Herpes simplex virus type 1 infection prevents detachment of nerve growth factor-differentiated PC12 cells in culture

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In tissue culture, rat pheochromocytoma (PC12) cells differentiated with nerve growth factor (NGF) cease division, extend neuritic processes and acquire many properties characteristic of neuronal cells. In previous work, we have shown that NGF-differentiated PC12 cells can survive infection with herpes simplex virus type 1 (HSV-1) and maintain the viral genome in a quiescent but reactivatable state. In this study, we report that uninfected NGF-differentiated PC12 cells uniformly and predictably detach from the culture flask substratum after approximately 7 weeks. Although uninfected cells were uniformly lost from the culture by 7 weeks, surprisingly HSV-1-infected cells survived beyond 10 weeks, the time limit of the study. The detachment of uninfected cells was not the result of cell death or apoptosis, as determined by viability assays performed on cells after detachment. Expression of the HSV-1 latency associated transcript (LAT) gene and virus replication was not necessary for the virus to suppress the ‘detachment’ phenomenon, since NGF-differentiated PC12 cells infected with either wild-type, DNA polymerase mutant or LAT null mutant virus survived in culture for similar lengths of time. Viral gene expression does appear to be necessary for the suppression, however, since cells infected with UV-inactivated virus were lost from culture with kinetics similar to those of uninfected cells. These findings indicate that de novo viral gene synthesis mediates changes to the host NGF-differentiated PC12 cells, which results in prevention of detachment.

Introduction

Herpes simplex virus type 1 (HSV-1) is a neurotropic herpesvirus that causes a variety of human illnesses. One characteristic of HSV-1 infection in people is the establishment of latent infections in sensory ganglia (Roizman and Sears, 1996). The virus remains latent in the neurones for the life of the host and can be reactivated to cause lesions at or near the initial site of infection. The only detectable viral transcript expressed in latently infected neurones is the latency-associated transcript (LAT) (Deatly et al., 1987; Rock et al., 1987; Stevens et al., 1987). LAT has been shown to play an important role in the establishment, maintenance, or reactivation of latency (Hill et al., 1990; Lieb et al., 1991; Perng et al., 1994, 2000a, b; Sawtell and Thompson, 1992; Steiner et al., 1989; Trousdale et al., 1991), possibly by suppressing lytic viral gene expression (Chen et al., 1997; Garber et al., 1997), suppressing apoptosis (Ahmed et al., 2002; Inman et al., 2001; Perng et al., 2000a, b), or by some other unknown mechanism. Infection with wild-type HSV-1 influences a variety of host cellular functions (Bruni and Roizman, 1998; Everett and Maul, 1994; Hammarsten et al., 1996; Hill et al., 1995; Hobbs and DeLuca, 2001; McLauchlan et al., 1989; Mossman et al., 2001; Phelan et al., 1993; Sandri-Goldin et al., 1995; Stingley et al., 2000). Such alterations to infected cells are assumed to assist HSV-1 in completing a productive virus replication cycle.

PC12 cells are derived from a rat pheochromocytoma (Green and Tischler, 1976). They have high affinity receptors for nerve growth factor (NGF) and, in response to NGF, cease division, extend neuritic processes and acquire many properties characteristic of neuronal cells. In previous work, we have shown that NGF-differentiated PC12 cells can survive infection with herpes simplex virus type 1 (HSV-1) and maintain the viral genome in a quiescent but reactivatable state. In this study, we report that uninfected NGF-differentiated PC12 cells uniformly and predictably detach from the culture flask substratum after approximately 7 weeks. Although uninfected cells were uniformly lost from the culture by 7 weeks, surprisingly HSV-1-infected cells survived beyond 10 weeks, the time limit of the study. The detachment of uninfected cells was not the result of cell death or apoptosis, as determined by viability assays performed on cells after detachment. Expression of the HSV-1 latency associated transcript (LAT) gene and virus replication was not necessary for the virus to suppress the ‘detachment’ phenomenon, since NGF-differentiated PC12 cells infected with either wild-type, DNA polymerase mutant or LAT null mutant virus survived in culture for similar lengths of time. Viral gene expression does appear to be necessary for the suppression, however, since cells infected with UV-inactivated virus were lost from culture with kinetics similar to those of uninfected cells. These findings indicate that de novo viral gene synthesis mediates changes to the host NGF-differentiated PC12 cells, which results in prevention of detachment.

