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Establishment of a quiescent herpes simplex virus type 1 infection in neurally-differentiated PC12 cells

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Rat pheochromocytoma (PC12) cells differentiated with nerve growth factor (Nd-PC12) were used to investigate the establishment of a non-productive herpes simplex virus type 1 (HSV-1) infection that is reversible. The results of this work are as follows: (i) Nd-PC12 cultures could be maintained as long term (>7 weeks) non-dividing cultures only when plated on collagen-coated dishes in the absence of serum; (ii) Infection of Nd-PC12 with HSV-1 strains KOS and 17 in the transient presence of acycloguanosine (ACV) resulted in all cultures free of detectable levels of infectious virus at the time of ACV removal and ACV was not needed to maintain the non-productive quiescent state in the subsequent 8 weeks; (iii) These persistently infected and quiescent (QIF)-PC12 cultures demonstrated both spontaneous and forskolin-inducible virus production, at low (5%) and high frequencies (92–100%), respectively during the first 2 weeks post-ACV withdrawal. (iv) In contrast to other *in vitro* models, HSV-1 failed to reactivate following removal of nerve growth factor. (v) A high percentage of QIF-PC12 cultures (50–100%) produced virus in response to forskolin treatment as long as 7 weeks post-ACV withdrawal. (vi) Expression of HSV-1 productive genes (i.e. α0, α4, α27, U_L30 and U_L18) dropped precipitously in the presence of ACV and remained undetectable or continued to decline following its removal, whereas the levels of LAT and the host gene G3PDH remained relatively constant throughout the 31 day study period as measured by RT–PCR. These results indicate that QIF-PC12 cells offer a novel, neuronal cell culture system that may enhance our ability to study HSV-1 reactivation from a cryptic, latent-like, non-productive state in the absence of replication inhibitors.

Keywords: cell culture model; herpes simplex virus, viral latency

Introduction

The lack of a universally accepted neural cell-line that supports HSV-1 latency has restricted our understanding of the molecular events involved in reactivation from latency. As a result, animals and tissue culture have served to provide an understanding of the mechanisms of this event. Animal models, however, are limited by difficulties. These include: (i) latency and reactivation events that are influenced by viral strains with different primary growth phenotypes; (ii) the limited number of neurons latently infected in animal models (Bloom *et al*, 1996; Hill *et al*, 1996; Maggioncalda *et al*, 1996; Mehta *et al*, 1995; Ramakrishnan 1994; Sawtell, 1997; Sawtell *et al*, 1998; Thompson and Sawtell, 1997), and (iii) inaccurate quantitation of reactivation events when measuring virus production at the recurrent site as a result of influences of transport, replication in epithelium, and the immune response.

A major advantage of tissue and cell culture models includes the ability to observe virus at the single cell level without the overlay of immunological events that modulate the eventual appearance of virus in the host. Tissue culture models derived from neuronal and sympathetic ganglia have properties of the *in vivo* system including: (i) restricted transcription of the HSV genome (Doerig *et al*, 1991;

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Halford *et al*, 1996; Smith *et al*, 1992, 1994); (ii) lack of virus production following removal of the inhibitory agent (Wilcox and Johnson, 1988); (iii) the presence of latency-associated transcripts (LATs) (Doerig *et al*, 1991; Smith *et al*, 1994); (iv) impaired reactivation of thymidine kinase negative virus (Wilcox *et al*, 1992), and (v) inducible reactivation (Halford *et al*, 1996; Moriya *et al*, 1994; Smith *et al*, 1992; Wilcox and Johnson, 1987, 1988; Wilcox *et al*, 1990). Nevertheless, preparation of dissected ganglia is inconvenient, material is limited, animal use is required, and axotomy introduces traumatic factors that influence reactivation of virus.

For the above reasons, cell culture models are important for studying the molecular details of the establishment, maintenance and reactivation stages of latency. Cell culture also allows for an unlimited supply of a defined host cell and the ability to manipulate genetic material. Over the past 25 years, cell culture systems using fibroblast cultures (Harris and Preston, 1991; Jamieson *et al*, 1995; O'Neill, 1977; O'Neill *et al*, 1972; Russell *et al*, 1987; Scheck *et al*, 1989; Wigdahl *et al*, 1982a,b, 1983) and lymphocytes (Hammer *et al*, 1981; Youssoufian *et al*, 1982) have enabled the study of HSV-1 during a latent-like state. These models, however required low input multiplicities and/or the use of replication inhibitors such as anti-viral agents, inhibitory temperatures, or the use of a mutant virus, to prevent virus production.

