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Herpes simplex virus latency in isolated human neurons

[herpesviruses/neuron-specific marker/human leukocyte interferon/(*E*)-5-(2-bromovinyl)-2′-deoxyuridine]

BRIAN WIGDAHL, CAROL A. SMITH, HELEN M. TRAGLIA, AND FRED RAPP*

Department of Microbiology and Cancer Research Center, The Pennsylvania State University College of Medicine, Hershey, PA 17033

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ABSTRACT **Herpes simplex virus is most probably maintained in the ganglion neurons of the peripheral nervous system of humans in a latent form that can reactivate to produce recurrent disease. As an approximation of this cell–virus interaction, we have constructed a herpes simplex virus latency *in vitro* model system using human fetus sensory neurons as the host cell. Human fetus neurons were characterized as neuronal in origin by the detection of the neuropeptide substance P and the neuron-specific plasma membrane A2B5 antigen. Virus latency was established by blocking complete expression of the virus genome by treatment of infected human neurons with a combination of human leukocyte interferon and (*E*)-5-(2-bromovinyl)-2′-deoxyuridine for 7 days. After removal of inhibitors, virus latency was maintained for at least 9 days. This *in vitro* model will provide a system to analyze, in a primary human neuron, the state of the herpes simplex virus genome during establishment and maintenance of experimental latency.**

Herpes simplex virus (HSV) infection of humans can produce diseases ranging in severity from mild gingivostomatitis to life-threatening encephalitis (1–4). In addition, HSV can remain latent in sensory and autonomic peripheral ganglia of humans, with subsequent virus reactivation often culminating in recurrent epithelial lesions (5, 6). To examine the mechanisms involved in the establishment and maintenance of HSV latency and virus reactivation, experimental approaches have included analyses of (*i*) central and peripheral nervous system tissue isolated from human cadavers (5, 7, 8); (*ii*) *in vivo* animal models (9, 10); (*iii*) animal nervous system explantation models (11); and (*iv*) *in vitro* cell culture models (12–15).

Because the peripheral ganglion neuron most probably maintains the HSV genome during virus latency, thereby playing a central role in latent and recurrent infection, we have constructed several *in vitro* systems to approximate this cell–virus interaction (16–22). We initially described a prototype HSV latency system established by treatment of human embryo lung fibroblast (HEL-F) cells infected with a low multiplicity of virus [0.1 plaque-forming unit (pfu) per cell] with 1-β-D-arabinofuranosylcytosine; virus latency was maintained after inhibitor removal by increased temperature (16–19). To increase the fraction of the latently infected cell population containing an HSV genome that could be activated into a productive replication cycle and consequently facilitate molecular analysis of virus latency, a more effective inhibitor combination consisting of (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVdUrd) and human leukocyte interferon (IFN-α) was used to block complete expression of the virus genome after a high multiplicity HSV type 1 (HSV-1) infection (2.5 pfu per cell) of HEL-F cells (20). In addition, virus latency was also established in isolated rat fetal neurons after treatment of similar high-multiplicity infections with combined BVdUrd and IFN-α (20). In both systems, virus latency was maintained after inhibitor removal by increasing the incubation temperature from 37°C to 40.5°C, and virus replication was reactivated by decreasing the temperature (20, 22). As determined by DNA blot hybridization, the latently infected HEL-F cell and neuron populations contained detectable quantities of most, if not all, HSV-1 *Hin*dIII, *Xba* I, and *Bam*HI DNA fragments (21). Furthermore, there was no detectable alteration in size or molarity of the HSV-1 junction or terminal DNA fragments obtained by *Hin*dIII, *Xba* I, or *Bam*HI digestion of DNA isolated from latently infected HEL-F cells or neurons (21). The data suggested that the predominant form of the HSV-1 genome in either latently infected cell population was nonintegrated, linear, and unit-length DNA (21). We now report an *in vitro* latency model using isolated primary human neurons as the host cell type.

