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Herpesvirus quiescence in neuronal cells: Antiviral conditions not required to establish and maintain HSV-2 quiescence

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We previously described a novel *in vitro* model of a non-productive herpes simplex virus type 1 (HSV-1) infection in neurally differentiated (ND)-PC12 cells that allows for inducible virus replication upon forskolin and heat stress (HS) treatment. In this research, we further characterized the model with respect to HSV-2 strain 333. We found that: (i) ND-PC12 cells are non-permissive to HSV-2 replication; (ii) HSV-2 can establish a quiescent infection, like HSV-1, in ND-PC12 cells with the transient use of acycloguanosine (ACV); however unlike HSV-1, anti-viral conditions are not obligatory to establish and maintain a quiescent state; (iii) the quiescent state is maintained in the presence of Vero cell cocultivation indicating that such cultures are free of infectious virus; and (iv) a high percentage of quiescently infected (QIF)-PC12 cell cultures (80–100%) produce HSV-2 in response to forskolin and HS (43°C, 3 h) treatment for as long as 4 weeks post infection. These findings indicate that ND-PC12 cells can harbor HSV-2 in a cryptic and non-productive state that is reversible. This model has appealing features for studying gene expression during the establishment, maintenance and reactivation phases of the HSV-2 quiescent state in cell culture. *Journal of NeuroVirology* (2000) **6,** 296–302.

Keywords: PC12 cell culture model; herpes simplex virus; latency; reactivation

Introduction

Herpes simplex virus types 1 and 2 (HSV-1 and -2) are alphaherpesviruses with similar, but unique molecular (Kieff *et al*, 1971, 1972), biological and clinical features (reviewed in Whitley, 1996). The genomes are approximately 150 kb in size and each contains corresponding sets of 74 genes (Dolan *et al*, 1998). Both viruses infect epithelium, with HSV-1 having a predilection for orofacial sites and HSV-2 preferentially infecting genital surfaces. During the primary infection, HSV invades local nerve endings and travels to sensory ganglia where it can colonize neuronal nuclei and establish a latent state (Hill *et al*, 1972; Stevens and Cook, 1971). Reactivation of HSV from latency occurs intermittently as a result of stressful stimuli (e.g., trauma and heat). Reactivated virus are responsible for causing recurrent epithelial infections that can occur in up to 89% of infected individuals (Benedetti *et al*, 1994).

The lack of a universally accepted neural cell culture model that supports HSV latency restricts our understanding of the molecular events involved in reactivation from latency. Although animal models reproduce certain aspects of HSV-2 latency in humans (Al-Saadi *et al*, 1988; Bourne *et al*, 1994; Croen *et al*, 1991; Krause *et al*, 1995; Kurata *et al*, 1978; MacLean *et al*, 1991; Martin and Suzuki, 1989; Mitchell *et al*, 1990; Stanberry *et al*, 1982; Stephanopoulos *et al*, 1988; Wang *et al*, 1997; Yoshikawa *et al*, 1996), limitations in these models make interpretation of reactivation data challenging. Tissue and cell culture models of HSV-2 latency have been developed in an attempt to overcome limitations of animal models. However, antiviral conditions have been required to maintain and/or establish the latent-like state (Colberg-Poley

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et al, 1979, 1981; Harris *et al*, 1989; Kondo *et al*, 1990; O'Neill, 1977; O'Neill *et al*, 1972; Russell *et al*, 1987; Russell and Preston, 1986; Wigdahl *et al*, 1981; Wilcox and Johnson, 1988; Wilcox *et al*, 1990; Yura *et al*, 1986). Development of a cell culture model with neuronal characteristics that lacks these restrictive requirements would be advantageous for understanding the molecular mechanisms of HSV reactivation.

Previously, we reported that neurally differentiated (ND)-PC12 cells can harbor HSV-1 in a quiescent, yet reversible state (Danaher *et al*, 1999a). These quiescently infected ND-PC12 cultures (QIF-PC12) demonstrate forskolin- and heat stress (HS)-inducible virus production in a high percentage (50–100%) of cultures for up to 8 weeks after infection, whereas mock-induced cultures maintain the quiescent viral state in the majority of infected cultures (Danaher *et al*, 1999b). In this study, we demonstrate that ND-PC12 cells permit establishment of an HSV-2 quiescent state, like HSV-1, following transient acycloguanosine (ACV) treatment. Unlike HSV-1, antiviral conditions are not required for the establishment of the HSV-2 quiescent state. In addition, we found that quiescent cultures could be maintained in the presence of Vero cells, and the presence of Vero cells enhanced the sensitivity to detect HSV-2 produced spontaneously and following induction (i.e., forskolin and HS treatment). These findings indicate that ND-PC12 cells can harbor HSV-2, like HSV-1, in a cryptic and non-productive state that is reversible, and this model has appealing features for studying gene induction during activation of HSV-2 from a non-productive state.