A cell line that has neural morphology and physiology, can survive infection and permit HSV-1 production, allow establishment of a long term nonproductive HSV-1 infection, and support HSV-1 in a state suitable for reactivation studies would be more desirable. The rat pheochromocytoma (PC12) cell line is a potential candidate because they are of neural crest origin and can be morphologically differentiated with the addition of nerve growth factor (Greene and Tischler, 1976). These cells have been shown to be permissive to HSV-1 infection (Bloom and Stevens, 1994; Rodahl and Haar, 1997; Rubenstein and Price, 1983a,b, 1984), and have been used to examine HSV-1 gene regulation and expression (Frazier *et al*, 1996a,b; Jordan *et al*, 1998; Leib *et al*, 1991; Xie *et al*, 1996) and the function of HSV origins of DNA replication (Hardwicke and Schaffer, 1997). The relevance of these studies, however remains incomplete since the ability of these cells to harbor HSV-1 in a 'latent-like' state has not been demonstrated (Block *et al*, 1994).

In this report, we demonstrate that neurally-differentiated PC12 cells can be infected with HSV-1 in a manner that supports a long term non-productive infection. This model when compared to others is unique in that (1) quiescent infection is established in a neuronal cell line that has features reminiscent of ganglionic neurons; (2) an artificial inhibitor of DNA synthesis is needed only to establish the quiescent state, not maintain it; (3) an inhibitor of HSV-1 gene expression is not required to establish the quiescent state; (4) the non-productive state is reversible with spontaneous and inducible reactivation, and (5) quiescence is long term. This model should serve as a suitable system for studies of HSV-1 reactivation from a non-productive state.

Results

Conditions for maintenance of long term, non-dividing differentiated PC12 cultures

Previous studies have demonstrated NGF-differentiated PC12 (Nd-PC12) cells can be maintained as non-dividing cultures both in the presence and absence of serum (Greene, 1978; Greene and Tischler, 1976). These cultures have been studied on non-coated (Block *et al*, 1994) and collagen coated dishes (Greene and Tischler, 1976). Preliminary studies in our laboratory, however, indicated that a significant portion of PC12 cells cultured in the presence of serum continued to divide and is consistent with the findings of others (Goodman *et al*, 1979; Ignatius *et al*, 1985). Because a differentiated, non-dividing and adherent cell population was desired, we first determined whether continued growth of cells was the result of high plating density that may have exhausted the NGF supplied (Gunning *et al*, 1981). To make this determination, cells were plated at two different densities on collagen and non-collagen coated dishes. Cells plated at low density were NGF

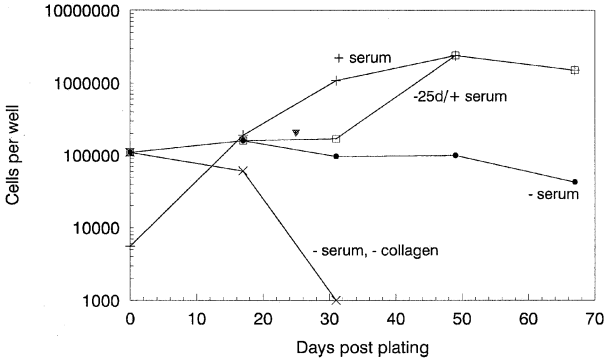

Figure 1 Growth characteristics of long-term cultures of NGF differentiated PC12 cells. PC12 cells were plated at 5.5×10^3 cells (low density) and 1.1×10^5 cells (standard density) per 3.8 cm^2 well in RPMI 1640 media with serum in 12-well culture plates. NGF differentiation was initiated by the addition of NGF (50 ng/ml) in RPMI+serum or RPMI+BSA (−serum) for low and standard cell density plates, respectively, 2 days after plating. NGF was maintained throughout the duration of the assay and culture medium was replaced every 2 days. On day 25, post-plating, select (−serum) cultures were switched to RPMI+serum+NGF (−25d/+serum). Cultures maintained on non-collagen coated plates are indicated (−collagen). Each data point represents the average number of cells/well from duplicate wells.

differentiated in the presence of serum (+serum) whereas those plated at standard density were differentiated in the absence of serum (−serum). Figure 1 shows that Nd-PC12 cells proliferated under serum containing conditions. In collagen coated wells containing media supplemented with serum, the cell density increased more than 30-fold (i.e. from 5.5×10^3 to 1.9×10^5 cells per well) by 17 days post-plating (p.p.) with an additional 12-fold increase (to 2.4×10^6 cells per well) in the following 32 days. The cell density of parallel cultures grown on non-coated plates also continued to divide maintaining cell densities of about 50% of those cultured on collagen-coated dishes (data not shown). When Nd-PC12 cells were grown on coated dishes in the absence of serum (−serum) a relatively constant cell number was maintained for 7 weeks with a subsequent loss of about 50% of the cells during the following 18 days. As shown, parallel cultures maintained in serum free conditions did not adhere well to non-coated dishes. The addition of serum to minus-serum Nd-PC12 cultures on day 25 p.p. resulted in a 70% increase in cell number within 6 days and an additional 14-fold increase in the following 18 days achieving equivalent cell densities as +serum cultures. These data demonstrate that a relatively constant number of Nd-PC12 cells could be maintained as non-dividing cultures only when cultured in the absence of serum and that cell growth arrest is reversible upon the addition of serum to the media for up to 25 days p.p.