MATERIALS AND METHODS

Cells, Viruses, and Infectious Virus Determination. Monolayer cultures of HEL-F cells and primary rabbit kidney (PRK) cells were grown and maintained as previously described (16–19). HSV-1 (strain Patton) was grown in HEL-F cells infected at 0.01 pfu per cell as previously described (16–19). HSV-1 stocks with titers between 1 and 2×10^9 pfu/ml, as assayed on either HEL-F or PRK cells, were stored at −70°C. After disruption of cells by freezing and thawing three times, total infectious HSV-1 present in infected human neuron cultures was quantitated by plaque assay on PRK cells as described (16, 17). To quantitate infectious virus in HSV-1-infected human fetus neurons during combined treatment with BVdUrd and IFN-α, the inoculum was removed from indicator cells after a 2-hr adsorption period and the infected cells were washed twice with Tris-buffered saline prior to overlay with 0.5% methylcellulose (20).

Isolation and Purification of Human Fetus Neurons. All human fetus nervous system tissue was isolated from elective, nontherapeutic, suction-aborted fetuses after 6–16 weeks of gestation. Abortion specimens were maintained in Liebovitz L-15 buffer prior to aseptic microscopic surgical removal of nervous system tissue initiated between 45 min and 2 hr after pregnancy termination. The dorsal root ganglia (DRG) were removed by making a midline incision through the vertebrae of the entire spinal column followed by surgical removal of individual ganglia from their attached spinal roots. With abortion-terminated fetal specimens obtained at approximately 6–9 weeks gestation, a time prior to extensive development of the dorsal root nerve, the DRG were removed by negative pressure in conjunction with a glass capillary tube. During the isolation and prior to dissociation, the DRG were maintained in Eagle's basal medium with Earle's salts, gluta-

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Abbreviations: HSV, herpes simplex virus; HSV-1, HSV type 1; BVdUrd, (*E*)-5-(2-bromovinyl)-2′-deoxyuridine; IFN-α, human leukocyte interferon; HEL-F, human embryo lung fibroblast; PRK, primary rabbit kidney; pfu, plaque-forming unit(s); DRG, dorsal root ganglia; CPE, cytopathic effect; IU, international units.

*To whom reprint request should be addressed.

mine (0.03%), glucose (0.6%), sodium bicarbonate (0.17%), and 2% fetal calf serum in a 5% CO_2 atmosphere at 37°C. After removal, the DRG were washed with isolation medium, collected by gravitational force, and dissociated as described (23, 24) with several modifications. The DRG were resuspended in calcium- and magnesium-free Hanks' buffered saline containing 0.02% collagenase type IIS and 0.004% bovine pancreatic DNase I. After a 0.5-hr incubation at 37°C, porcine pancreatic trypsin, type II, was added to a final concentration of 0.25% and the suspension was incubated for an additional 0.5 hr. The resultant cell suspension was subjected to centrifugation at 230 × *g* for 10 min and the cell pellet was washed and resuspended in maintenance medium consisting of Eagle's basal medium containing glutamine (0.03%), glucose (0.6%), sodium bicarbonate (0.17%), chicken embryo extract (1%), nerve growth factor (0.05 μg/ml), 10% fetal calf serum, and 10% heat-inactivated horse serum and supplemented with the mitotic inhibitors, 1-β-D-arabinofuranosylcytosine (10 μM), 5-fluoro-2′-deoxyuridine (10 μM), and uridine (10 μM). After a single-cell suspension had been obtained by trituration through a 23-gauge needle, the cells were plated on collagen-coated glass coverslips in medium with mitotic inhibitors to minimize contamination from dividing cell types, predominantly fibroblasts. After an initial 36-hr treatment with mitotic inhibitors, neuronal cultures were alternated at 24-hr intervals between maintenance medium and maintenance medium containing mitotic inhibitors. After three such treatments, the essentially pure neuronal cultures were given daily medium changes for 3 days to minimize the effect of mitotic inhibitors on subsequent experimentation. Only human fetus DRG neuron cultures of greater than 99% purity were used to initiate latency experiments.

