Characterization of Nerve Growth Factor-Dependent Herpes Simplex Virus Latency in Neurons In Vitro

CHRISTINE L. WILCOX[†]* AND EUGENE M. JOHNSON, JR.

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

Received 22 June 1987/Accepted 28 October 1987

Primary sympathetic neuronal cultures were maintained for up to 5 weeks after inoculation with herpes simplex virus (HSV) without evidence of viral infection. Treatment with acyclovir for the first 7 days after viral inoculation prevented lytic infections in 100% of the cultures and resulted in viral latency in 100% of the cultures; reactivation occurred as the result of nerve growth factor (NGF) deprivation. Treatment of the cultures with several different inhibitors of viral DNA polymerase (acyclovir, aphidicolin, and phosphonoacetic acid) for 7 days after viral inoculation did not prevent the establishment of latency, which suggests that viral DNA replication was not required. During the latent phase of the infection, viral antigens were not detected with HSV-specific immunohistochemistry. However, 24 h after NGF deprivation, viral antigens were detected in essentially all of the neurons, indicating that the majority of neurons harbored latent HSV. The establishment of latency was not strain or type specific since latency was established with HSV type 2 and four strains of HSV type 1 and reactivation occurred in response to NGF deprivation.

Herpes simplex virus (HSV) causes diseases in humans which range in severity from the benign common cold sore to life-threatening encephalitis. HSV has the unusual capability of establishing a latent phase of the infection during which the virus is present in neurons in an apparently nonreplicating state. The latent virus periodically reactivates, which can produce cutaneous lesions and transmissible virus. Considerable progress has been made in understanding the molecular biology of HSV, yet little is known regarding the mechanisms involved in the establishment, maintenance, and reactivation of HSV latency.

Recently, we reported the establishment of HSV type 1 (HSV-1) latency in sympathetic neurons in vitro and demonstrated that deprivation of nerve growth factor (NGF) results in reactivation (38). Sympathetic and neural crest-derived sensory neurons in vivo and in vitro require NGF to maintain normal function and, depending on age and cell type, to survive (34). NGF is synthesized and released by the neuronal target tissue, is bound specifically to receptors on the nerve terminals, and is retrogradely transported to the neuronal cell body (12, 34). The requirement of NGF for the maintenance of latency in vitro is consistent with several observations: (i) the only clearly documented sites of HSV latency (1, 2, 6, 17, 19, 23, 25, 36) are NGF-dependent neurons (22, 34); and (ii) treatments which produce a reduction or cessation of NGF retrograde transport, such as axotomy (28) and central rhizotomy (15), result in the reactivation of latent HSV (5, 26, 35).

Previously, we described the establishment of HSV-1 latency in neuronal cultures by infecting with a low multiplicity of infection (MOI) (38). With an infecting MOI of 0.03, the initial loss of the cultures is 37%, and of the surviving cultures, 12% harbor latent HSV. We found that by treating the cultures with HSV-neutralizing antiserum for 2 weeks after HSV inoculation, the MOI can be increased to 1, which will produce 63% survival of the cultures with 53%

of the survivors harboring latent HSV. However, with an infecting MOI of 2, even in the presence of anti-HSV serum, 100% of the cultures were destroyed to lytic infections (unpublished data). These results suggested that if viral spread from lytic infections were blocked, then it might be possible to increase survival and, possibly, the number of cultures with latent HSV.

In this report we have demonstrated that treatment of neuronal cultures, for 7 days after HSV inoculation, with drugs which inhibit viral DNA polymerase (acyclovir [ACV], aphidicolin, or phosphonoacetic acid) resulted in the survival of 100% of the cultures and the establishment of NGF-dependent HSV latency in 100% of the cultures. The results indicate that viral DNA replication is not required for the establishment of latency. Viral antigens were not detected during the latent phase of the infection but became evident on essentially all of the neurons within 24 h after NGF deprivation, indicating that the majority of neurons harbored latent virus. We show that the establishment of latency was not HSV strain or type specific.