Establishment of a quiescent nonproductive infection

To determine if Nd-PC12 cultures could harbor HSV-1 in a non-replicating state, Nd-PC12 cells were established in serum free conditions. Cells were infected in the presence of acycloguanosine (ACV) on day 7 or 15 following initiation of neural differentiation by NGF with KOS at MOI of 27 ± 3 to favor infection of every cell. Cultures were supplemented with ACV since cultures infected in the absence of ACV continued to produce virus for greater than 2 months (unpublished observation; Block *et al*, 1994). ACV was maintained from day −1 to +9 p.i. as described by Wilcox and Johnson (1988). Under these conditions, all cultures were free of detectable levels of infectious virus in the supernatants at the time of ACV withdrawal and virus was detected in only 4% of cultures during the 8 days following ACV withdrawal.

To determine whether virus was present in a recoverable form during the period following drug removal, cells maintained in NGF and serum-free media were subjected to forskolin, an activator of adenylate cyclase known to reactivate virus from latently infected tissue (Smith *et al*, 1992). Figure 2 shows that forskolin treatment resulted in detectable virus in 92% of 7-day Nd-cultures and 100% of 15-day Nd-cultures within 9 days post-treatment,

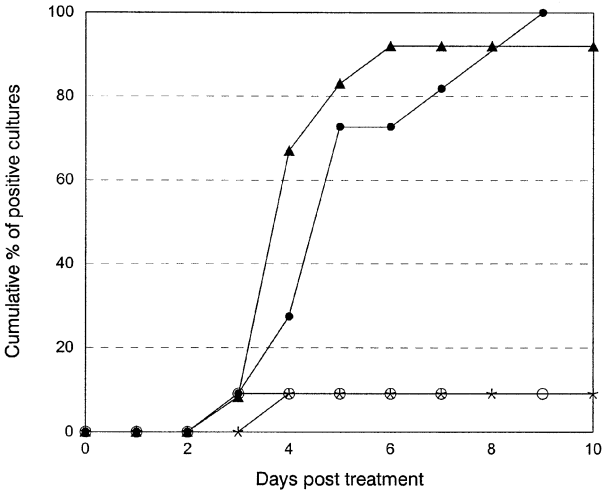

Figure 2 Induced HSV-1 production from quiescent-infected PC12 cell cultures. PC12 cells were plated at 1.1×10^5 cells/$3.8\,cm^2$ well and maintained in RPMI supplemented with BSA and NGF in 12-well plates. Media was replaced every 2 days. Cultures were infected with KOS at a MOI of 27 ± 3 on day 7 and 15 following the initiation of neural differentiation with NGF. ACV (50 μM) was maintained in the medium from −1 day to 9 day p.i. Cells were treated with forskolin (50 μM) on day 8 post-ACV withdrawal. Culture medium was analyzed for released virus daily by direct plaque assay and replaced with fresh medium every 2–3 days. Control cultures infected on day 7 and day 15 p.p. are represented by (⋆ and ○), respectively. Forskolin-induced cultures infected on day 7 and 15 p.p. are represented by (▲ and ●), respectively.

compared to only one positive well (9%) in the untreated infected control sets. Cytopathic effect was not seen microscopically in these reactivating cultures. These findings indicate that a quiescent, nonproductive HSV-1 infection can be established in PC12 cells that have undergone a 7 or 15 day neural differentiation period. In addition, virus persists in Nd-PC12 cells in a recoverable state, and reactivation may be occurring in only a minority of the infected cells.

Reactivation from long-term (50 days) HSV-1 infected, quiescent PC12 cultures

To determine whether Nd-PC12 cells could harbor HSV-1 in a recoverable state for long periods of time following removal of ACV, Nd-PC12 cultures were infected with strain KOS at a MOI of 27 ± 3 on day 15 p.p. and maintained as quiescently infected cultures (QIF-PC12) as described in Figure 3. Under these conditions, all cultures were free of detectable levels of infectious virus in the supernatants at the time of ACV withdrawal. Virus was detected in only one of 35 cultures during the following 8 days, and virus was not detected from total cell lysates of four infected control wells on day 16 post-ACV withdrawal (data not shown). To determine if NGF withdrawal would induce virus production, as described by Wilcox and Johnson (1987), NGF was withdrawn on day 8 post-ACV withdrawal. Virus

was detected by day 8 post-treatment in 100% of forskolin-induced control cultures, 8% NGF-free cultures, and 5% of infected untreated control cultures (Figure 3A). Except for the detection of virus in two of the remaining 14 infected untreated control wells on day 17 post-ACV withdrawal, virus was not detected in infected control wells for the remaining 39 days. To determine whether these virus negative (non-producing) cultures were still capable of producing virus, four of 12 cultures were treated with forskolin on day 38, and four of eight were treated with forskolin on day 49 post-ACV withdrawal. Induction with forskolin at these later dates resulted in the detection of virus in 75% and 50% of cultures, respectively, compared with 0% in the remaining infected untreated control cultures (Figure 3B and C). These data indicate that virus can be recovered from long-term QIF-PC12 cultures when induced with forskolin.