Establishment of Virus Latency in Isolated Human Fetus DRG Neurons. The procedure for establishing virus latency in human fetus DRG neurons was similar to that described for attaining HSV latency in isolated rat fetus neurons (22). Essentially pure human fetus DRG neurons were pretreated for 24 hr with BVdUrd [10 μg/ml; kindly provided by E. De Clercq (Rega Institute for Medical Research, Katholieke Universiteit Leuven, Belgium)] and IFN-α [125 international units (IU)/ml, 6.5×10^5 IU/mg of protein; kindly provided by Life Sciences Research Laboratories (St. Petersburg, FL)], infected with HSV-1, and treated daily for 7 days with the same inhibitor combination at 37°C. HSV-1 infections were performed with 5×10^4 pfu per well (2 cm^2). If the well contained a confluent monolayer of HEL-F cells, this input quantity of HSV-1 would be equivalent to a multiplicity of infection of 0.5 pfu per cell. However, because the essentially pure human fetus neuron cultures occupied only the center third of each well (approximately 2000 neurons per well) and existed as a matrix composed of neuron cell bodies and neuritic extensions, an accurate determination of an effective multiplicity of infection was not possible.

Biotin-Avidin-Coupled Indirect Immunofluorescence Microscopy. Uninfected or HSV-1-infected human fetus DRG neurons were fixed with acetone at room temperature for 10 min. After rehydration, cultures were treated in succession with (*i*) normal blocking serum prepared in the same animal in which the biotinylated secondary antibody was prepared; (*ii*) primary monoclonal antibody (mouse anti-A2B5 IgG, rat anti-substance P IgG, or mouse anti-HSV-1 ICP-8); (*iii*) biotinylated secondary antibody (biotinylated horse anti-mouse IgG for the A2B5 and HSV-1 ICP-8 monoclonal antibodies and biotinylated rabbit anti-rat IgG for the substance P monoclonal antibody); and (*iv*) either rhodamine- or fluorescein-conjugated avidin. The normal blocking serum was removed from the fixed cells by blotting and, after each additional step, the cultures were washed with 50 mM Tris·HCl buffer, pH 7.6, as recommended by the supplier (Vector Laboratories, Burlingame, CA). Incubations with each reagent were performed at room temperature for 20 min. The A2B5 and substance P monoclonal antibodies were obtained from Accurate Chemicals (Westbury, NY) and the HSV-1 ICP-8 monoclonal antibody was generously provided by M. Zwieg (National Cancer Institute, Frederick, MD). Immunofluorescence was observed with a Zeiss photosystem III fluorescence microscope.

RESULTS

Characterization of Isolated Human Fetus DRG Neurons. As previously observed by light microscopic analysis (23–25), cell cultures prepared from human fetus DRG contained predominantly two cell types; very large, flat, fibroblastoid cells and round phase-bright cells with long extended processes. When mitotic inhibitors were not added to the neuronal cultures, the proliferation of the fibroblastoid cells resulted in the formation of a confluent cell monolayer within 3–6 days after isolation. However, as described for preparation of neurons from rat fetus DRG (15, 22), when the cell cultures were treated with mitotic inhibitors to inhibit cell proliferation, essentially pure human fetus DRG neuron cultures were obtained. As shown by light microscopic analysis (Fig. 1 *A* and *B*), mitotic inhibitor-treated cultures used for experimentation were greater than 99% neurons, consisting of a dense matrix of neuritic processes and neuron-like single cells or multiple cell aggregates, with only a few contaminating fibroblast or Schwann cells. Furthermore, the contaminating cells had aberrant nuclear and cytoplasmic morphology as a result of treatment with mitotic inhibitors. The network of processes was more evident when viewed by scanning electron microscopy (Fig. 1*C*). In addition, the entrance of the processes into the body of the neuron-like cells was evident at a higher scanning magnification (Fig. 1*D*).

In addition to the examination of cell morphology by light and scanning electron microscopy, the neuron-like cells underwent examination for the presence of characterized neuron-specific biochemical markers: the neuron cell body plasma membrane A2B5 antigen and neuropeptide substance P. As shown by immunofluorescence microscopy using monoclonal antibodies directed against these neuron-specific determinants, cells that morphologically resembled neurons also contained the neuron-specific plasma membrane A2B5 antigen and substance P (Fig. 1 *E–H*). As previously reported (23–26), the A2B5 antigen was localized within the plasma membrane of the neuron cell body and was not present, or was present in low amounts, in the neuritic extensions (Fig. 1*E*), whereas substance P (27) was found in the cell body and neuritic processes (Fig. 1*G*). In addition, the differential specificity of the A2B5 monoclonal antibody was demonstrated by the intense staining of the cell body of the neuron-like cells as opposed to the background staining of the surrounding fibroblastoid cells (Fig. 1*E*).