MATERIALS AND METHODS

Preparation of viruses. HSV-1 (115), a recent clinical isolate obtained from Charles Reed (Virology Laboratory, St. Louis Children's Hospital, St. Louis, Mo.), was used unless otherwise indicated. HSV-1 (F), HSV-1 (McIntyre), HSV-2 (G), and Vero cells were purchased from the American Type Culture Collection (Rockville, Md.). HSV-1 (KOS) and HSV-1 (HRM) were provided by L. I. Pizer (University of Colorado Health Sciences Center, Denver). HSV-1 (HRM) is a host range mutant prepared by Berge Hampar (Laboratory of Molecular Virology, National Cancer Institute, Frederick, Md.) by passage of wild-type strain 14-120 in the presence of bromodeoxyuridine. Viruses were grown and quantitated by plaque assay on confluent Vero cells.

Neuronal cultures. Sympathetic neuronal cultures were prepared from superior cervical ganglia from prenatal rats (Zivic-Miller Laboratories, Inc., Zelienople, Pa.) essentially as described by Johnson and Argiro (16). Briefly, the ganglia were removed aseptically and incubated for 1 h at 37°C with

* Corresponding author.

[†] Present address: Department of Microbiology, University of Colorado Health Sciences Center, 4200 E. Ninth Ave., Denver, CO 80262.

1 mg of collagenase (Cooper Biomedical, Inc., West Chester, Pa.) per ml. After rinsing with medium, the ganglia were mechanically dissociated and plated onto 15-mm, four-well culture dishes (Nunc, Roskilde, Denmark) coated with rat-tail collagen at a density of 5,000 to 8,000 neurons per culture. The cultures were maintained with Eagle minimal essential medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 1.4 mM glutamine (Sigma, St. Louis, Mo.), 10% fetal calf serum (GIBCO Laboratories), and 50 ng of 2.5S mouse NGF per ml, prepared by the method of Bocchini and Angeletti (4), and incubated at 36°C in an atmosphere of 5% CO₂. The culture medium was renewed every 3 to 5 days. For the first 10 to 12 days after plating, the cultures were treated with 20 μ M fluorodeoxyuridine (Sigma), which reduced the nonneuronal cell population to less than 5%, but did not affect the neurons (16).

Establishment and reactivation of HSV latency. At 10 to 12 days after plating, ACV (Burroughs Wellcome Co., Research Triangle Park, N.C.), aphidicolin (Calbiochem, La Jolla, Calif.), or phosphonoacetic acid (Sigma) was added, at the concentrations indicated in the table and figure legends, to the cultures 12 h before inoculation with HSV. The MOI of HSV used is indicated in the table and figure legends. Seven days after inoculation, the drug treatment was discontinued by washing with several changes of the standard medium. Cultures were tested, by using the culture supernatants in plaque-forming assays, for the presence of infectious virus at various times after inoculation. Samples of culture supernatant, directly and serially diluted, were allowed to adsorb on confluent monolayers of Vero cells for 1 h. After the adsorption period, the inoculum was removed, and the monolayer was rinsed with medium and overlaid with medium containing 5% Methocel (Dow Chemical Co., Midland, Mich.). After 3 days, the plaques were counted after the Vero cells were fixed and stained. Because plaque-forming assays were to be performed on culture supernatants containing antiviral drugs, we compared the effects of the absence and presence of 50 μ M ACV during the absorption period and found this to have minimal effects on the number of plaques detected.

Fourteen days or longer after inoculation with virus, the cultures were evaluated for the presence of latent virus. To reactivate latent virus, cultures were deprived of NGF by adding to the culture medium 1% heat-inactivated, anti-NGF serum (titer of 32,000, defined as the reciprocal of the highest dilution preventing neurite outgrowth in the standard embryonic chick dorsal root ganglia bioassay [9]). Neutralizing antibodies to NGF were obtained by immunizing guinea pigs with mouse NGF in complete Freund adjuvant as described previously (30). Cultures harboring latent virus were treated with 1% heat-inactivated, nonimmune guinea pig serum as a control.