Detection of viral transcripts during the establishment and maintenance of the quiescent phase of QIF-PC12 cultures

To study viral gene expression during the establishment and maintenance phases of a quiescent infection, QIF-PC12 cells were established under conditions that minimize the effects of high copy number of viral genomes. This was achieved using strain 17 because it reactivates more efficiently in this model than KOS (data not shown). The infections were performed at an MOI of 0.5. The rate of virus reactivation from QIF-PC12 cells established under these conditions are presented in a subsequent manuscript (Danaher *et al*, 1999).

RNAs from duplicate QIF-PC12 cell cultures were collected at the indicated time points throughout the 31 day period and analyzed by RT–PCR in duplicate. The specificity of the RT–PCR products were confirmed by Southern blot analysis. Primers and probes used in this analysis are shown in Table 1. Figure 4 shows representative results from one of four samples from each time point examining the viral and host genes (G3PDH gene). A precipitous decline in transcripts occurred from the selected representatives of the three classes of the herpes virus genes (i.e. immediate early [α], early [β], late [γ]) tested following day 1 p.i. Detection of transcripts from α genes extended to as long as 10 days after ACV removal (i.e. 20 days p.i.), as typified by the α4 product. Transcripts from the U_L30 (DNA polymerase gene), representing the β genes, became undetectable by the day of ACV withdrawal (i.e. day 5 p.i.). Gamma genes, as represented by U_L18 (VP23), were below the level of detection by day 10 p.i. In contrast to the precipitous decline in all classes of viral gene transcripts represented, the stable LAT transcript persisted, although somewhat reduced compared to day 1 p.i. levels, for the length of the assay. From Figure 4, it is apparent that the products of the representative viral genes, except for LAT, diminished significantly throughout the 31 day period assayed. Expression of the host cell gene G3PDH was constant throughout the time period measured.

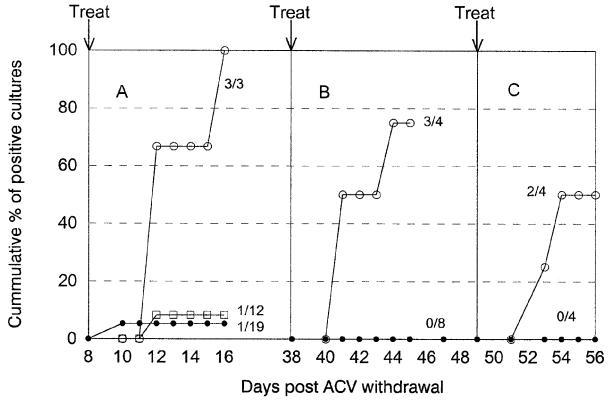

Figure 3 Induction of HSV-1 from long-term QIF-PC12 cells. PC12 cells were plated at 1.1×10^5 cells/3.8 cm^2 well in RPMI+serum in 12-well plates. Beginning on day 2 p.p., cells were maintained in RPMI supplemented with BSA and NGF, with media changes every 2 days. Cells were infected with HSV-1 strain KOS (M) at a MOI of 27 ± 3 on day 15 post plating. Culture medium was supplemented with ACV (50 μM) from days -1 to 8 p.i. Cultures were treated as indicated and monitored for released virus as described in Figure 2. Represented are forksolin treated cultures (○), cultures subjected to NGF-withdrawal (□), and control cultures (●). (A) represents treatment on day 8, (B) represents treatment on day 38, and (C) represents treatment on day 49.

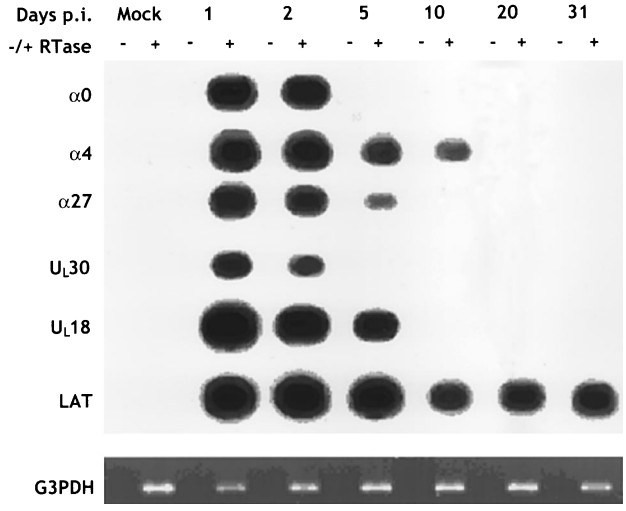

Figure 4 Southern analysis of HSV-1 transcripts produced during the establishment and maintenance of the quiescent phase of infection. PC12 cells were plated at 1.1×10^6 cells/9.6 cm^2 tissue culture wells in RPMI supplemented with BSA and NGF. On day 4 post plating, media was switched to RPMI+serum supplemented with NGF for 2 days, followed by RPMI supplemented with BSA, NGF and ACV. Cells were infected with HSV-1 strain 17+ at a MOI of 0.5 on the following day and maintained in the presence of ACV (100 μM) for 10 days. Duplicate culture wells were collected at the indicated times for RT–PCR analysis.