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division, extend long neuronal processes that can support action potentials and have been used as models of neuronal cells maintained in tissue culture (Danaher et al., 2000; Greene and Tischler, 1976; Su et al., 1999; Thomselli et al., 1990). We have previously demonstrated that after 2 weeks of NGF differentiation, infection of PC12 cells with HSV-1 results in a limited productive infection, which is followed by the establishment of a ‘quiescent’ state. The quiescent HSV genomes inside the nucleus can become active and release infectious progeny following treatment with reactivation stimuli (Su et al., 1999). The NGF-differentiated PC12 culture system provides an opportunity to study HSV-1–neuronal cell interactions in a homogeneous population of cells in tissue culture.

Here, we report that compared with uninfected cells, and contrary to expectations, HSV-1-infected neuronal-like PC12 cells in culture remained attached to the culture flask substratum longer than uninfected cells. Following HSV-1 infection, NGF-differentiated PC12 cell detachment was prevented or delayed, and the infected culture adhered to the culture flask beyond 10 weeks of seeding. Studies with an LAT null mutant, a DNA polymerase mutant and UV-inactivated viruses suggested that detachment prevention was dependent on de novo gene expression but it appeared to be LAT- and viral DNA replication-independent. Although the precise mechanism whereby HSV-1 prevents PC12 detachment is not yet known, the observation is striking. Several important facts are clear: LAT gene expression and virus replication are dispensable, yet viral gene expression appears to be necessary.

Methods

**Cells.** PC12 cells [American Type Culture Collection (ATCC), Rockville, MD] were grown in RPMI 1640 supplemented with 10% heat-inactivated horse serum and 5% heat-inactivated fetal bovine serum (PC12 medium). CV-1 cells (ATCC), PolB3 cells [an HSV-1 DNA polymerase-expressing cell line, kindly provided by Dr Charles Hwang, State University of New York (Hwang et al., 1997)] and CHO–HveA cells (stably transfected CHO cells with a β-galactosidase (β-gal) reporter under the control of the ICP4 promoter, a gift from Professor Patricia Spear, Northwestern University, Chicago, IL) were maintained in Eagle’s minimal essential medium plus 5% calf serum.

**Viruses and virus stock preparation.** HSV-1 strain 17, the McKrae strain and the LAT null mutant (dLAT2903) and its rescuant, dLAT2903R (Perng et al., 1994), were grown in CV-1 cells. To prepare virus stock, CV-1 cells were infected with virus at an m.o.i. of 0.1 and harvested when 95% of the infected culture displayed a cytopathic effect. Infected cells were frozen, thawed, sonicated and then aliquoted. Virus titre was determined by a standard plaque assay on CV-1 monolayers under methylocellulose. DNA polymerase mutant HP66 (Marcy et al., 1990), kindly provided by Dr Donald Coen (Harvard Medical school, Boston, MA) was grown and titred in the PolB3 cell line.

**Differentiation of PC12 cells.** To differentiate PC12 cells, 1 × 10⁵ cells were seeded on 25 cm² culture flasks coated with poly-l-ornithine (Sigma). The following day, cells were incubated in PC12 medium containing 100 ng/ml of 255 NGF (Collaborative Biomedical Products) for 1 week. The medium was replaced every 3 days. On day 7, fluoroodeoxyuridine (5-Flu) (Sigma) was added to a concentration of 20 μM for 3 days to eliminate undifferentiated PC12 cells. This 3 day treatment has been tested previously and shown to eliminate all cells in cultures of undifferentiated PC12 cells (data not shown). Fresh NGF-supplemented medium was replaced thereafter.

**Establishment of long-term quiescent HSV-1 infection.** Differentiated PC12 cultures were infected with HSV-1 strain 17 at an m.o.i. of 20 (2 × 10⁸ p.f.u./flask). Following a 1 h incubation at 37 °C, cultures were treated with 3 ml sodium citrate buffer, pH 3, for 30–60 s to inactivate residual virus, as previously described (Su et al., 1999). The buffer was removed and flasks were rinsed once with PC12 medium. After low-pH treatment, cultures were incubated at 37 °C with fresh medium containing NGF.