Replication of HSV-1 in Human Fetus DRG Neurons. To determine whether the essentially pure human fetus neuron population obtained by treatment with mitotic inhibitors would be permissive for the synthesis of progeny HSV-1, cultures were infected with HSV-1 48 hr after removal of mitotic inhibitors. As shown in Fig. 2 *A* and *B*, virus-induced cytopathic effect (CPE) was very evident within 24 hr after infection; infected cells became refractile to light and developed nuclear granulation, axonal processes became fragmented, and, ultimately, neurons detached from the collagen matrix. As determined by indirect immunofluorescence using a primary HSV-1 monoclonal antibody in concert with a biotinylated secondary antibody and fluorescein-conjugated avidin, the HSV-1 early polypeptide, ICP-8, was detected in the nucleus of neuron-like cells (Fig. 2*C*), as reported previously for other cell types (28, 29). In addition to virus-specific CPE and expression of an HSV-1-specific polypeptide in

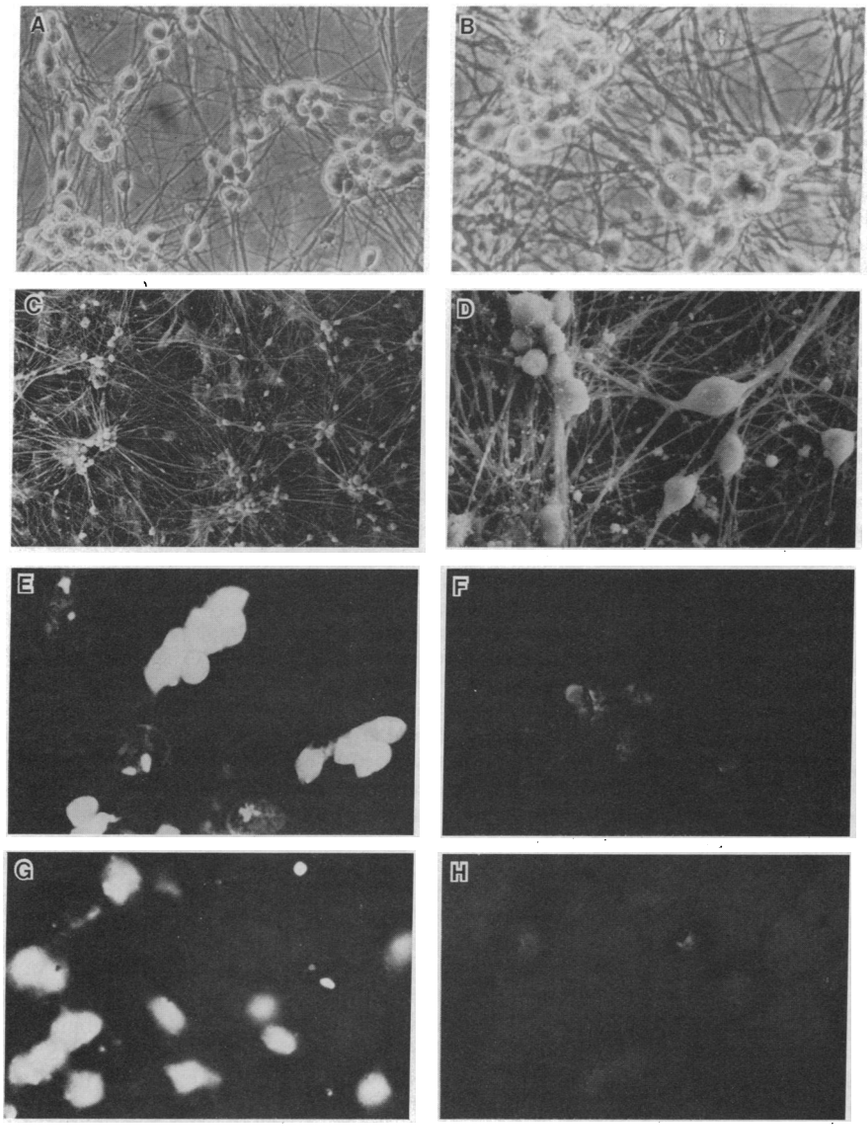

FIG. 1. Morphological and biochemical analyses of human fetus DRG neurons. Neurons were partially purified by treatment with mitotic inhibitors and morphological and biochemical parameters were examined 3 days after treatment termination. (*A* and *B*) Light microscopic analysis, ×69 and ×190, respectively. (*C* and *D*) Scanning