JOURNAL OF VIROLOGY, July 1987, p. 2311–2315
0022-538X/87/072311-05$02.00/0

Vol. 61, No. 7

Nerve Growth Factor Deprivation Results in the Reactivation of Latent Herpes Simplex Virus In Vitro

CHRISTINE L. WILCOX AND EUGENE M. JOHNSON, JR.*

Department of Pharmacology, Washington University School of Medicine, St. Louis, Missouri 63110

Received 29 January 1987/Accepted 30 March 1987

Primary sympathetic neuronal cultures were maintained for up to 5 weeks after inoculation with herpes simplex virus (HSV) without evidence of viral infection. Upon deprivation of nerve growth factor, the cultures produced infectious HSV, indicating that the cultures harbored latent HSV. This study demonstrates a function of nerve growth factor in the maintenance of HSV latency.

Herpes simplex virus (HSV) resides in a latent state in neurons (6, 12, 14) of sensory (1, 2, 18) and sympathetic ganglia (20, 29). During latency, infectious virus is not detectable; however, the latent virus can reactivate to produce recurrent lesions and transmissible virus. The mechanisms involved in the establishment, maintenance, and reactivation of latent HSV are not understood. The study of the HSV infection in isolated neurons, the cells in which the viral genome resides during latency, may provide critical information for understanding HSV latency and reactivation. Price and Schmitz (22) suggested that the loss of trophic support provided to the neuron by the peripheral target results in the reactivation of latent HSV. The only neurotrophic agent isolated to date is nerve growth factor (NGF) (26). NGF is synthesized and released by the target tissue, binds specifically to receptors on the nerve terminals, and is retrogradely transported to the neuronal soma (9). Sympathetic and neural crest-derived sensory neurons in vivo and in vitro require NGF for maintaining normal function and, depending on age and the cell type, for survival (26).

Some of the stimuli reported to cause reactivation of latent HSV appear unrelated, e.g., UV irradiation (3) and fever (30). However, some reactivating stimuli, such as central rhizotomy (5) and axotomy (22, 28), produce a reduction or cessation in retrogradely transported neurotrophic support from the neuronal targets (10, 15, 23). These observations, combined with the fact that the neuronal cell types shown to harbor latent HSV are NGF dependent, led us to develop methods to establish latent HSV in neurons in vitro and to determine whether NGF deprivation can influence the latent state of HSV.

Previous attempts by others to establish an in vitro model of HSV latency have been unsuccessful (7, 13, 17, 19, 21, 27, 31, 34). One in vitro approach consists of HSV inoculation of primary neuronal cultures (33) or nonneuronal cell lines (16, 32), followed by a combination of treatment with arabinosyl cytosine, interferon, and elevated temperature (39.5 to 40.5°C). Lowering the temperature to 37°C results in the expression of infectious virus. This model has been criticized because the treatments used probably block replication of the virus rather than establish latency (19).

In this report, we demonstrate that, in cultures of primary sympathetic neurons inoculated with HSV at low multiplicities of infection, a significant percentage of neuronal cultures survived and showed no evidence of viral infection. However, a percentage of these cultures harbored latent HSV. NGF deprivation of these cultures resulted in the reactivation of the latent HSV.

Sympathetic neuronal cultures were prepared from superior cervical ganglia from neonatal rats, as described previously (11). Briefly, the ganglia were incubated for 1 h at 37°C with 1 mg of collagenase per ml (Cooper Biomedical, Inc., West Chester, Pa.), mechanically dissociated, and plated onto rat-tail collagen-coated four-well culture dishes (Nunc, Roskilde, Denmark). Approximately 0.7 ganglion was plated per culture. The culture medium was Eagle minimal essential medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 1.4 mM glutamine–10% fetal calf serum–50 ng of 2.5S mouse NGF per ml, prepared by the method of Bocchini and Angeletti (4). The cultures were maintained at 36°C in an atmosphere of 5% CO_2. For the first 10 to 12 days after plating, the cultures were treated with 20 μM fluorodeoxyuridine, which reduced the nonneuronal cell population to less than 5% but did not affect the neurons (11). Neuronal densities were approximately 5,000 to 8,000 per culture.