**Terminal deoxynucleotidyltransferase-mediated UTP end-labelling (TUNEL) staining.** Detached NGF-differentiated PC12 cells were sedimented on poly-l-ornithine-coated slides, fixed with 4% formaldehyde, permeabilized with 0.2% Triton X-100 in PBS, and then stained with the Fluorescein-labelled Apoptosis Detection System (Promega), according to manufacturer’s specifications. Cells were observed and photographed using fluorescence microscopy.

**Preparation and characterization of UV-irradiated viruses.** CV-1 cells were infected with HSV-1 strain 17 at an m.o.i. of 0.1. Forty-eight hours later, infected cells were scrapped off into culture medium, centrifuged, frozen, thawed and sonicated, as described above. Cell-free virions were isolated by centrifugation in a 20% sucrose gradient. Virions resuspended in 1 × PBS were filtered via a 0.45 μm filter and then aliquoted into four wells. Each well of virions was subjected to UV irradiation (12000 J/m²) for 0, 1.5, 2 or 2.5 min, using a UV-Stralinker (Stratagene). After UV-irradiation, each aliquot was tested for: (i) infectivity on a CV-1 cell monolayer by standard plaque assay; (ii) the ability to transactivate the ICP4 promoter activity in CHO–HevA cells as below; and (iii) the ability to prevent detachment. CHO–HevA cell cultures were prepared and infected with each aliquot of UV-treated viruses at an m.o.i. of 1 (dilution based on the virus titre obtained for 0 min UV aliquot). Six hours after infection, cultures were fixed and stained for β-gal activity, as previously described (Su et al., 2000). The number of positive-stained (blue) cells was counted in an area of approximately 3 mm² for each infected culture under a microscope. The number of positive-stained cells in the 0 min aliquot was considered to be 100%.

Results

**HSV-1 infection prevents detachment of NGF-differentiated PC12 cells from the culture surface.** In the course of studying NGF-differentiated PC12 cells, we found that HSV-1-infected NGF-differentiated PC12 cells consistently remained attached in culture for longer periods of time than the uninfected controls. Uninfected NGF-differentiated PC12 cells began to detach from the culture flasks 5 weeks after seeding, whereas infected NGF-differentiated cells remained attached to the culture flasks. To quantify this observation, a single ‘end state’ time point (using four culture flasks per group) was analysed 48 days after seeding. NGF-differentiated PC12 cells were infected at an m.o.i. of 20 with HSV-1 strain 17, or left uninfected. Uninfected and HSV-1-infected cells that remained in the culture flask on day 48 after...
HSV-1 prevents neuronal-like PC12 detachment

Fig. 1. For legend see facing page.

Seeding were trypsinized, collected and counted by trypan blue staining. It was found that an average of 92% of the uninfected, NGF-differentiated PC12 cells were lost from the surface of the culture flask after 7 weeks of seeding. In contrast, only 11% of the HSV-1 strain 17-infected cells were lost from the surface of the culture flask.

Since a single ‘end state’ time point showed a significant difference in the adherence of uninfected and infected NGF-
differentiated PC12 cells, it was of interest to determine the kinetics of the detachment of uninfected PC12 cells detaching from the culture flask and further quantify the results. The kinetics of cells detaching from the flasks was studied by observing the change in number of cells in pre-selected areas over time. NGF-differentiated PC12 cultures were infected with HSV-1 strain 17 at an m.o.i. of 20, or left uninfected. Morphology and cell numbers were followed by serial
Fig. 1. Percentage of PC12 cells remaining in culture as a function of time. NGF-differentiated PC12 cells were prepared as described in Methods. On day 10, cultures were treated with the anti-mitotic agent 5-Flu for 3 days. One day after the removal of 5-Flu (day 13 after seeding), cultures were photographed and then infected with $4 \times 10^6$ p.f.u./flask of HSV-1 strain 17, or left uninfected. To monitor the percentage of cells remaining in the flask, each flask was marked randomly with two distinct dots (as shown at the corner of each photo in panel A), and each frame was photographed and the cells counted on the indicated days post-seeding. Representative photos of the same field at different days after infection are shown in panel A. (B) The percentage of cells remaining in the flask was calculated based on the number of cells in the flask on the day of infection. The average of ten frames ± standard deviation of each group is shown.