Table 1 Primer and probe sequences used to amplify and analyze cDNA obtained from HSV-1 infected PC12 cells

Gene	*Primer and probe sequence*[a]		*Sequence location*[b]	*Kinetic class*	*Size of PCR product*	*Reference*
α0	α0-1	ACGGACACGGAACTGTTCGAGA	← 123,151	α	262 bp	Nichol *et al*, 1996
	α0-2	TGTTGCGCAATTGCATCCAGGT	122,890→			Nichol *et al*, 1996
	α0 probe	CGTCGCCCTCGTCGCTCCCC	123001→			This study
α4	α4-1	GGCGGGAAGTTGTGGACTGG	←127308	α	138 bp	Devi-Rao *et al*, 1994
	α4-2	CAGGTTGTTGCCGTTTATTGCG	127171→			Devi-Rao *et al*, 1994
	α4 probe	GTCCCGGCCCGTTACAGCAC	127221→			This study
α27	α27-1	TTTCTCCAGTGCTACCTGAAGG	114922→	α	283 bp	Devi-Rao *et al*, 1994
	α27-2	TCAACTCGCAGACACGACTCG	←115204			Devi-Rao *et al*, 1994
	α27 probe	CTGGACGAACTGTGTTCGCG	114961→			This study
U_L30	Pol-1	GAACACGGACTATTACTTCTCC	66274→	β	227 bp	Devi-Rao *et al*, 1994
	Pol-2	CAAAGGCTCTATGCAACATTCG	←66500			Devi-Rao *et al*, 1994
	Pol probe	GCGTGCGTGACATTCAAGGC	66311→			This study
U_L18	VP23-1	TGAACCCCAGCCCCAGAAACC	←35564	γ	149 bp	Devi-Rao *et al*, 1994*
	VP23-2	CGAGTAAACCATGTTAAGGACC	35416→			Devi-Rao *et al*, 1994*
	VP23 probe	CGGCGTTTACGGGACCGAGT	35451→			This study
LAT	LAT-1	GACAGCAAAAATCCCCTGAG	120702→	Latency	195 bp	Lynas *et al*, 1989
	LAT-2	ACGAGGGAAAACAATAAGGG	←120896			Lynas *et al*, 1989
	LAT probe	CGACACGGATGGGCTGGTGT	120794→			This study
G3PDH	G3PDH-1	GAATCTACTGGCGTCTTCACC			239 bp	Halford *et al*, 1986
	G3PDH-2	GTCATGAGCCCTTCCACGATGC				Halford *et al*, 1986

[a]Primer and probe sequences are shown 5′ to 3′. Upstream (mRNA sense) and downstream (mRNA antisense) primers of each set are referred to as 1 and 2, respectively.
[b]Location indicates the position of the 5′ base of each oligonucleotide according to the HSV-1 sequence of strain 17*syn*+ (McGeoch *et al*, 1986, 1988; Perry and McGeoch, 1988). Only the locations in the long and short internal repeats are indicated for the LAT, α0, and α4 genes
*Previously published as primer that amplifies U_L19.

Table 2 Analysis of gene expression during the establishment and maintenance phases of quiescent HSV-1 infection in Nd-PC12 cells

Gene	*Average cDNA copies/cell (± standard deviation)*						*Limit of detection*
	Day 1	*Day 2*	*Day 5*	*Day 10*	*Day 20*	*Day 31*	
α0	4.6 (1.9)	3.3 (1.7)	0.30 (0.26)	0.076[b] (0.11)	nd	nd	0.032
α4	3.4 (2.2)	2.2 (1.5)	0.56 (0.30)	0.23 (0.099)	<[b]	<[c]	0.078
α27	4.4 (0.75)	3.1 (0.46)	0.48 (0.11)	0.098 (0.031)	<[b]	nd	0.058
U_L30	0.47 (0.19)	0.17 (0.042)	0.030[a] (0.021)	nd	<[c]	nd	0.022
U_L18	>1.1	0.80 (0.20)	0.32 (0.29)	0.015[a] (0.010)	0.014[a] (0.0084)	<[b]	0.010
LAT	0.12 (0.011)	0.12 (0.016)	0.067 (0.047)	0.056 (0.020)	0.049 (0.0023)	0.038 (0.014)	0.014
G3PDH	5.5 (0.51)	7.2 (0.69)	9.8 (0.55)	9.2 (0.57)	8.2 (1.7)	7.4 (0.51)	

cDNA samples from duplicate wells were subjected to amplification by PCR and analyzed by Southern blot (HSV-1 genes) or ethidium bromide staining (host gene) in duplicate. Unless otherwise indicated product was detected in all four samples analyzed. Standard deviations are indicated in parentheses. All PCR reactions of experimental samples were within the linear range except U_L18 on day 1 p.i.
[a]Product detected in 3/4 samples.
[b]Product detected in 2/4 samples.
[c]Product detected in 1/4 samples.
<Indicates that average cDNA copies was less than the limit of detection
nd Not detected in any sample.