At 10 to 12 days after plating, the neuronal cultures were rinsed with several changes of the medium during 24 h to remove the antimitotic agent and were then infected with HSV type 1 (F) (American Type Culture Collection, Rockville, Md.), prepared and processed for determination of titers on Vero cells (American Type Culture Collection) at the multiplicity of infection as indicated. After a 2-h absorption period, the viral inoculum was removed and replaced with either medium or medium containing 2% anti-HSV antiserum (human antiserum, neutralizing antibody titer of 64,000 as determined by a plaque reduction assay). At 14 days after inoculation with HSV, surviving cultures treated with anti-HSV antiserum were rinsed extensively to remove anti-HSV antibodies.

Cultures were evaluated for the presence of HSV by several methods. Plaque-forming assays were used to detect infectious HSV. Cell-associated virus was assayed after the disruption of the cells by several cycles of rapid freeze-thawing. Virus released from cells was detected in assays of the culture media. Sympathetic neurons productively infected with HSV develop a characteristic cytopathic effect (CPE) (21), which could be observed under phase-contrast microscopy. Productive HSV infections were confirmed by detection of virus-specific thymidine kinase activity (25).

Neutralizing antibodies to NGF were obtained by immunizing guinea pigs with mouse NGF in complete Freund adjuvant, as described previously (24). The antibody titer

* Corresponding author.

TABLE 1. Effects of varying the multiplicity of infection of HSV and the addition of antibodies to HSV on the survival of neuronal cultures and the frequency of HSV reactivation after NGF deprivation

Multiplicity of infection (PFU/cell)	Anti-HSV antibodies added	% Survival[a] (no. of surviving cultures/no. of inoculated cultures)	% Reactivation[b] (no. of HSV-positive cultures[c]/no. of cultures tested)
0.03	−	53 (32/60)	6 (1/16)
	−	54 (13/24)	14 (2/24)
	−	48 (25/52)	16 (4/25)
	+	100 (120/120)	8 (5/60)
	+	100 (120/120)	10 (6/60)
0.10	−	0 (0/144)	
	+	100 (52/52)	19 (5/26)
0.50	+	93 (119/128)	26 (15/54)
	+	94 (120/128)	24 (15/63)
1.00	+	63 (38/60)	53 (10/19)

[a] Survival of cultures was evaluated morphologically.
[b] Reactivation was produced by the addition of antibodies to NGF to the surviving cultures.
[c] Cultures were considered HSV positive when infectious virus was demonstrated.

was 32,000, determined as the highest dilution of the antibody able to block NGF activity in the embryonic chicken dorsal root ganglia bioassay (8). Nonimmune guinea pig serum was used as a control. Anti-NGF serum or nonimmune serum was used at 1%.

The effects of varying the multiplicity of infection of HSV on the survival of cultures after HSV inoculation are shown in Table 1. Without the addition of antibodies to HSV after virus inoculation, many cultures were destroyed by lytic infection, the percentage of which increased with greater multiplicities of infection. The addition of antibodies to HSV, although not necessary to establish latency (see below), greatly reduced the number of cultures destroyed by lytic infections.

The time course and frequency of the lytic viral infection were examined. After inoculation with 0.5 PFU of HSV per cell followed by the addition of anti-HSV serum, cultures were assayed daily for infectious virus by harvesting the cells after rinsing extensively to remove anti-HSV antibodies. Infectious virus was detected in a total of 6% of the cultures (6 of 104) only between days 4 and 7 postinoculation; thereafter, infectious virus was not detected (Table 2). Cultures have been monitored for up to 5 weeks postinoculation without any detectable cell-associated virus (0 of 60). In similar experiments, cultures were monitored for viral thymidine kinase activity or CPE; again, evidence of productive viral infection was observed only between days 4 and 7 postinoculation (data not shown). The appearance of CPE always presaged eventual destruction of all of the neurons in the culture. In cultures which had no evidence of CPE, there was no apparent loss in cell numbers and no detectable infectious virus was released into the medium at any time. The longest time at which the cultures were assayed for infectious virus was 5 weeks postinoculation (0 of 108).

TABLE 2. Time course of initial HSV infection after inoculation of neuronal cultures

Day postinoculation[a]	No. of HSV-positive cultures/total[b]
1–3	0/32
4	2/8
5	1/8
6	2/8
7	1/8
8–14	0/48

[a] Neuronal cultures were inoculated with 0.5 PFU of HSV per cell, followed by the addition of anti-HSV serum.
[b] After being washed three times with standard medium, the cells were lysed and assayed for infectious HSV.