Results

HSV-2 establishes a quiescent infection in ND-PC12 cells that is reversible

Preliminary data in our laboratory indicated that QIF-PC12 cell cultures established with HSV-2 produce virus following heat stress and forskolin induction (data not shown). However, unlike previous findings with HSV-1, minimal amounts of virus (i.e., only a single plaque forming unit) were detected in the majority of QIF-PC12 cell cultures determined to be positive for HSV-2 production following induction. Furthermore, cocultivation of HSV-2 established QIF-PC12 cell cultures with Vero cells did not increase the proportion of cultures producing virus, but increased the amount of virus detected from such cultures. This indicated that HSV-2 was not induced from QIF-PC12 cell cultures by cocultivation with Vero cells, and Vero cells could be used to increase the sensitivity of the system (see below).

Based on these findings, we analyzed in more detail forskolin and HS induced HSV-2 reactivation from QIF-PC12 cell cultures that were cocultivated with Vero cells. QIF-PC12 cell cultures were established with strain 333 at MOI of 10 as described in the Materials and methods using transient ACV treatment. Cultures were cocultivated with Vero cells at a ratio of 1 : 1 three days before induction with forskolin or HS (day 15 p.i.). Virus production was monitored from day 10 through 23 p.i. Prior to induction, HSV-2 was detected infrequently (2.8%; 1/36) in cocultivated cultures. The 35 cultures that were not shedding virus were used in induction assays. Following induction treatment (Figure 1), virus was detected in 90 to 100% of HS and forskolin induced cocultivated cultures, and 8.3% (1/12) of mock-induced cocultivated cultures. These data indicate that quiescently infected cultures can be established with HSV-2 and virus production can be induced by both physical and chemical stimuli.

The possibility that Vero cells influenced HSV induction from QIF-PC12 cell cultures was assessed in two ways. First, parallel neuronal cultures established with HSV-2 were cocultivated with Vero cells 2 days after induction with forskolin. This allowed for comparison of virus production occurring before and after cocultivation. Second, induction of HSV-1 established cultures was analyzed in the presence and absence of Vero cell cocultivation, as detection of HSV-1 progeny from such cultures does not require Vero cells (Danaher *et al*, 1999a). In HSV-2 established cultures (Figure 1), virus was detected in a similar proportion of cultures by day 4 post-forskolin treatment whether cultures were cocultivated before or after induction.

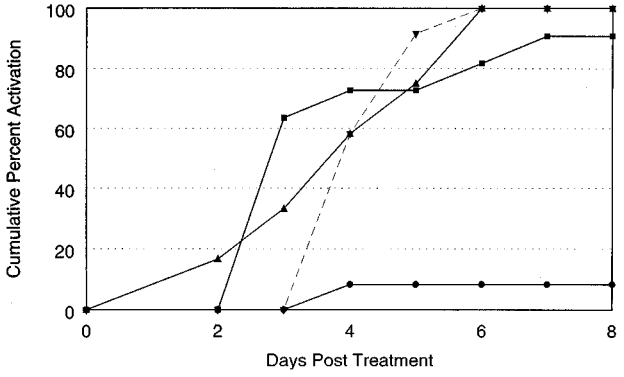

Figure 1 Induction of HSV-2 from QIF-PC12 cell cultures. QIF-PC12 cell cultures were established with HSV-2 strain 333 at MOI of 10 with the transient use of ACV as described in the Material and methods. Cultures were cocultivated with Vero cells at a 1 : 1 ratio 3 days before induction. Nonproductive cultures were subjected to 50 μM forskolin (▲), HS (43°C, 3 h) (■, or mock-induction (●) on day 15 p.i. Parallel cultures were also cocultivated with Vero cells 2 days after the forskolin induction (- - -▼- - -). Cultures were monitored for virus production using culture supernatants in the direct plaque assay. Virus was detected in 2.8%; (1/36) of cultures cocultivated before induction and 0% (0/12) of cultures cocultivated after induction between day of ACV withdrawal (day 8 p.i.) and day of induction.