Table 2 shows the values obtained from duplicate Southern analysis from duplicate cultures at each time point of viral RT–PCR products quantified by densitometry during the 31-day period. The sensitivity in all cases was greater than 0.1 target sequences per cell, with a range of 0.010–0.078 represented by U_L18 and α4, respectively. Consistent with the results shown in Figure 4, a precipitous decline in viral transcripts associated with productive infection was determined following day 1 p.i., whereas levels of LAT remained relatively constant. Transcript production from the α0 and U_L30 (DNA polymerase gene) declined the fastest during the first 5 days p.i. By day 10 p.i. (day of ACV withdrawal), the level of U_L18 (VP23) and α gene expression was down 93–99%, and the level of U_L30 expression fell below the level of detection. Following the withdrawal of ACV, the expression of these genes continued to decline and by day 20 the average cDNA copies for the α genes were below the level of detection. By day 20 and thereafter, the average cDNA copies for U_L18 were at the level of

detection and below. In contrast, LAT transcripts were reduced 53% by day 10 p.i. but remained relatively constant thereafter, and never dropped near, or below, the level of detection assay. Host G3PDH RNA levels remained relatively constant throughout the assay period. Viral specific product was not detected in uninfected control cultures or in minus reverse-transcriptase controls.

To strengthen the belief that the above analysis was undertaken on cells that were truly quiescent for HSV-1 genome, parallel 12-well cultures, that were determined to be non-productive, were treated with forskolin on day 20 p.i. and reactivated with similar kinetics to that of KOS, as reported in the previous section, with 75% reactivation by 7 day post-forskolin treatment. This experiment was done under similar conditions described in Figure 2.

Discussion

Data from the experimental use of rat pheochromocytoma cells presented here indicate that Nd-PC12 cells can be used to establish a long term, non-productive HSV-1 infection that is reversible. The model is unique in that an artificial inhibitory agent is used to establish, but not maintain, the quiescent infection in a neuronal cell line, and the non-productive state is long-term, yet reversible with features of spontaneous and inducible reactivation. The reader's attention is turned to the details that support these points.

By applying information reported by Rubenstein and Price (1983a) and Block *et al* (1994), we found that it was crucial to the establishment of a non-productive HSV-1 infection that the cultures be: (i) non-dividing; (ii) neurally-differentiated, and (iii) temporally maintained in ACV. Cultures became non-dividing by removing serum from the media and by supplementing the media with NGF (Greene and Tischler, 1976). Despite the absence of serum, neurally-differentiated cells remained viable throughout the 2 month study period as evident by their microscopic appearance and ability to produce virus on treatment (i.e. induction). Maintenance of long-term non-dividing cultures was achieved only when cells were grown in serum-free medium and were plated on collagen-coated dishes.

The antiviral properties of ACV effected the switch to a non-permissive state. We found that ACV treatment for at least 8 days similar to Wilcox and Johnson (1988) resulted in cultures free of detectable levels of virus from all cell lysates and most culture supernatants tested as measured by direct plaque assay after ACV removal. The non-productive state was established and maintained despite high levels of viral inoculum that favored infection of virtually every cell. This is in contrast to the cell culture models used by Wigdahl *et al* (1982a,b, 1983) that required low input multiplicity to maintain a non-productive state.

The non-productive state in QIF-PC12 cells reported herein was characterized by the inability to detect virus in cell lysates examined and the vast majority of culture supernatants examined, and infected cells that showed no visible microscopic features of lytic infection. This is in contrast to infection of Nd-PC12 in the absence of ACV that fails to result in a non-productive infection, but instead results in a persistent infection characterized by low level virus shedding as described by Block *et al* (1994). The ability of ACV to diminish virus replication and allow for the establishment of a quiescent state following its removal is consistent with evidence that processes that limit the acute viral infection favor the establishment and maintenance of a long-term non-productive HSV-1 infection in neurons (Nichol *et al*, 1996; Wilcox and Johnson, 1988). The fact that ACV was not needed to maintain quiescence is in contrast to previous cell culture models that required the continued presence of inhibitory conditions (O'Neill, 1977; Wigdahl *et al*, 1982a,b, 1983) or HSV-1 mutants (Harris and Preston, 1991; Jamieson *et al*, 1995; Russell *et al*, 1987) to maintain repression of viral growth.