Immunohistochemistry. Cultures were grown as described on collagen-coated cover slips (Miles Laboratories, Naperville, Ill.). Cultures were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min. The cover slips were dried and mounted on microscope slides. The slides were processed for indirect immunohistochemistry by using the avidin-biotin-enzyme complex method (Vectastain; Vector Laboratories, Burlingame, Calif.). The cells were permeabilized by treatment with 0.5% Nonidet P-40 (Sigma) for 10 min. Two primary antibodies were used. A polyclonal human anti-HSV serum (anti-HSV) (50% neutralization titer against HSV-1 of 64,000) was diluted 1:200 in PBS containing 1% bovine serum albumin, fraction V (Sigma). Anti-ICP4, a monotypic rabbit antiserum prepared as described

(20) against the immediate-early gene ICP4 which is overproduced by the mutant HSV-1 (*tsK*) at the nonpermissive temperature, was diluted 1:50 in PBS containing 1% bovine serum albumin fraction V. The primary antibodies were incubated with the slides overnight at 4°C. The slides were washed three times with cold PBS and then incubated at room temperature for 1 h with the biotinylated antibody (Vector Laboratories) diluted 1:200 in PBS. The cultures were rinsed and incubated at room temperature with the avidin-biotin-glucose oxidase complex (Vector Laboratories) for 30 min. For the final reaction product, the slides were incubated with Nitro Blue Tetrazolium (Substrate Kit I, Vector Laboratories) for 30 min at 37°C and rinsed with distilled water; cover slips were mounted with glycerol-PBS.

RESULTS

HSV latency in the presence of ACV. Results from our previous study indicated that as we increase the MOI, the number of surviving neuronal cultures is reduced, yet the percentage of cultures with HSV latency is increased (38). This suggested that latency could be established in a higher proportion of the cultures by increasing the MOI, if the initial lytic infection were suppressed. As the cultures contain a small percentage of nonneuronal cells (5%), viral replication in the nonneuronal cells is a potential source of infectious virus. Cytopathic effect (CPE) was consistently observed in the nonneuronal cells after inoculation with HSV (unpublished data). Additionally, from the previous study, we found that treatment of the cultures with anti-HSV serum added 1 h after viral inoculation increases the survival of the cultures (38). However, at an MOI of 2, the anti-HSV serum was unable to prevent overwhelming lytic infections in the cultures (unpublished data). To suppress the production of infectious virus, we studied the effects of ACV on the establishment of latency.

Neuronal cultures were treated with ACV from 12 h before and for 7 days after inoculation with HSV; thereafter, ACV was removed. ACV had no apparent toxic effects on either mock-infected or HSV-infected neurons. ACV was not needed to maintain latency. The drug was removed 7 days after inoculation, and cultures were maintained for up to 5 weeks postinoculation without any evidence of productive infection, as determined by the absence of detectable infectious virus or CPE. It was found that with the use of ACV during the inoculation period, the MOI could be increased, within limits, without the development of lytic infection. Consequently, both the survival of cultures and the percentage of cultures harboring HSV were increased to 100% (Table 1). Figure 1 shows the time course of the expression of infectious HSV released into the culture medium at various times after inoculation with 1 PFU of HSV per cell. The virus was most likely rapidly absorbed or inactivated, as indicated by the small fraction of the initial virus inoculum detected in supernatants removed from the cultures immediately after the absorption period. Thereafter, cultures were followed after the inoculation without detecting infectious virus or CPE. Latent cultures, deprived of NGF by adding anti-NGF serum to the cultures, resulted in 100% of the cultures developing CPE and producing infectious HSV (Fig. 1 and Table 1). Infectious virus was detected in the culture medium 48 to 72 h after the addition of anti-NGF serum (Fig. 1). Cultures with latent virus treated with nonimmune serum never produced infectious HSV (Fig. 1 and Table 1). With an MOI of 20, even the presence of 500 μ M ACV, a dose well above the reported concentra-