This indicated that the Vero cells did not contribute to induction of HSV-2, but merely allowed for detection of progeny virus. Results shown in Figure 2 demonstrate that cocultivation of HSV-1 established QIF-PC12 cell cultures with Vero cells did not induce HSV-1 production or alter the efficiency of the response to forskolin and HS. Therefore, the findings from these two experiments demonstrate that cocultivation with Vero cells did not contribute to the reactivation response. Furthermore, HSV-1 and -2 established QIF-PC12 cell cultures can maintain a non-productive state in the presence of Vero cells. This indicates that low amounts of infectious virus are not being chronically shed and the cultures are truly quiescent.

Long-term HSV-2 quiescent infection in PC12 cells cocultivated with Vero cells

The ability of HSV-2 to maintain a short-term quiescent infection in ND-PC12 cells suggested such a state could be maintained long-term in the presence of Vero cell cocultivation. To assess this, QIF-PC12 cell cultures were established with HSV-2 as described above. Cultures were cocultivated with Vero cells at a ratio of 1 : 1 within 10 days of ACV withdrawal. Induction was performed with forskolin on day 30 p.i. Cultures were monitored for virus production using culture supernatants. Over the 3 week period between ACV withdrawal and induction, 71% (17/24) of the HSV-2 infected cultures maintained quiescence. HSV-2 was produced from 100% (8/8) of forskolin induced cultures and 0% (0/9) of mock induced cultures by 8 days post-induction (data not shown). These data indicate that long-term HSV quiescence can be maintained in QIF-PC12 cell cultures in the presence of Vero cell cocultivation and absence of ACV, and these cultures reactivate virus when induced with forskolin 30 days p.i.

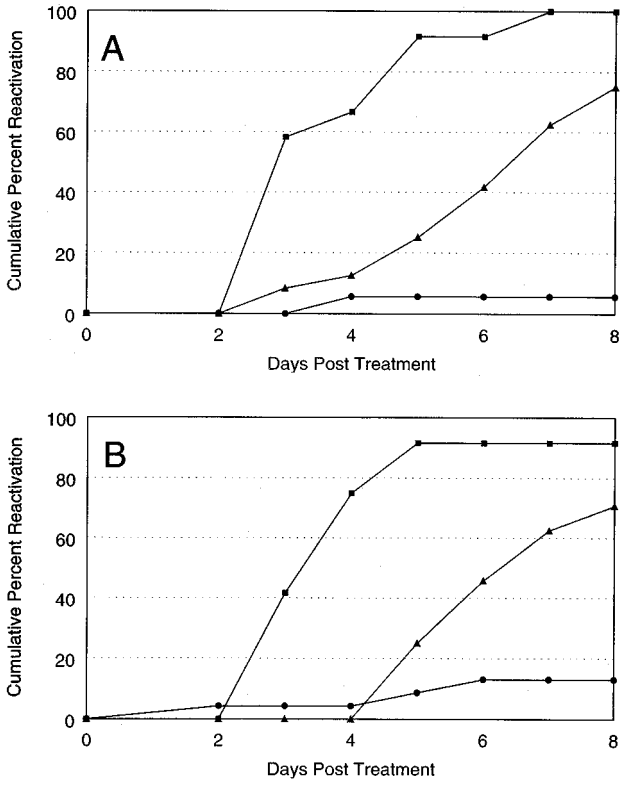

Figure 2 Effect of cocultivation on induction of HSV-1 from QIF-PC12 cell cultures. QIF-PC12 cell cultures were established with HSV-1 strain 17^+ at MOI of 1 with the transient use of ACV as described in the Material and methods. Cultures were either mock cocultivated (**A**), or cocultivated with Vero cells at a 1 : 1 ratio 3 days before induction (**B**). Nonproductive QIF cultures were subjected to 50 μM forskolin (▲), HS (43°C, 3 h) (■), or mock-induction (●) on day 15 p.i. Cultures were monitored for virus production using culture supernatants in the direct plaque assay. Virus was detected in 0% (0/72) mock cocultivated and 1.7% (1/60) Vero cocultivated cultures between the day of ACV withdrawal (day 8 p.i.) and day of induction.