To address the hypothesis that loss of trophic support results in the reactivation of latent HSV, NGF deprivation was produced by adding NGF antibodies 2 weeks postinoculation to cultures which had had no evidence of viral infection. Treatment with anti-NGF resulted in the reactivation of HSV in 6 of 53% of the cultures, depending on the conditions used to establish the latent infection (Table 1). The percentage of cultures harboring latent HSV which

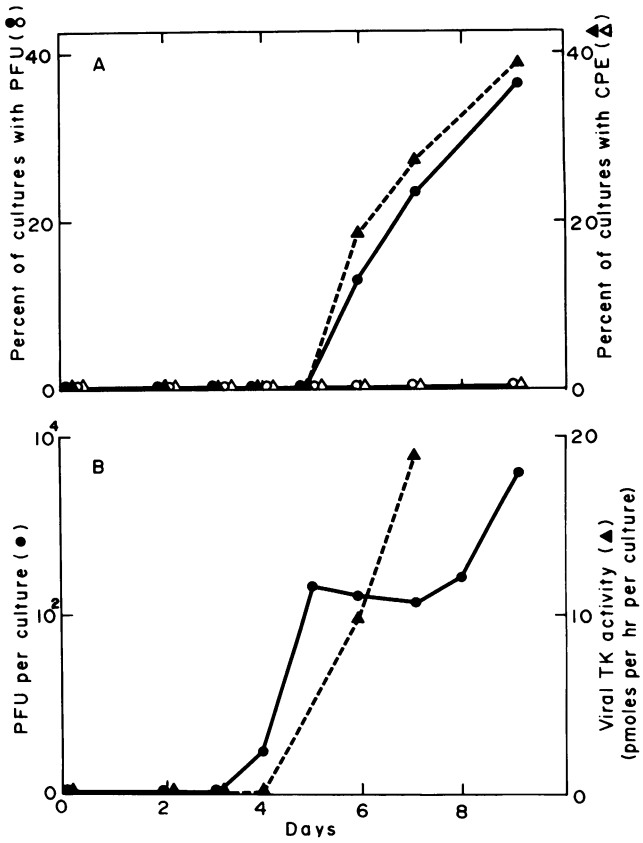

FIG. 1. Reactivation of latent HSV from superior cervical ganglion neurons in culture after treatment with anti-NGF. Neuronal cultures were inoculated with 0.5 PFU of HSV per cell and treated with 2% anti-HSV serum. The cultures which had no evidence of virus infection 14 days after inoculation with HSV were rinsed with medium to remove antibodies to HSV and were treated with anti-NGF. (A) At the times indicated after treatment with anti-NGF or normal serum, cultures were observed for HSV-induced CPE and culture supernatants were assayed for infectious HSV released into the media. The percentage of cultures which had CPE (▲, △) or infectious virus (●, ○) was determined after treatment with 1% anti-NGF serum (closed symbols) or 1% normal serum (open symbols). For each treatment, 60 cultures were monitored. (B) Cultures prepared as described above were assayed for cell-associated virus on the days indicated after initiation of treatment with anti-NGF. Infectious virus was measured in plaque-forming assays (●), or viral thymidine kinase (TK) activity (▲) was measured. Values shown represent the mean of either two or three samples; values were within 10% of the mean.