It was noted that cell migration occurred following NGF treatment and continued for 3–4 weeks after seeding. Therefore, cell number per field varied by as much as ± 20% within the first 3 weeks. However, the cell number was relatively stable 4 weeks after seeding, and remained so until detachment began. To reduce the confounding influence of cell migration on counts of cell number per field, ten separate fields in five culture flasks were counted per experimental group and the average numbers of cells were calculated with a standard deviation. At the end of the experiment, the amount of total DNA in each flask was measured to corroborate the results obtained from the cell counting.

As shown in Fig. 1(B), limited cell division continued after NGF treatment (compare day 10 to day 0), as a small population of PC12 cells did not respond optimally to NGF treatment. This is expected and replicating cells were eliminated by 5-Flu treatment on day 10, as previously described (Su et al., 1999). Elimination of mitotic cells by 5-Flu accounts for the drop in cell number from day 10 to day 13. To obtain a similar baseline for both uninfected and infected cultures, cell counts were taken prior to viral infection on day 13 after seeding. The number of cells counted at day 13 was taken as 100%, as day 13 was when infection took place. As expected, there was no appreciable cell death or gross morphological alteration observed in HSV-1-infected cultures after infection (Fig. 1B, days 13–19), despite modest HSV replication during the first week following infection ($10^5$ p.f.u./25 cm² flask) (Su et al., 1999).

Compared with uninfected cells, HSV-1 infection enabled PC12 cells to remain adherent in culture flasks for a significantly longer period of time than the uninfected counterparts (Fig. 1B, days 40–54). Uninfected NGF-differentiated PC12 cells detached from the observation fields between 37 and 54 days after seeding, while no significant loss was seen in the HSV-1-infected cultures (Fig. 1A). In fact, more than 90% of the HSV-1-infected, NGF-differentiated PC12 cells remained attached to the culture flask 102 days after seeding, at which point the experiment was terminated.
Table 1. Viability of detached NGF-differentiated PC12 cells

NGF-differentiated PC12 cells were prepared and infected with HSV-1 strain 17 or left uninfected, as in Methods. On day 42 after seeding, when most of the uninfected cells had detached from the culture flask, detached cells were collected and stained with trypan blue or by TUNEL staining. As a control, HSV-1-infected cells were forced to detach by tapping on the edges of flasks. The percentage of positive-stained cells was the average of four different fields of microscope observation with the standard deviation.

<table>
<thead>
<tr>
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<th>Percentage of trypan blue ‘positive’ cells</th>
<th>Percentage of TUNEL-staining ‘positive’ cells</th>
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<tr>
<td>Uninfected</td>
<td>5.2 ± 3.9</td>
<td>3.2 ± 2.7</td>
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<tr>
<td>HSV-1 infected</td>
<td>4.7 ± 5.9</td>
<td>4.2 ± 3.8</td>
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These results were further quantified by isolating total DNA remaining in the flasks. The amount of DNA, and therefore the relative number of cells, was more than tenfold higher in the HSV-1-infected NGF-differentiated PC12 cultures, as compared with the uninfected NGF-differentiated PC12 cultures (data not shown). This was similar to the results obtained by direct cell counts (Fig. 1B).

The above results were observed in six independent experiments conducted in the same manner. Thus, it appears that the HSV-1 infection prevented cell detachment from the culture flask surface that would otherwise occur by day 50 following NGF differentiation of PC12 cells.

Viability of detached PC12 cells

Since the number of uninfected NGF-differentiated PC12 cells remaining attached to the culture flask dropped by week 7 after seeding to less than 10% of the number seeded, it was of interest to determine whether detachment was caused by cell death. Therefore, on day 42 after seeding, uninfected cells that had spontaneously detached from the culture flask and HSV-1-infected cells that had been mechanically detached from the flask were collected and stained with trypan blue. Surprisingly, only 5% of the uninfected cells stained positive with the trypan blue (Table 1), which indicated that the majority of the cells were physically viable. This was similar to the results obtained with mechanically detached HSV-1-infected cells, where 5% of cells also stained positive with trypan blue. As a positive control, undifferentiated PC12 cells, permeabilized with 0.2% Triton X-100 and incubated with trypan blue, were uniformly positively stained.