Reactivation of HSV-2 is MOI dependent

We next determined whether the efficiency of HSV-2 activation from quiescence was MOI-dependent. QIF-PC12 cell cultures were established with HSV-2 strain 333 at MOIs of 3 and 30 and cocultivated with Vero cells on day 12 p.i. as above. Nonproductive cultures harboring a quiescent infection on day 15 p.i. were subjected to forskolin (50 μM) or mock induction. Virus production was monitored using cultured supernatants as described above. Between the period of ACV withdrawal and induction, virus was detected in 4.2% (1/24) of cultures for each MOI. Figure 3 shows that viral MOI influenced the efficiency of HSV-2 activation from quiescence. Cultures infected at MOI of 30 activated virus at a higher rate (i.e., 1 – 2 days faster) and degree (100%; 12/12 by day 6) in response to forskolin, than cultures infected at MOI of 3 (82%; 9/11 by day 7).

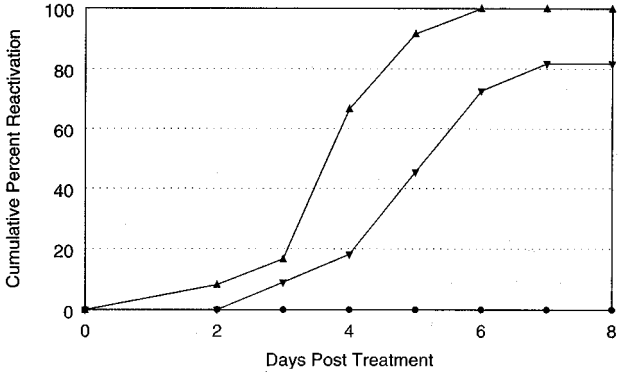

Figure 3 Reactivation of HSV-2 is MOI-dependent. QIF-PC12 cell cultures were established with HSV-2 strain 333 at MOIs of 3 and 30 as described in Figure 1. Cultures were cocultivated with Vero cells at a ratio of 1 : 1 on day 12 p.i. Nonproductive cultures were subjected to 50 μM forskolin (▼, MOI=3), (▲, MOI=30), or mock-induction (●) on day 15 p.i. Cultures were monitored for virus production by direct plaque assay of supernatants. Virus was detected in 4.2% (1/24 at each MOI) of cultures between day 8 p.i. (ACV withdrawal) and day of induction.

In the mock-induced QIF-PC12 control cultures, spontaneous virus production was not detected (0%; 0/11, 0/12) at either MOI. These data indicate that the efficiency of forskolin induced HSV-2 activation from QIF-PC12 cell cultures is MOI-dependent.

ACV is not required for the establishment of a quiescent infection with HSV-2

Since HSV-2 was found to be non-permissive in ND-PC12 cells (data not shown), we assessed the ability of HSV-2 to establish a quiescent infection without the use of ACV. QIF-PC12 cell cultures were established with HSV-2 strain 333 in the absence of ACV in parallel to the above experiment. Following infection, the culture media was changed daily for 3 days p.i. and thereafter every 2 to 3 days until induction. The majority of cultures maintained quiescence at MOI of 3 (88%, 21/24) and 30 (79%, 19/24) until the day of induction (day 15 p.i.). Figure 4 shows that HSV-2 was produced from 91% (10/11) of forskolin induced and 10% (1/10) of mock induced cultures established at MOI of 3 by day 6 post-induction. Parallel cultures established at MOI of 30 demonstrated a similar level of forskolin induced reactivation (90%, 9/10) and slightly higher (22%, 2/9) spontaneous virus production than cultures established at MOI of 3 (data not shown). In addition, the mock induced MOI of 3 cultures that were non-productive on day 23 were maintained for an additional 7 days, and on day 30 p.i. were mock or forskolin induced. Virus was recovered from 100% (4/4) of forskolin induced cultures and 0% (0/4) of mock induced cultures. These data indicate that HSV-2 can establish a long-term (i.e., 30 days) quiescent state in ND-PC12 cell cultures without the use of ACV, and virus reactivation results from forskolin induction.