electron microscopic analysis, ×30 and ×250, respectively. (*E–H*) Immunofluorescence microscopic analysis, ×230. (*E* and *F*) Primary monoclonal antibody directed against the neuron-specific plasma membrane A2B5 antigen and no-antibody control, respectively. (*G* and *H*) Monoclonal antibody directed against substance P and no-antibody control, respectively.

neuron-like cells, the synthesis of HSV-1 progeny, as determined by plaque assay on PRK cells, was detectable within 20 hr after infection, with virus production continuing through 50 hr after infection (Table 1).

Repression of HSV-1 Replication in Human Fetus DRG Neurons. Because human fetus DRG neurons when cultured *in vitro* were permissive for HSV-1 replication, a combination of BVdUrd (30, 31) and IFN-α was used to block the

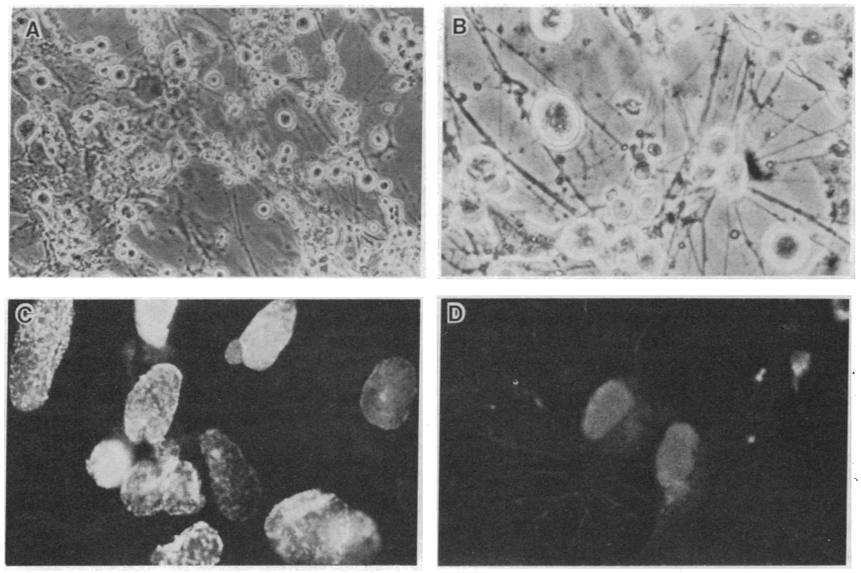

FIG. 2. Light and immunofluorescence microscopic analysis of HSV-1-infected human fetus DRG neurons. Three days after removal of mitotic inhibitors, partially purified populations of human fetus DRG neurons were infected with 5×10^4 pfu of HSV-1 per well; the neurons were analyzed 36 hr after infection. (*A* and *B*) Light microscopic analysis, ×82 and ×220, respectively. (*C* and *D*) Immunofluorescence microscopic analysis, monoclonal antibody directed against HSV-1 ICP-8 and no-antibody control, respectively; ×240.