To further explore the physiology of the cell by examining the number of apoptotic cells in the culture, a TUNEL assay was performed on detached, uninfected, NGF-differentiated PC12 cells. Detached, uninfected, NGF-differentiated PC12 cells were tested for apoptosis immediately after detachment by a fluorescein-labelled apoptosis detection system. As with trypan blue staining, less than 5% of the uninfected NGF-differentiated PC12 cells examined scored positive for TUNEL staining (Table 1). This percentage of TUNEL staining was also seen in the infected NGF-differentiated PC12 cells, which had been detached by mechanical force. Since the majority of detached NGF-differentiated PC12 cells were not stained with trypan blue and scored negative in the TUNEL assay, there was no evidence that the uninfected NGF-differentiated PC12 cells were detaching as a consequence of cell death.

HSV-1 LAT null mutant infection of NGF-differentiated PC12 cells

The LATs are the only viral gene family consistently detected during HSV-1 neuronal latency (Rock et al., 1987; Stevens et al., 1987) and NGF-differentiated PC12 cell long-term quiescent infection (Su et al., 1999). LAT gene expression has been suggested to have various effects on host cellular function and viral gene regulation. It was of interest to determine whether LAT expression was necessary for HSV-1 to prevent detachment of infected NGF-differentiated PC12 cells. dLAT2903, an HSV-1 LAT null mutant that does not produce LAT RNAs (Perng et al., 1994), was used to determine the role of LAT expression in infected PC12 cells. NGF-differentiated PC12 cells were infected with HSV-1 strain 17, wild-type McKrae strain (parent strain of dLAT2903), dLAT2903, dLAT2903R (rescued dLAT2903), or left uninfected. Morphology and cell number were observed as a function of time by serial photography of ten pre-selected fields in five culture flasks each (as described in Fig. 1A).

As expected, after 7 weeks in culture, the majority of uninfected NGF-differentiated PC12 cells had detached from the culture flask, whereas HSV-1 strain 17-infected NGF-differentiated PC12 cells remained attached to the culture flask over the time observed (Fig. 3). Interestingly, McKrae-, dLAT2903- and dLAT2903R-infected NGF-differentiated PC12 cells also did not detach from the culture flask (Fig. 2). It was noted that there appeared to be a 25% increase in cell number in McKrae-infected cultures between day 13 and day 15. However, this difference was not statistically significant and was probably due to cell migration following NGF treatment, as mentioned above. This finding indicates that LAT expression is not needed to prevent detachment and that prevention of detachment is not HSV-1 strain-specific.

Role of de novo virus gene expression in mediating ‘longevity’ of PC12 cells in culture

Infection with human cytomegalovirus, a member of the Herpesviridae family, has been reported to result in activation of signal transduction pathways, through the receptor–ligand engagement (Singh et al., 2001; Yurochko et al., 1997; Zhu et al., 1997) in the absence of de novo viral gene expression. Thus,
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Fig. 2. HSV-1 LAT null virus prevents PC12 cell detachment. PC12 cells were differentiated with NGF and treated with 5-Flu, as described in Methods. One day after removal of 5-Flu (day 13 after seeding), cultures were infected with $4 \times 10^6$ p.f.u./flask of HSV-1 strain 17 (17+), dLAT2903 (an LAT null mutant derived from McKrae), marker-rescued virus (dLAT2903R), wild-type strain McKrae, or left uninfected. To monitor the number of cells remaining in the flask, each flask was marked randomly with two distinct dots and each frame was photographed using digital microscopy and counted on indicated days after seeding. The percentage of cells remaining in the flask was calculated based on the number of cells in the flask on the day of infection (day 13). The average of ten frames ± standard deviation of each group is shown.

it remained possible that viral gene expression was unnecessary for HSV-1 to prevent NGF-differentiated PC12 cell detachment from culture flasks. It has been shown that UV-inactivated HSV-1 results in virus that retains virion-associated functions, but does not induce viral gene expression and is unable to produce progeny (Schaffer et al., 1973). Therefore, UV-inactivated HSV-1 strain 17 was used to determine the role of de novo gene expression.