TABLE 1. Effect of ACV concentration versus the MOI of HSV on survival and the establishment of latency

ACV concn ^a (μ M)	MOI (PFU/cell)	% Survival ^b (no. of positive cultures/ no. tested)	% Reactivation ^c (no. of positive cultures/no. tested) after treatment with:	
			Anti-NGF serum	Normal serum
10	0.05	100 (36/36)	22 (4/18)	0 (0/18)
10	0.5	100 (36/36)	100 (18/18)	0 (0/18)
10	5	0 (0/36)		
50	1	100 (72/72)	100 (36/36)	0 (0/36)
50	5	100 (176/176)	100 (36/36)	0 (0/36)
50	10	0 (0/8)		
500	10	75 (6/8)	100 (3/3)	0 (0/3)
500	20	0 (0/8)		

^a Neuronal cultures were treated with ACV at the concentrations indicated for 7 days after inoculation with HSV-1 (strain 115); thereafter, the drug was removed from the culture medium.

^b Cultures were evaluated 2 weeks postinoculation for viability based on neuronal morphology, and culture supernatants were tested in plaque-forming assays for infectious HSV.

^c Two weeks postinoculation, surviving cultures were treated with either 1% guinea pig anti-NGF serum or 1% nonimmune guinea pig serum. Cultures were evaluated for CPE and were harvested on day 5 after serum treatment to test for the presence of infectious virus by plaque-forming assays. Cultures were considered positive when both CPE and infectious virus were detected.

tion producing 50% inhibition (8), was unable to prevent the destruction of the cultures (Table 1).

Cultures harboring latent virus were deprived of NGF by several washings with medium lacking NGF. Under these conditions, reactivation from 100% of the cultures (16 of 16) was observed. There was a delay of approximately 24 h before virus was detected as compared with results obtained using anti-NGF antibodies, consistent with the known difficulty in removing NGF, which nonspecifically binds to many substrates (34). These data demonstrate that the signal for reactivation was the removal of NGF and was not a nonspecific function of the antiserum.

Viral antigens during the latent phase of infection. Indirect immunohistochemistry was used to determine whether detectable levels of viral antigens were expressed during the latent phase of the infection. The results of immunohistochemical staining, obtained by using the polyclonal anti-HSV serum (Fig. 2 and 3) and monotypic anti-ICP4 serum (Fig. 2), are shown. In three experiments (eight cultures in each), there was no positive immunostaining with either primary antibody during the latent phase of the infection in cultures treated with nonimmune serum. However, when cultures harboring latent virus were treated with anti-NGF serum, immunoreactive staining using the polyclonal anti-HSV serum was evident on the neuronal soma within 12 h after the addition of anti-NGF serum (not shown). By 24 h after anti-NGF treatment, staining was extensive and present on both the neuronal cell bodies as well as the neurites (Fig. 2 and 3). Immunoreactive staining was present only on neurons 24 h after anti-NGF treatment, and essentially all of the neurons in the cultures had extensive staining (Fig. 3).

Establishment and reactivation of latent HSV: strain or type specificity. Based on evidence that certain HSV strains are restricted in neurovirulence (31) and that HSV-1 and -2 are unique biologically and biochemically, as well as exhibiting apparent neurotropisms as to the sites of latency (e.g., while either type can produce primary infections without apparent restriction, latent HSV-1 is found predominantly in trigeminal ganglia and HSV-2 generally establishes latent infections in sacral dorsal root ganglia [29]), we determined whether the establishment of HSV latency was restricted in vitro by HSV strain or type. Four additional strains of HSV-1 and one strain of HSV-2 were tested for their ability to establish latent infections and to reactivate in response to NGF deprivation. Under the conditions studied, HSV-2 (G) and

three of the four strains of HSV-1 established latent infections, and reactivation occurred in response to NGF deprivation (Table 2). This indicates that our results are not unique to a particular strain or type of HSV. HSV-1 (McIntyre) resulted in the lytic destruction of the cultures. McIntyre was the only syncytium-forming strain tested, and this characteristic, increased neurovirulence or other properties may explain the inability to prevent lytic destruction of the neurons.

