chronically infected trigeminal ganglia of mice. Virology **89**:102-111.
27. **Rajcani, J. and F. Ciampor.** 1978. Experimental pathogenesis of nonlethal herpes virus infection and the establishment of latency. Acta Virol. **22**:278-286.
28. **Rodda, S., I. Jack, and D. O. White.** 1973. Herpes simplex virus from trigeminal ganglion. Lancet **1**:1395-1396.
29. **Scriba, M.** 1975. Herpes simplex virus infection in guinea pigs: an animal model for studying latent and recurrent

herpes simplex virus infection. Infect. Immun. **12**:162-165.

30. **Sim, C., and D. H. Watson.** 1975. The role of type specific and cross reacting structural antigens in the neutralization of herpes simplex virus type 1 and type 2. J. Gen. Virol. **19**:197-233.
31. **Stevens, J. G.** 1975. Latent herpes simplex virus and the nervous system. Curr. Top. Microbiol. Immunol. **70**:31-50.
32. **Stevens, J. G.** 1978. Latent characteristics of selected herpes viruses. Adv. Cancer Res. **26**:227-256.
33. **Stevens, J. G., and M. L. Cook.** 1971. Latent herpes simplex virus in spinal ganglia of mice. Science **173**:843-845.
34. **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.
35. **Stevens, J. G., A. B. Nesburn, and M. L. Cook.** 1972. Latent herpes simplex virus in the central nervous system of rabbits and mice. Nature (London) New Biol. **235**:216-217.
36. **Vahlne, A., and E. Lycke.** 1978. Herpes simplex virus infection of *in vitro* cultured neuronal cells (mouse neuroblastoma C1300 cells). J. Gen. Virol. **39**:321-332.
37. **Waltz, M. A., R. W. Price, and A. L. Notkins.** 1974. Latent infection with herpes simplex virus types 1 and 2: viral reactivation *in vivo* after neurectomy. Science **184**:1185-1187.
38. **Wilcox, K. W., A. Kohn, E. Sklyanskaya, and B. Roizman.** 1980. Herpes simplex virus phosphoproteins. I. Phosphate cycles on and off some viral polypeptides and can alter their affinity for DNA. J. Virol. **33**:167-182.
39. **Zur Hansen, H., and H. Schulte-Holthausen.** 1975. Persistence of herpes virus nucleic acid in normal and transformed human cells—a review, p. 117-123. *In* G. de The, M. A. Epstein, and H. Zur Hansen (ed.), Oncogenesis and herpesviruses II, part 1. International Agency for Research on Cancer, Lyon.