Cell-free HSV-1 virions were subjected to sufficient UV light to reduce infectivity by more than 6 logs, but retain virion-associated transactivation function (Fig. 4A). NGF-differentiated PC12 cells were infected with HSV-1 strain 17 (0 min of UV inactivation), UV-inactivated virus HSV-1 strain 17 (2 min of UV inactivation), or left uninfected. After infection, morphology and cell numbers were observed by serial photomicroscopy of pre-selected areas (as in Fig. 1A). As expected, after 55 days in culture most of the uninfected PC12 cells had detached from the culture flasks (Fig. 4B), while the majority of the HSV-1-infected cells remained adhered. Notably, most of the PC12 cells in flasks inoculated with UV-inactivated virus were lost by 55 days. Thus, the cells in the UV-inactivated flasks behaved similarly to those in uninfected cultures. Since UV-inactivated virus is defective in viral gene expression, this data suggests that de novo viral gene expression is necessary for HSV-1 to prevent the detachment of NGF-differentiated PC12 cells.

Role of HSV-1 replication in prevention of detachment

Because HSV-1 replication is dependent on de novo viral gene expression, and data from the UV-inactivated HSV-1 implied that some gene expression was necessary to prevent detachment of infected NGF-differentiated PC12 cells, it was of interest to test whether virus replication was, itself, necessary to prevent detachment. An HSV-1 DNA polymerase mutant (HP66), lacking the ability to replicate, was investigated for its ability to prevent cell detachment. NGF-differentiated PC12 cells were infected with either HSV-1 strain 17, HP66, or left uninfected. Morphology and cell number were observed as a function of time by serial photography of ten pre-selected fields in five culture flasks each (as in Fig. 1A). As a control for the infectivity of HP66 in NGF-differentiated culture, the content of HSV-1 DNA inside nuclei was determined by Southern hybridization 6 h after infection. The amount of HP66 DNA uncoated in the nucleus was comparable with that of HSV-1 strain 17 (data not shown). Thus, the ability of HP66 and HSV-1 strain 17 to enter NGF-differentiated PC12 cell culture was comparable.
As shown in Fig. 4, less than 10% of HP66- and HSV-1-infected PC12 cells had detached from the culture flask by day 55 after seeding, while 90% of the uninfected cells had detached by that time. Since HP66-infected NGF-differentiated PC12 cells remained attached to the culture flasks, it appears that HSV-1 replication is not needed to prevent detachment.

Discussion

One striking observation described here is that quiescent HSV-1 infection prevented 6–7-week-old neurone-like NGF-differentiated PC12 cells from detaching from the culture flask. Although NGF-differentiated PC12 cells have been widely used as a neuronal-like culture in various studies, the length of time that the cells can be kept adherent in this post-mitotic state in culture flasks has not been extensively studied. This length of time probably varies with culture conditions as well as the homogeneity of cultures. In our experiments, cultures are routinely transiently treated with 5-Flu for 3 days after the first week of NGF incubation to eliminate any residual PC12 cells that are still dividing. This treatment is important in studies involving viral infection. Under these conditions, cultures incubated with 100 ng/ml of 2.5S NGF in serum-containing medium maintained a constant cell number for 6–7 weeks (Fig. 1). A previous report (Danaher et al., 2000) showed that when PC12 cells were cultured with NGF and serum-containing medium, but with no anti-mitotic agent, the NGF-differentiated PC12 cells remained in culture for longer than 8 weeks. Under conditions lacking anti-mitotic agent treatment, cell division continued with a 3-log increase in cell number by day 40 of culture. It is possible that a higher cell density and more heterogeneously differentiated cells provided various growth factors that maintained cells in an ‘indefinite’ adherent status. However, under the conditions we described here, the life of attached, relatively homogeneous, well-differentiated PC12 cells was limited in culture. Strikingly, HSV-1 quiescent infection was able to prevent the detachment of well-differentiated PC12 cells in culture.

The neurone provides an unusual post-mitotic environment. During normal development, as many as 50% of the neurones are eliminated by apoptosis (reviewed in Milligan et al., 2000; Morrison & Hof, 1997). Thus, apoptosis and gene regulation appear to be managed and used to achieve specific cell destinies. Neuronal cell death may be tactical, as a part of development or to hinder pathogen infection, or pathological, due to injury or aging. Survival and sustenance of developing neurones is dependent on binding neurotrophic factors, such as NGF, and associating with proper extracellular matrix (ECM). Interestingly, results from both trypan blue and TUNEL staining suggested that the majority of detached cells were viable at the time of their detachment. However, following detachment, the surviving neurone-like PC12 cells were noted to be de-differentiating morphologically and resuming the ‘rounded’ phenotype characteristic of the loosely adherent parental PC12 cells, even in the presence of NGF-containing medium. Since PC12 cell are transformed, pluripotent cells, detaching from an extracellular matrix might not result in apoptosis, but in morphological de-differentiation into a cell type that can survive in a matrix-free setting.