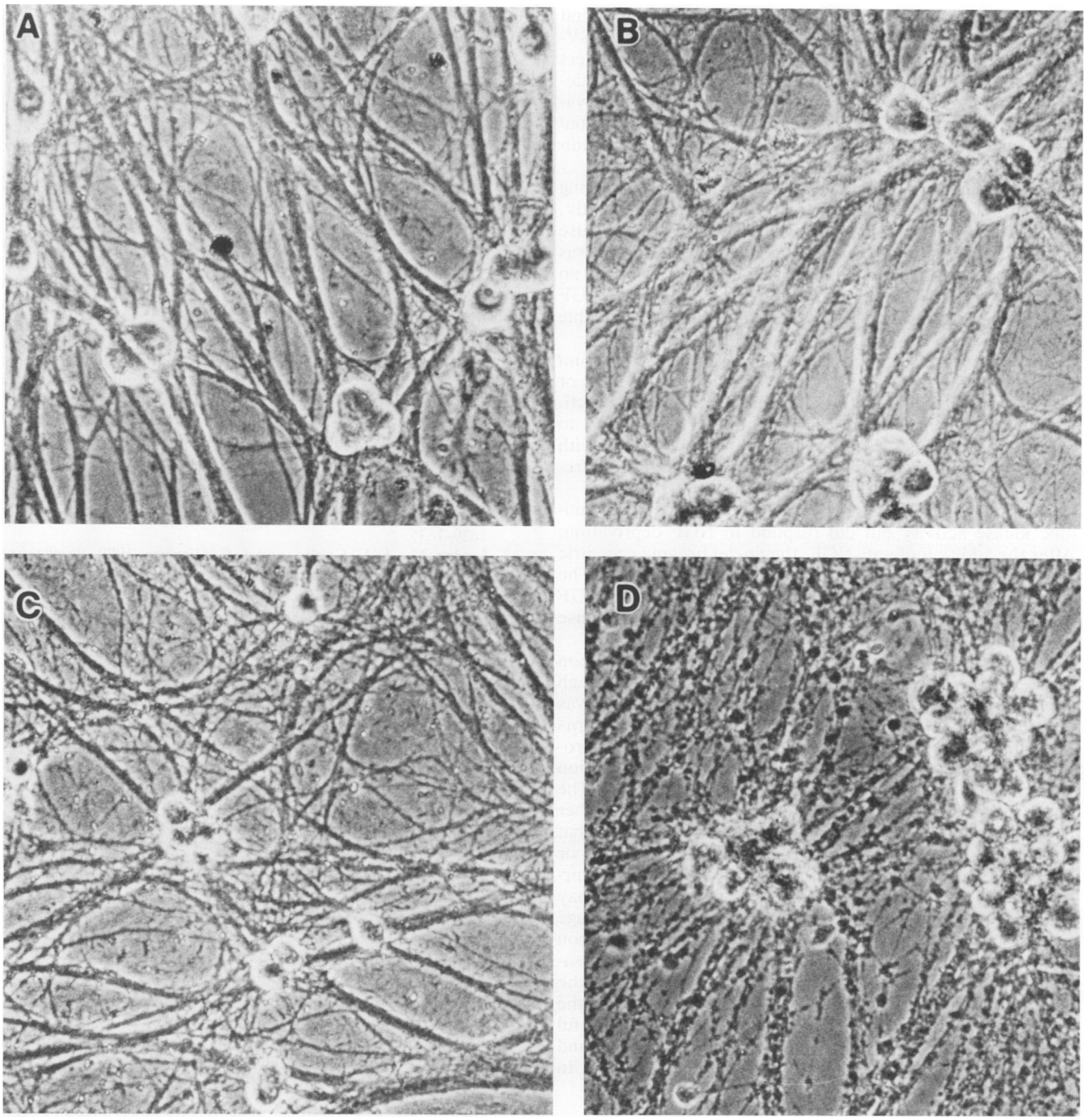

FIG. 2. Phase-contrast micrographs of HSV-infected and mock-infected neuronal cultures after treatment with nonimmune serum or anti-NGF serum. Neuronal cultures, 14 days after inoculation with 0.5 PFU of HSV per cell and treatment with 2% anti-HSV serum or mock infection, were treated for 9 days either with 1% nonimmune serum or with 1% anti-NGF serum. (A) Mock-infected neurons treated with nonimmune serum. (B) HSV-inoculated culture treated with nonimmune serum. (C) Mock-infected neurons treated with anti-NGF. (D) HSV-inoculated neurons treated with anti-NGF, showing HSV-induced CPE.

reactivated after anti-NGF treatment was related to the multiplicity of infection of HSV. With increased PFU of HSV per cell, greater losses of the cultures occurred within week 1 postinoculation, but the percentage of surviving cultures which had latent HSV was also increased (Table 1). Viral latency was stable; inoculation with 0.5 PFU of HSV per cell followed by the addition of anti-HSV serum 5 weeks before anti-NGF treatment resulted in reactivation in 25% of the cultures, a frequency indistinguishable from that of virus which reactivates after anti-NGF treatment 2 weeks after HSV inoculation.

Figure 1A shows the time course of infectious HSV released into the culture media and the appearance of CPE from latent cultures after anti-NGF treatment. Infectious

virus was initially detected in the media when CPE was first observed (day 6 after the addition of anti-NGF). HSV-induced CPE spread throughout the cultures as viral titers increased (Fig. 1A and 2D). Viral titers subsequently decreased as the cells were lysed. Additionally, the reactivation time course was confirmed in assays for cell-associated virus and viral thymidine kinase activity on the days indicated after anti-NGF treatment (Fig. 1B).