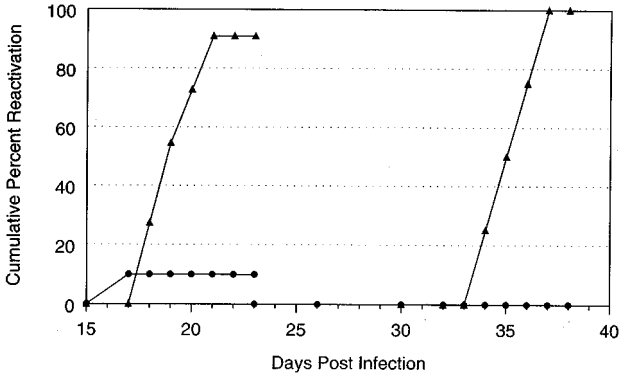

Figure 4 Reactivation of HSV-2 from QIF-PC12 cell cultures established without ACV. QIF-PC12 cell cultures were established with HSV-2 strain 333 at MOI of 3 without the use of ACV as described in the text. Cultures were cocultivated with Vero cells at a ratio of 1:1 on day 12 p.i. Nonproductive cultures were subjected to 50 μM forskolin (▲) or mock-induction (●) on days 15 and 30 p.i. Cultures were monitored for virus production by direct plaque assay of supernatants. Virus was detected in 3/24 (12%) of cultures between day 8 p.i. and day of initial induction.

Discussion

The mechanisms that govern HSV-2 reactivation from latency remain unknown. An obstacle to understanding HSV-2 latency and reactivation has been the lack of *in vitro* models that permit analysis of HSV-2 quiescence and induction at the neuronal and clonal cell level. In this study we utilized our model for HSV-1 quiescent infection in ND-PC12 cells to investigate quiescent and reactivation properties of HSV-2. The data presented here demonstrate that: (1) cocultivation of QIF-PC12 cell cultures with Vero cells does not induce virus production; (2) such cultures can be induced to produce virus following both physical and chemical stimuli; (3) the presence of Vero cells is not required for induction but rather serves to increase the sensitivity of the assay; (4) reactivation from HSV-2 established cultures is MOI-dependent; and (5) the use of antiviral conditions are not required to establish QIF-PC12 cell cultures with HSV-2.

We found that Vero cells did not signal virus production from HSV infected non-productive-PC12 cells. This permitted us to exploit the permissive nature of Vero cells to enhance sensitivity of the system to study HSV-2 induction. Induction assays were performed only using cocultivated cultures that were not shedding virus. As expected from our preliminary data, virus was detected from only a limited proportion of mock induced cocultivated QIF-PC12 cell cultures. In contrast, virus was readily detectable from the majority of cocultivated QIF-PC12 cell cultures following forskolin and HS induction. It is important to note that a similar proportion of cultures produced virus by day 4 whether cultures were cocultivated before or after induction. Furthermore, induction from control QIF-PC12 cell cultures established with HSV-1 was similar whether or not cultures were cocultivated with Vero cells. Together these findings indicate that Vero cells do not contribute to the induction of HSV-2 from the quiescent state in our model.

The studies presented here demonstrate that HSV-2 has quiescent and activation properties following induction by forskolin and HS similar to those described here and previously for HSV-1 (Danaher *et al*, 1999a,b). Virtually all cultures transiently treated with ACV permitted establishment of HSV-2 quiescence. Virus production following induction was MOI-dependent. That is, QIF-PC12 cell cultures established with ACV at

higher MOI yielded HSV-2 sooner and from a higher proportion of cultures following induction than those established at a lower MOI. A majority of these cultures maintained HSV-2 in a quiescent state for at least 30 days and virus was inducible thereafter with forskolin.

An important finding was that antiviral conditions were not required to establish a HSV-2 quiescent state in ND-PC12 cell cultures. The establishment of quiescence under these conditions was favored at lower MOI. These findings suggest that the acute HSV-2 infection is self-clearing in the majority of ND-PC12 cell cultures and inhibitory viral growth conditions are not required to maintain a quiescent state, even for weeks, when cultures are cocultivated with Vero cells. The ability of HSV-2 to establish a quiescent state without the requirement of antiviral agents is likely the result of the relative non-permissivity of ND-PC12 cells for HSV-2 growth (data not shown).

The fact that Vero cell cocultivation did not stimulate HSV-1 production from QIF-PC12 cell cultures is in contrast to the results of Su *et al* (1999) who found that African green kidney monkey (CV-1) cells induced stimulation of HSV-1 from quiescently infected PC12 cell cultures. Although the reason(s) for these contrasting results are not altogether clear, the difference could be the result of contrasting methods used to establish quiescence. To be specific, the method of infection, use of serum-free conditions, and the temporal presence of ACV could provide a cryptic HSV state unique from that reported by Su *et al* (1999). Also, the cell type used for cocultivation could be important, as Su *et al* demonstrated that the ability to induce HSV is cell line specific.