Since the virus stocks used here were prepared as total cell lysates, it is possible that cell factors contained in the virus stocks might be responsible for the prevention of detachment. However, for the UV experiment, cell-free gradient-purified HSV-1 strain 17 virions were used. The 0 min UV treatment gradient-purified virus prevented detachment (Fig. 3) as well as the total cell extract virus stocks used in the other experiments.
array analysis (Mergen Ltd) was performed to compare the initial viral infection on cell attachment, a rat RNA gene number of ten frames per frame and each frame was photographed using digital microscopy and counted on indicated days post-seeding. The percentage of cells remaining in the flask was calculated based on the number of cells in the flask on the day of infection (day 13). The average standard deviation of each group is shown.

Thus, it is unlikely that contaminating cell factors were responsible for HSV-1 infection preventing detachment of PC12 cells.

Experiments to determine the role of HSV-1 in the prevention of detachment using UV-inactivated virus suggested that de novo gene synthesis was important in the prevention of detachment. The use of the UV-inactivated virus also suggested that a ligand–receptor mediated event, host cell response to foreign DNA and virion-associated function were not sufficient to prevent detachment. Experiments with the HSV-1 DNA polymerase mutant (Fig. 4) and LAT null mutants (Fig. 2) have demonstrated that neither HSV-1 replication nor LAT gene expression were necessary for the prevention of detachment.

Collectively, the data reported here suggest that HSV-1 de novo gene expression exerts a prolonged, sustained effect in infected NGF-differentiated PC12 cells. The detachment phenomena occurred more than 20 days after infection, when the only detectable viral gene transcript was LAT (Su et al., 1999). However, LAT was not needed to prevent detachment (Fig. 2). The effect of detachment prevention must therefore be the result of an event occurring early after infection.

Infection with HSV-1 has been shown to influence a variety of host cell factors and metabolic pathways (Bruni & Roizman, 1998; Everett & Maul, 1994; Hammarsten et al., 1996; Hill et al., 1995; Hobbs & DeLuca, 2001; McAulchan et al., 1989; Mossman et al., 2001; Phelan et al., 1993; Sandri-Goldin et al., 1995; Stingley et al., 2000). To investigate the effect of an initial viral infection on cell attachment, a rat RNA gene array analysis (Mergen Ltd) was performed to compare the cellular gene profiles of uninfected and HSV-1-infected NGF-differentiated PC12 cultures, 24 days after infection (day 37 after seeding). Out of 1100 genes studied by gene array analysis, there were clearly some significant differences at the level of cellular gene profiles (data not shown). The majority of genes modulated by HSV-1 infection 37 days after seeding were cell-cycle related. It is known that HSV-1 infection interacts with cell-cycle related proteins during the productive infection (Bruni & Roizman, 1998; Hobbs & Deluca, 2001; Lomonte & Everett, 1999; Song et al., 2000; Davido et al., 2002; Jordan et al., 1999). Interestingly, our preliminary microarray data suggested that HSV-1 can interfere with the cell cycle of quiescent long-term-infected cells and, furthermore, that this interaction enhances cell attachment. Further studies are in progress to evaluate the microarray data and the possible correlation between the cell-cycle modulation and the neuron-like cell attachment.

Although the molecular and biochemical mechanisms of HSV-1 de novo gene expression in the prevention of detachment are not known, our results strongly suggest that quiescent HSV-1 infection prolongs neuronal-like cell life in culture. The implication of this interesting observation for in vivo systems will require investigations using animal models. None the less, this novel finding suggests another possible mechanism by which HSV-1 might prolong the life of latently infected neurones in vivo other than LAT-mediated neuronal survival.

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Fig. 4. HSV-1 replication is not required to prevent NGF-differentiated PC12 cell detachment. PC12 cells were differentiated with NGF and treated with 5-Flu, as described in Methods. One day after the removal of 5-Flu (day 13), cultures were infected with 4 × 10⁶ p.f.u./flask of DNA polymerase mutant HP66, HSV-1 strain 17 (17+) or left uninfected. To monitor the number of cells remaining in the flask, each flask was marked randomly with two distinct dots and each frame was photographed using digital microscopy and counted on indicated days post-seeding.
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