Viral DNA replication in establishment of latent infections. The results of several in vivo studies suggest that viral DNA replication is not a prerequisite for the establishment of latent HSV infections. The establishment of latency in the

FIG. 1. Expression of infectious virus during the establishment of latent infections and during the reactivation of HSV after NGF deprivation from neuronal cultures inoculated with HSV in the presence of ACV. Neuronal cultures were inoculated with HSV-1 (115) at an MOI of 1 in the presence of 50 μ M ACV. At 24 h after inoculation, virus was not detected in the presence of ACV or at later times after the removal of the drug. One hundred percent of the cultures survived after viral inoculation with no apparent loss of neurons in the cultures. When NGF deprivation was produced by the addition of 1% anti-NGF serum to the neuronal cultures ($\blacktriangle$), virus was detected in the culture supernatants from 100% of the cultures 72 h after the addition of anti-NGF. Neuronal cultures treated with 1% nonimmune serum ($\blacksquare$) did not produce detectable virus. Results are expressed as the mean of the number of PFU recovered in the medium from four cultures per time point ($\pm$ standard error of the mean).

FIG. 2. Immunohistochemical staining with anti-HSV serum and anti-ICP4 serum of HSV-infected and mock-infected neuronal cultures. Twenty-four hours after treatment with 1% nonimmune or anti-NGF serum, neuronal cultures were inoculated at an MOI of 1 with HSV-1 (115) in the presence of 50 μ M ACV. ACV was removed from the cultures after 7 days. Fourteen days after viral inoculation, HSV-infected and mock-infected cultures were treated with anti-NGF or nonimmune serum. Twenty-four hours after the initiation of treatment, the cultures were fixed and processed as described in the text. Immunohistochemical staining was performed using a general anti-HSV serum (upper panels) or a monotypic anti-ICP4 serum (lower panels) as the primary antibodies. The photomicrographs shown represent the results obtained with (A) HSV-inoculated neurons treated with nonimmune serum, (B) mock-infected neurons treated with anti-NGF, and (C) HSV-infected neurons treated with anti-NGF serum. Magnification, $\times 192$.

presence of ACV also suggested that viral DNA synthesis was not required. Several additional agents, which inhibit HSV DNA polymerase at unique sites (13), were tested for their effects on the establishment of latency. Cultures were treated for 12 h before and for 7 days after inoculation with HSV; thereafter, the drug was removed from the culture medium by several changes of the medium. At the concentrations of aphidicolin and phosphonoacetic acid used, there were no apparent toxic effects, and there was no apparent loss in the number of neurons. Treatment of the cultures with aphidicolin or phosphonoacetic acid resulted in the survival of 100% of the cultures, and 100% of the cultures harbored latent virus (Table 3). Reactivation was observed as the result of NGF deprivation; nonimmune serum did not produce reactivations. After the addition of anti-NGF serum, cultures developed CPE and released infectious virus following a time course similar to that observed with ACV-treated cultures as described above (data not shown).