In conclusion, this report presents data that extends our knowledge of the characteristics and utility of the ND-PC12 cell model for study of the quiescent HSV state. The model finds its basis in the development of other successful cell culture models (Block *et al*, 1994; Hammer *et al*, 1981; Harris and Preston, 1991; O'Neill *et al*, 1972; Russell *et al*, 1987; Scheck *et al*, 1989; Wigdahl *et al*, 1982). We suggest this model represents a refinement, and molecular studies are underway that will determine viral gene expression during quiescence and reactivation. Advantages of the QIF-PC12 cell culture model include: (1) establishment and maintenance of a HSV-2 quiescent infection in a high proportion of PC12 cell cultures with and without the transient use of ACV; (2) the ability to produce HSV-2 from a quiescent state in response to forskolin and HS treatment for as long as 4 weeks p.i.; and (3) the ability to discriminate between quiescence, spontaneous reactivation and inducible reactivation using a range of MOIs. These features should enable the analysis of reactivation events of a cryptic HSV genome at the single neural cell level *in vitro*.

Material and methods

Virus and cells

Rat pheochromocytoma (PC12) and Vero (African green monkey kidney) cells were obtained from ATCC (Rockville, MD, USA). All culture media and supplements were purchased from Gibco BRL (Gaithersburg, MD, USA) unless otherwise indicated. PC12 cells were grown in RPMI 1640 media containing 5% fetal bovine serum (FBS) and 10% heat-inactivated horse serum. Vero cells were grown and maintained in M199 medium containing 5% FBS. Cells were incubated at 37°C in a humidified incubator with 5% CO_2. All media was supplemented with penicillin (100 units/ml) and streptomycin (100 μg/ml). HSV-1 strain 17^+ was a kind gift of N Fraser (Wistar Institute, Philadelphia, PA, USA). HSV-2 strain 333 was a kind gift from Philip R Krause (Food and Drug Administration, Bethesda, MD, USA). Viral stocks were prepared in Vero cells and maintained at -85°C.

Morphologic differentiation

For morphologic differentiation, PC12 cells were maintained in RPMI 1640 supplemented 0.1% bovine serum albumin, fraction V (BSA) and 50 ng/ml of 2.5S mouse nerve growth nerve factor (NGF) (Becton Dickinson) (maintenance media) throughout the studies, unless indicated, beginning on the day of plating. PC12 were plated, following two rinses with RPMI 1640 containing 0.1% BSA and dissociation by passage through a 22-gauge needle, in 12-well tissue culture dishes (Becton Dickinson Labware, Franklin Lakes, NJ, USA) coated with rat tail collagen type 1 (Becton Dickinson) at 1.1×10^5 cells/well in maintenance media. Collagen was applied as recommended by the supplier. Following 4 days of differentiation in maintenance media, cultures were maintained in RPMI 1640 supplemented with 10% horse serum (heat-inactivated) and 5% FBS, and 50 ng/ml NGF for 2 days. The following day cultures received maintenance media supplemented with 100 μM acycloguanosine (ACV) when indicated, purchased from Sigma (St. Louis, MO, USA). Morphologic differentiation was confirmed by microscopic visualization of dendritic processes. Media was changed every 2 to 3 days unless indicated.

Establishment of a quiescent infection

Neurally-differentiated PC12 cells (ND-PC12) were infected in a volume of 0.4 ml/well in 12-well plates without agitation at the indicated multiplicity of infection (MOI) overnight at 37°C. When used, ACV was maintained in the medium from 1 day prior to infection 8 days post-infection (p.i.). After ACV withdrawal, a quiescent state (i.e., free of detectable infectious virus in culture supernatants) was maintained for at least 7 days prior to induction. At the indicated times, Vero cells were trypsinized,

washed twice with RPMI and introduced into the QIF-PC12 cell cultures at a ratio of 1:1 in maintenance media.

Induction stimuli and assay of virus production
HSV QIF-PC12 cells, that were free of detectable infectious virus, were subjected to heat stress (43°C for 3 h in an incubator), or maintenance media supplemented with or without 50 μM forskolin (Sigma) as previously described (Danaher *et al*, 1999a,b). Virus production was determined using 25% volume of supernatants from infected PC12 cultures in a direct plaque assay (DPA) on monolayers of Vero cells as previously described (Miller and Smith, 1991). Cultures were subsequently replenished with fresh maintenance media.

Acknowledgements

Citations Danaher *et al*, 1999a and Danaher *et al*, 1999b are the first two of this series. We thank Katja Dohrendorf and Steve Lubbe for their technical support, and James M Hill for technical reading. This work was supported by National Institute of Dental and Craniofacial Research, National Institutes of Health, grant DE11104 to CS Miller.

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