An important finding was that the non-productive HSV-1 infection in Nd-PC12 cells is reversible. It is reversible in that virus is consistently induced and recovered from non-productive QIF-PC12 cultures that show no evidence of residual cell associated virus during the quiescent infection. Induction of virus from these cultures occurs following forskolin treatment. Forskolin has a wide range of effects including activation of adenylate cyclase (Huang *et al*, 1982; Seamon and Daly, 1981, 1986), activation of viral gene expression (Ikeda *et al*, 1996), induction of virus reactivation (Smith *et al*, 1992), and inhibition of voltage-sensitive calcium channels (Park and Kim, 1996). Forskolin induced 50–100% of non-productive QIF-PC12 cultures to produce detectable levels of released virus for as long as 51 days post-ACV withdrawal (59 days p.i.) from cultures that were free from spontaneous reactivation throughout the study period. This is in concert with the findings of Smith *et al* (1992) who reported that forskolin induced HSV-1 reactivation from explanted primary fetal sensory ganglia that were latently infected *in vitro*. Although NGF deprivation resulted in virus production in their tissue culture systems (Wilcox and Johnson, 1987, 1988; Wilcox *et al*, 1990) and enhanced virus replication in their PC12 system (Block *et al*, 1994), this treatment failed to release virus from our QIF-PC12 cultures. This suggests that our PC12 model has unique properties for HSV-1 reactivation compared with immature, explanted sensory neurons (Wilcox and Johnson, 1987, 1988; Wilcox *et al*, 1990) and PC12 cells harboring a low-

grade chronic infection (Block *et al*, 1994). Reasons for the difference with respect to the Wilcox *et al* (1990) model might be explained by the dependence of fetal neurons on neurotrophic factors, requirements for cell viability following axotomy, or different cell types used. Difference in the observations seen between our system and Block *et al* (1994) could be the result of the altered states of infection in the two models (i.e. quiescence *versus* shedding).

Changes in gene expression during the establishment and maintenance stages (i.e. following removal of ACV) of the non-productive infection were identified to demonstrate similarities and differences with features reported for latency. Gene expression was examined by synthesizing cDNA from total RNA prepared from cultures terminated on the various days p.i. using RT–PCR. The intent of this analysis was to examine relative changes in the levels of gene expression and to identify times that are free from, or demonstrate low, gene expression that would enhance the significance of reactivation studies. The values reported represent average cDNA copy number generated per cell with an overall sensitivity of greater than 0.1 HSV-1 target sequences per cell.

Analysis of HSV-1 gene expression during the establishment of the non-productive state as measured by RT–PCR revealed a precipitous drop in the level of transcription of representative α (α0, α4 and α27), β (U_L30), and γ (U_L18) genes during the establishment phase. This result could be predicted since ACV inhibition of DNA replication reduces HSV-1 productive infection gene expression in neurons (Kosz-Vnenchak *et al*, 1993) and α gene expression has been shown to peak between 2 and 12 h p.i. (Bloom and Stevens, 1994; Nichol *et al*., 1996). The continual decline in productive gene expression after ACV withdrawal (i.e. maintenance phase) is consistent with evidence that initiation of DNA synthesis is a key regulatory event between lytic and latent events (Nichol *et al*, 1996). The low levels of message by day 10 p.i. (i.e. day of ACV withdrawal) and thereafter was indicative of a quiescence state and that these low levels of gene expression were insufficient to allow the virus to resume the productive cycle. In contrast, levels of LAT transcripts were detected throughout the 31-day study period and fell only threefold from day 1 p.i. levels. Although the level of LAT expression was lower than expected, it is consistent with the work of Rodahl and Haarr (1997) who demonstrated that LAT expression requires viral DNA replication in PC12 cells. These findings indicate that the transient treatment with ACV was sufficient to result in low levels, and the shutdown, of α and β gene products that favored the establishment of a non-productive state, and that levels of LAT transcripts remained readily detectable during the maintenance phase of infection in Nd-PC12 cells.

In summary, we determined that HSV-1 infection of Nd-PC12 in the temporal presence of ACV results in a non-productive infection that is reversible. These findings were consistent for two HSV-1 strains (KOS and 17). Using strain 17+ in the presence of ACV, gene expression (i.e. α0, α4, α27, U_L30, U_L18 and LAT) was minimal on day 1 p.i. and precipitously declined, except for LAT, throughout the maintenance (i.e. quiescent) phase. Our results indicate that QIF-PC12 cells offer a non-productive and reversible state (i.e. inducible to produce infectious progeny) that should enhance our ability to study processes involved in HSV-1 reactivation from a cryptic, latent-like, non-productive state in the absence of replication inhibitors.