DISCUSSION

The mechanisms responsible for the maintenance of HSV latency have been extremely difficult to study. Recently, we reported that it is possible to establish and maintain HSV

latency in dissociated sympathetic neurons by inoculating with very low MOI, and we demonstrated that NGF is required for the maintenance of latency (38). In that study of HSV-1 infection of neuronal cultures, we found that the highest MOI that could be used which resulted in cultures surviving without any treatments was 0.03 PFU/cell, and of the 53% surviving cultures, only 12% reactivated after NGF deprivation (38). We also showed that, by adding anti-HSV serum immediately after the adsorption period and for 14 days subsequently, the MOI could be increased to 1. This procedure resulted in 63% of the cultures surviving, and in 53% of the surviving cultures reactivation of latent HSV occurred after NGF deprivation (38). In the present study, treatment with ACV 12 h before and for 7 days after virus inoculation allowed infection with an MOI of 1 (i.e., 300-fold more virus) without any evidence of lytic infections in the cultures. With this procedure, latent infections were established in 100% of the cultures. The studies using anti-HSV antiserum cannot be compared directly with the studies with ACV; the anti-HSV serum eliminates more residual virus and, thereby, actually results in less virus gaining access to the neurons. We suggest that ACV treatment does not potentiate latency in neurons, but rather prevents the lytic infection of the neurons by inhibiting viral gene expression,

FIG. 3. Immunohistochemistry, using anti-HSV antibodies, of HSV-1-infected cultures 24 h after treatment with 1% nonimmune or anti-NGF serum. Neuronal cultures were inoculated at an MOI of 1 with HSV-1 (115) in the presence of 50 μ M ACV. ACV was removed from the cultures after 7 days. Fourteen days after viral inoculation, the cultures were treated with nonimmune or anti-NGF serum. Twenty-four hours after the initiation of anti-NGF treatment, the cultures were fixed and processed for immunohistochemistry as described in the text. The photomicrographs shown are latent cultures 24 h after treatment with (A) nonimmune serum and (B) anti-NGF serum. Magnification, $\times 16$.

by preventing secondary infection from productively infected cells, or both.

The fact that the cultures were destroyed after infection with 20 PFU/cell, even in the presence of a high concentration of ACV, suggests a relationship between the MOI and whether the infection becomes lytic or latent. This effect may result from the number of virions which enter the cell and express their genes, or may be the result of the effects of virion-associated protein(s) on cellular metabolism or viral gene expression. Virion-associated proteins have been shown to shut down host cell protein synthesis (10, 21) and to enhance the expression of the immediate-early proteins (3, 24). There may be a critical concentration of virion protein(s) required to activate viral genes or act on the cell; above these concentrations the cell may be committed to a lytic infection.

The ability of the virus to establish latency in the presence of drugs which block viral DNA polymerase and the lack of detectable infectious virus suggest that viral DNA replication is not required for the establishment of latent HSV infections *in vitro*. Results from *in vivo* studies also indicate that viral DNA replication is not necessary for the establishment of latency. Temperature-sensitive mutants reported to be unable to replicate viral DNA at the nonpermissive body temperature are capable of producing latency (18, 37).

Specific immunohistochemical staining was evident on essentially all of the neurons by 24 h after addition of

TABLE 2. Percentage of survival and establishment of latent infections in neuronal cultures after infection with herpes simplex viruses

Virus ^a	% Survival ^b (no. of positive cultures/no. tested)	% Reactivation ^c (no. of positive cultures/no. tested) after treatment with:	
		Anti-NGF serum	Nonimmune serum
HSV-1 (F)	100 (72/72)	100 (36/36)	0 (0/36)
HSV-1 (HRM)	100 (16/16)	100 (8/8)	0 (0/8)
HSV-1 (KOS)	100 (16/16)	100 (8/8)	0 (0/8)
HSV-1 (McIntyre)	0 (0/16)		
HSV-2 (G)	100 (16/16)	100 (12/12)	0 (0/4)

^a Superior cervical ganglia neuron cultures were infected at an MOI of 1 in the presence of 50 μ M ACV. One week postinoculation, the drug was removed from the cultures.

^b Cultures were evaluated morphologically 2 weeks postinoculation. Additionally, culture supernatants were tested for infectious virus in plaque-forming assays.