Reactivation was also achieved by using medium lacking NGF. The time course of reactivation was delayed slightly, but the frequency was the same as that observed with the use of anti-NGF antibodies (data not shown). The delay was probably caused by residual NGF in the cultures. NGF is an inherently "sticky" molecule, which is the reason anti-NGF antibodies have traditionally been used to eliminate available NGF (26).

Cultures with latent virus treated with nonimmune serum never produced infectious virus, CPE (Fig. 1A and 2B), or viral thymidine kinase activity. Upon completion of reactivation experiments, latent control cultures were shown to harbor latent virus, since treatment of the cultures with anti-NGF resulted in reactivation with the expected frequency and time course (data not shown).

Mock-infected cultures produced neither virus nor thymidine kinase activity but did respond to NGF deprivation. After the addition of anti-NGF, the nuclei became eccentric and the cell size was significantly reduced (Fig. 2C). The neurons remained phase bright during the 10-day anti-NGF treatment period, indicating that the protocol did not cause significant cell death.

In summary, NGF deprivation resulted in the reactivation of latent HSV in sympathetic neuronal cultures. Although approximately 5% of the cell population in the cultures was nonneuronal cells, the cell type that harbored the latent virus was almost certainly the neuron. The stimulus which produced reactivation, antiserum to NGF, is a perturbation which would affect only the neurons in the cultures. The nonneuronal cells, fibroblasts and Schwann cells, are neither NGF dependent nor NGF responsive. The data suggest that NGF may be important in vivo in regulating, directly or indirectly, the latent state of the virus. While other perturbations which result in the reactivation of latent HSV may act through other mechanisms, perturbations that damage either the nerve terminal or the terminal field of the neuron may cause reactivation by producing a reduction or cessation in NGF normally retrogradely transported to the neuronal cell body. This in vitro model, which satisfies the operational definition of HSV latency (19) should be useful for examining factors involved in the establishment and reactivation of latent HSV and may provide a system in which to study latency at the molecular level.

This work was supported by Public Health Service grants NS 18071 and 5-T32-HL07275 from the National Institutes of Health and by a grant from the March of Dimes Birth Defects Foundation.