Materials and methods

Media, cell lines and virus

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 (HS). Vero cells were grown and maintained in M199 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 KOS (M) was a kind gift of R Thompson (University of Cincinnati, Cincinnati, OH, USA). HSV-1 strain 17+ was a kind gift of N Fraser (Wistar Institute, Philadelphia, PA, USA). Viral stocks were prepared in Vero cells and maintained at −85°C. Virus production was determined using supernatants from infected cultures in a direct plaque assay (DPA) on monolayers of Vero cells as previously described (Miller and Smith, 1991).

Morphologic differentiation

PC12 cells were collected from flasks, dissociated by passage through a 22-gauge needle and plated, as indicated in Figure and Table legends, on 6- or 12-well tissue culture dishes (Becton Dickinson Labware, Franklin Lakes, NJ, USA) coated with rat tail collagen type 1 (Becton Dickinson), unless otherwise indicated. Collagen was applied as recommended by the supplier. Morphologic differentiation was initiated with 50 ng/ml of 2.5S mouse nerve growth factor (NGF) (Becton Dickinson) in RPMI 1640 media containing 5% FBS and 10% HS or RPMI containing 0.1% bovine serum albumin, fraction V (BSA) as described by Green and Tischler (1976), Green (1978) and Gunning *et al* (1981). Morphologic differentiation was confirmed by microscopic visualization of dendritic processes. Media was changed every 2–3 days.

Establishment of a quiescent and persistent infection
Neurally-differentiated PC12 cells (Nd-PC12) were infected with virus, in a volume of 0.4 or 1.0 ml per well, for 12- and 6-well plates, respectively, without agitation at the indicated multiplicity of infection (MOI) overnight at 37°C. Acycloguanosine (ACV), purchased from Sigma (St. Louis, MO, USA) was added to the medium, at the indicated concentrations, from days −1 to the indicated time post-infection (p.i.). After ACV withdrawal, a quiescent state (i.e. free of detectable infectious virus in culture supernatants and lysates) was maintained for at least 7 days prior to induction.

Reactivation stimuli
HSV-1 quiescently infected PC12 cells (QIF-PC12), that were free of detectable infectious virus, were subjected to RPMI media containing BSA and NGF supplemented with 50 μM forskolin (Sigma) for 2 days at the indicated times. Forskolin was prepared in DMSO (Sigma) as recommended by Huang *et al* (1982).

RNA isolation and cDNA synthesis
Cells were harvested for RNA isolation from duplicate wells on the days indicated by scraping cell sheets in PBS followed by centrifugation at 2000 r.p.m. for 5 min. Cell pellets were washed with PBS, pelleted as above and stored at −85°C following removal of PBS. RNA was isolated using the Qiagen RNeasy Total RNA Kit as recommended by the manufacturer. Three μg of each RNA sample was treated with DNAse I (Boehringer Mannheim, Indianapolis, IN, USA) in 20 mM Tris-HCl (pH 8.4), 2 mM $MgCl_2$, 50 mM KCl, and 10 Units of RNase-free DNase I in a final volume of 20 μl at room temperature for 15 min. DNase I was inactivated by adding 2 μl EDTA (25 mM, pH 8.0) and heating at 65°C for 10 min. cDNA was generated with SuperScript II reverse transcriptase (Gibco BRL) and random primers (Gibco BRL) as recommended by the supplier. cDNA was stored at −20°C until use.

RT–PCR
PCR reactions containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.5 μM of each primer of the indicated set, 2.5 units Taq DNA polymerase (Gibco BRL) and cDNA derived from 1500 cells were prepared on ice. Samples were denatured at 94°C for 3 min followed by 35 cycles (94°C for 45 s, 55°C for 30 s, 72°C for 90 s), for HSV target sequences, and 26 cycles, for the host target. Control containing tenfold dilutions of HSV-1 DNA ranging from 1.25 fg to 12.5 pg (7.5–75,000 genome equivalents) or twofold dilutions of PC12 cellular DNA ranging from 0.5 to 4.0 ng (155 to 1240 haploid genome equivalents) were performed in parallel to assess the levels of cDNA in each sample. Experimental and control samples were performed in duplicate and triplicate, respectively. The specificity of viral specific PCR products were verified by Southern blot analysis as described by Brown (1993). In brief, equivalent amounts of PCR products were applied to 2.0% agarose gels and electrophoresed in 0.5 × TBE at 50 V for 45–60 min. DNA was transferred to Magna NT nylon membranes (Micron Separation Inc., Westboro, MA, USA) by capillary action following denaturation and neutralization. Membranes were hybridized to digoxigenin-labeled (DIG Oligonucleotides 3′ end labeling and DIG Luminescent Detection Kits [Boehringer Mannheim]) probes specific for HSV-1 gene sequences (see Table 1). The blots were scanned using the PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA) and analyzed with GPTools software (Biophotonics, Ann Arbor, MI, USA). Levels of the host glyceraldehyde-3-phosphate dehydrogenase (G3PDH) message were determined for each sample to monitor the reverse transcriptase reaction.

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