Table 1. HSV-1 replication in human fetus DRG neurons

Time after infection, hr	Infectious virus, pfu per well
0 (input)	10,000
3	9,800
6	6,800
9	6,600
12	20,000
15	45,000
24	99,000
30	140,000
54	210,000

Human fetus DRG neurons were infected with 10^4 pfu per well, incubated at 37°C, and harvested at the indicated time after infection. Total infectious virus was quantitated by plaque assay on PRK cells. Values shown are average of duplicate samples from a representative experiment.

synthesis of virus DNA and expression of late virus-specific genes and possibly a subset of immediate-early and early virus-specific genes (32). As previously described for the establishment of HSV-1 latency in isolated rat fetus neurons (22), human fetus DRG neurons were treated with combined BVdUrd (10 μg/ml) and IFN-α (125 IU/ml), infected with HSV-1, and then treated for 7 days with the same inhibitor combination. After 3 days of combined inhibitor treatment, virus was undetectable in HSV-1-infected human fetus DRG neuron cultures, whereas there was complete destruction of untreated HSV-1-infected neuron cultures, with extensive synthesis of progeny virus (Table 2). After 7 days of combined treatment, infectious HSV-1 remained undetectable (Table 2), with greater than 80% survival of neuron populations (data not shown). Treatment of HSV-1-infected neurons with BVdUrd (10 μg/ml) alone also effectively inhibited HSV-1 replication in human fetus DRG neurons, with infectious virus undetectable after 5 days of treatment (Table 2). In contrast, IFN-α (125 IU/ml) treatment of infected neuron cultures did not inhibit HSV-1 replication (Tables 1 and 2).

Maintenance of Virus Latency After Removal of BVdUrd and IFN-α. After 7 days of combined BVdUrd and IFN-α treatment, the HSV-1-infected human fetus DRG neurons were washed twice with phosphate-buffered saline and incubated at 37°C in maintenance medium without inhibitors. As shown in Table 2, infectious HSV-1 was undetectable after the 7-day combined inhibitor treatment. Individual HSV-1-infected neuron cultures maintained at 37°C without inhibitors were monitored daily for the presence of extracellular virus. In addition, infected neuron cultures were disrupted and harvested for total infectious virus determination at 24-hr intervals. Infectious HSV-1 was not detected in extracellular or total virus determinations during the 9-day interval after inhibitor removal and maintenance at 37°C (Table 3 and data not shown). In numerous infected human DRG neuron cultures, infectious virus was not detected for as long as 14 days after inhibitor removal (data not shown). In several infected neuron cultures, infectious virus was not detected in extracellular or total virus prior to complete deterioration of the neuron culture (15–20 days after removal of BVdUrd and IFN-α). To determine whether the 9-day virus latency interval after removal of inhibitors was due to the 7-day combined treatment with BVdUrd and IFN-α, human fetus DRG neurons were treated with combined BVdUrd and IFN-α, either mock- or HSV-1-infected (5×10^4 pfu per well), and then treated for 7 days with the same inhibitor combination. After removal of inhibitors on day 7, the mock- or HSV-1-infected cultures were washed twice with Tris-buffered saline, and infected with 100 pfu of HSV-1. Within 3 days, progressive virus-specific CPE was evident that once again culminated in the synthesis of progeny virus (data not shown).

Table 3. Infectious virus quantitation after 7-day combined BVdUrd and IFN-α treatment of HSV-1-infected human fetus DRG neurons

Time after inhibitor removal, days	Infectious virus, pfu per well
1	UD
2	UD
3	UD
4	UD
5	UD
6	UD
7	UD
8	UD
9	UD
10	110
11	45,000

Human fetus DRG neurons were treated with combined BVdUrd (10 μg/ml) and IFN-α (125 IU/ml), infected with 5×10^4 pfu per well, and treated daily for 7 days with the same inhibitor combination. On day 7, inhibitors were removed, cultures were washed twice with Tris-buffered saline, and infected cultures were incubated at 37°C without inhibitors. At the indicated time after infection, cultures were harvested and assayed for total infectious virus on PRK cells. Values are from one representative experiment. UD, undetectable (limit of detection ≦ 2 pfu per culture).