^c Cultures were evaluated morphologically, and culture media were tested in plaque-forming assays. Cultures were considered positive for reactivation when both CPE and detectable infectious virus were present.

anti-NGF to the latent cultures. The few nonneuronal cells present in the cultures had no evidence of viral antigen until at least 72 h after the cultures were treated with anti-NGF. The appearance of viral antigens on the neurons within 24 h of the addition of anti-NGF is consistent with the expression of resident virus and is too early for secondary infections to have occurred (14). This indicates that most, if not all, of the neurons harbored latent virus. *In situ* hybridizations will be required to provide further support for this conclusion.

The detection of viral antigens 24 to 48 h before the detection of infectious virus has several possible explanations. The method used to test for infectious virus, examinations of culture supernatants, required that infectious virus be released into the culture medium; viral release is a late event compared to the synthesis of viral proteins. As expression of herpesvirus genes is temporally regulated (14), viral proteins are synthesized before the assembly of virions. In addition, because neurons are postmitotic cells, replication of the viral DNA in neurons may be slower than replication in dividing cells, where the machinery for DNA

TABLE 3. Effects of inhibitors of viral DNA polymerase on the percentage of cultures surviving and with latent virus after HSV inoculation

Drug ^a (concn)	% Survival ^b (no. of positive cultures/no. tested)	% Reactivation ^c (no. of positive cultures/no. tested) after treatment with:	
		Anti-NGF serum	Normal serum
Aphidicolin (10 μ M)	100 (72/72)	100 (36/36)	0 (0/36)
Phosphonoacetic acid (100 μ g/ml)	100 (24/24)	100 (12/12)	0 (0/12)

^a Neuronal cultures were treated with the drug at the concentrations indicated for 7 days after inoculation at an MOI of 1 with HSV-1 (115); thereafter, the drug was removed from the culture medium.

^b Cultures were evaluated 2 weeks postinoculation for viability based on neuronal morphology, and culture supernatants were tested in plaque-forming assays for infectious HSV.

^c At 2 weeks postinoculation, surviving cultures were treated with either 1% guinea pig anti-NGF serum or 1% nonimmune guinea pig serum. Cultures were evaluated for CPE and were harvested on day 5 after serum treatment to test for the presence of infectious virus by plaque-forming assays.

replication is present. In neurons it is possible that certain viral proteins may be expressed long before viral DNA is synthesized and virions are assembled.

Expression of viral genes during latency, particularly the immediate-early genes, has been suggested. Green et al. (11), using immunofluorescence with a monospecific anti-ICP4 antiserum, found evidence of viral antigens in rabbit trigeminal ganglia during latency. Spontaneous reactivations occur in rabbits at a high rate, making interpretation of these data difficult. Stevens and Cook (32) did not detect viral antigens during latency in the spinal ganglia of mice. However, recent data, obtained by more sensitive techniques, indicate that viral transcription originating from the region of viral DNA coding for the immediate-early gene IE-1 (ICP0) occurs during latency (7, 27, 33). Stevens et al. (33) found that the only transcript detectable during latency is complementary to ICP0 mRNA. That we were unable to detect viral proteins during the latent phase of the infection may indicate that the amount of protein(s) was too low to detect by immunohistochemistry, or the antisera did not contain antibodies against a restricted set of viral proteins. Alternatively, as suggested by Stevens et al. (33), the transcripts made during latency may not leave the nucleus. We are currently using *in situ* hybridization to examine cultures harboring latent HSV for evidence of expression of viral transcripts.

In summary, we have shown that HSV latency is readily established by both HSV-1 and HSV-2 in the presence of drugs which block viral DNA polymerase and that these viruses are dependent on the continuous presence of NGF for the maintenance of latency. Although progress has been made, the cellular and viral functions responsible for the establishment, maintenance, and reactivation of HSV latency are not well understood. Studying HSV infection in the isolated neuron *in vitro* may provide information regarding the regulation of the complex interactions between cellular and viral functions during HSV latency and reactivation.

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