LITERATURE CITED

1. **Baringer, J. R., and P. Swoveland.** 1973. Recovery of herpes simplex virus from human trigeminal ganglions. N. Engl. J. Med. **288**:648–650.
2. **Bastian, F. O., A. S. Rabson, C. L. Yee, and T. S. Tralka.** 1972. Herpes hominis: isolation from human trigeminal ganglions. Science **178**:306–307.
3. **Blyth, W. A., T. J. Hill, H. J. Field, and D. A. Harbour.** 1976. Reactivation of herpes simplex virus infection by ultraviolet light and possible involvement of prostaglandins. J. Gen. Virol. **33**:547–550.
4. **Bocchini, V., and P. U. Angeletti.** 1969. The nerve growth factor: purification as a 30,000 molecular weight protein. Proc. Natl. Acad. Sci. USA **64**:787–794.
5. **Carton, C. A., and E. D. Kilbourne.** 1952. Activation of latent herpes simplex by trigeminal sensory-root section. N. Engl. J. Med. **246**:172–176.
6. **Cook, M. L., V. B. Bastone, and J. G. Stevens.** 1974. Evidence that neurons harbor latent herpes simplex virus. Infect. Immun. **9**:946–951.
7. **Feldman, L. A., R. D. Sheppard, and M. B. Bornstein.** 1968. Herpes simplex virus-host cell relationships in organized cultures of mammalian nerve tissues. J. Virol. **2**:621–628.
8. **Fenton, E. L.** 1970. Tissue culture assay of nerve growth factor and of the specific antiserum. Exp. Cell Res. **59**:383–392.
9. **Hendry, I. A., K. Stockel, H. Thoenen, L. L. Iversen.** 1974. The retrograde transport of nerve growth factor. Brain Res. **68**: 103–121.
10. **Johnson, E. M., Jr., and H. K. Yip.** 1985. Central nervous system and peripheral nerve growth factor provide trophic support critical to mature sensory neuronal survival. Nature (London) **314**:751–752.
11. **Johnson, M. I., and V. Argiro.** 1983. Techniques in the tissue culture of rat sympathetic neurons. Methods Enzymol. **103**: 334–347.
12. **Kennedy, P. G. E., S. A. Al-Saadi, and G. B. Clements.** 1983. Reactivation of latent herpes simplex virus from dissociated identified dorsal root ganglion cells in culture. J. Gen. Virol. **64**: 1629–1635.
13. **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.
14. **McLennan, 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 activation. J. Gen. Virol. **51**:233–243.
15. **Nja, A., and D. Purves.** 1978. The effects of nerve growth factor and its antiserum on synapses in the superior cervical ganglion of the guinea-pig. J. Physiol. (London) **277**:53–75.
16. **O'Neill, F. J., R. J. Goldberg, and F. Rapp.** 1972. Herpes simplex virus latency in cultured human cells following treatment with cytosine arabinoside. J. Gen. Virol. **14**:189–197.
17. **Pizer, L. I., S. U. Kim, P. Nystrom, and V. C. Coates.** 1978. Herpes simplex virus replication in pheochromocytoma cell line that responds to nerve growth factor. Acta Neuropathol. **44**: 9–14.
18. **Plummer. G.** 1973. Isolation of herpesviruses from trigeminal ganglia of man, monkeys, and cats. J. Infect. Dis. **128**:345–348.
19. **Price, R. W.** 1986. Neurobiology of human herpesvirus infections. Crit. Rev. Clin. Neurobiol. **2**:61–123.
20. **Price, R. W., B. J. Katz, and A. L. Notkins.** 1975. Latent infection of the peripheral ANS with herpes simplex virus. Nature (London) **257**:686–688.
21. **Price, R. W., R. Rubenstein, and A. Khan.** 1982. Herpes simplex virus infection of isolated autonomic neurons in culture: viral replication and spread in a neuronal network. Arch. Virol. **71**: 127–140.
22. **Price, R. W., and J. Schmitz.** 1978. Reactivation of latent herpes simplex virus infection of the autonomic nervous system by postganglionic neurectomy. Infect. Immun. **19**:523–532.
23. **Purves, D., and A. Nja.** 1976. Effect of nerve growth factor on synaptic depression following axotomy. Nature (London) **260**: 535–536.
24. **Rich, K. M., H. K. Yip, P. A. Osborne, R. E. Schmidt, and E. M. Johnson, Jr.** 1984. Role of nerve growth factor in the adult dorsal root ganglia neuron and its repsonse to injury. J. Comp. Neurol. **230**:110–118.
25. **Tenser, R. B., R. L. Miller, and F. Rapp.** 1979. Trigeminal ganglion infection by thymidine kinase-negative mutants of herpes simplex virus. Science **205**:915–917.
26. **Thoenen, H., and Y.-A. Barde.** 1980. Physiology of nerve growth factor. Physiol. Rev. **60**:1284–1335.
27. **Vahlne, A., E. Nilheden, and B. Svennerholm.** 1981. Multiplicity

activation of herpes simplex virus in mouse neuroblastoma (C1300) cells. Arch. Virol. **70**:345–356.

28. **Walz, M. A., R. W. Price, and A. L. Notkins.** 1974. Latent ganglionic infection with herpes simplex virus types 1 and 2: viral reactivation *in vivo* after neurectomy. Science **184**:1185–1187.
29. **Warren, K. G., S. M. Brown, Z. Wroblewska, D. Gilden, H. Koprowski, and J. Subak-Sharpe.** 1978. Isolation of latent herpes simplex virus from the superior cervical and vagus ganglions of human beings. N. Engl. J. Med. **298**:1068–1069.
30. **Warren, S. L., C. M. Carpenter, and R. A. Boak.** 1940. Symptomatic herpes, a sequela of artificially induced fever. J. Exp. Med. **71**:155–168.
31. **Whetsell, W. O., J. Schwartz, and T. S. Elizan.** 1977. Comparative effects of herpes simplex virus type 1 and 2 in organotypic cultures of mouse dorsal root ganglion. J. Neuropathol. Exp. Neurol. **36**:547–560.
32. **Wigdahl, B. L., A. C. Scheck, E. De Clercq, and F. Rapp.** 1982. High efficiency latency and activation of herpes simplex virus in human cells. Science **217**:1145–1146.
33. **Wigdahl, B. L., R. J. Ziegler, M. Sneve, and F. Rapp.** 1983. Herpes simplex virus latency and reactivation in isolated rat sensory neurons. Virology **127**:159–167.
34. **Ziegler, R. J., and R. S. Pozos.** 1984. Inability of various immune mechanisms to establish *in vitro* latent infection in sensory neurons infected with HSV-1. Arch. Virol. **79**:123–130.