DISCUSSION

To characterize the intracellular events associated with the establishment and maintenance of HSV latency and virus reactivation, we have reconstructed *in vitro* a series of model systems (16–22). These systems have utilized, as host cells, HEL-F cells and isolated rat fetal neurons, to initiate studies directed at examining virus- and cell-specific function(s) regulating virus latency and the physical state of the latent virus genome (16–22). As an essential experimental extension of

Table 2. Effect of antiviral inhibitors on HSV-1 replication in human fetus DRG neurons

Inhibitor	Infectious virus, pfu per well				
	1 day	2 days	3 days	5 days	7 days
None	160,000	540,000	170,000	ND	ND
IFN-α	200,000	800,000	600,000	300,000	ND
BVdUrd	14,000	51	UD	UD	UD
BVdUrd and IFN-α	24,000	390	27	UD	UD

Human fetus DRG neurons were treated for 24 hr with IFN-α (125 IU/ml), BVdUrd (10 μg/ml), combined BVdUrd (10 μg/ml) and IFN-α (125 IU/ml), or no inhibitor; infected with 5×10^4 pfu of HSV-1 per well; treated daily with the same inhibitor combination; and harvested at the indicated time after infection. Total infectious virus was quantitated by plaque assay on PRK cells. Values shown are average of duplicate samples from a representative experiment. ND, not determined, 100% CPE observed. UD, undetectable (limit of detection, ≦ 2 pfu per culture).

these studies, we now report the design of an *in vitro* HSV latency model using a primary human neuron as the host cell type. By using a morphologically and biochemically defined population of neurons isolated from human fetus DRG, virus latency was established by partially blocking virus gene expression by treatment of infected neurons with BVdUrd and IFN-α. After removal of inhibitors, virus latency was maintained for an interval of at least 9 days.

Recent studies by Kennedy *et al.* (25) have examined the virus-specific CPE and antigen expression after HSV-1 (Glasgow strain 17) infection of cell cultures prepared from human fetus DRG. These authors suggested that DRG neurons isolated from aborted human fetuses after 15–16 weeks of gestation were markedly nonpermissive for HSV-1 replication as compared to baby hamster kidney cells and were more restrictive to HSV-1 replication than astrocytes, Schwann cells, or fibroblasts isolated from human fetus nervous system tissue. However, these conclusions were drawn from observations made within 24 hr after infection and utilizing cultures containing a mixture of cell types. Consequently, it was not possible to quantitate the synthesis of progeny virus in human fetus DRG neurons. The studies reported herein agree with those of Kennedy *et al.* (25) in that the virus-specific CPE observed in HSV-1-infected partially purified populations of human fetus DRG neurons developed at a slower rate as compared with that observed in infected HEL-F cells (data not shown). However, the extensive synthesis of progeny virus in HSV-1-infected human fetus DRG neuron cultures suggested that the cell population was permissive for HSV-1 replication. These results do not exclude the possibility that a subpopulation of human fetus DRG neurons may be nonpermissive for HSV-1 progeny virus synthesis with or without associated synthesis of detectable virus antigen(s).

The fact that infection of human fetus DRG neurons resulted in extensive virus-specific CPE, progeny virus synthesis, and ultimately, destruction of the entire neuronal culture dictated the use of methodology to limit the expression of the virus genome subsequent to infection. Treatment of human fetus DRG neurons (infected with at least a moderate multiplicity of infection) with either BVdUrd or BVdUrd combined with IFN-α effectively limited HSV-1 genome expression and virus-specific CPE and resulted in greater than 80% neuron survival. Although infectious HSV-1 was undetectable after a 7-day treatment, it has not yet been determined (*i*) what fraction of the neuron population maintained the virus genome in a latent form that could or could not be activated after inhibitor removal or (*ii*) what fraction of the neuron population the virus genome was eliminated from during the inhibitor treatment. The detection of HSV-1 DNA sequences by DNA blot hybridization in the HSV-1-infected human fetus DRG neuron population after combined inhibitor treatment (data not shown) and the ability to recover infectious HSV-1 subsequent to a minimum of a 9-day incubation at 37°C after inhibitor removal suggest that at least a fraction of the neurons maintain the virus genome in a form that can be reactivated. This model system will facilitate detailed studies of the expression of HSV-1-specific RNA and protein and the state of the HSV-1 genome during establishment of *in vitro* virus latency and during virus reactivation in cultured neurons.

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