A5-Positive Primary Sensory Neurons are Non-Permissive for Productive Infection with Herpes Simplex Virus 1 In Vitro

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ABSTRACT

Herpes simplex viruses 1 and 2 (HSV-1, HSV-2) establish latency and express the latency-associated transcript (LAT) preferentially in different murine sensory neuron populations, with most HSV-1 LAT expression in A5+ neurons and HSV-2 LAT in KH10+ neurons. To study mechanisms regulating establishment of HSV latency in specific subtypes of neurons, cultured dissociated adult murine trigeminal (TG) neurons were assessed for relative permissiveness for productive infection. Unlike neonatal TG, the relative distribution of A5+ and KH10+ neurons in cultured adult TG was similar to that seen in vivo. Productive infection with HSV was restricted and only 45% of cultured neurons could be productively infected with either HSV-1 or HSV-2. A5+ neurons supported productive infection with HSV-2 but were selectively non-permissive for productive infection with HSV-1, which was not due to restricted viral entry or DNA uncoating since HSV-1 expressing β-galactosidase under the neurofilament promoter was detected in ~90% of cultured neurons with no preference for neuronal subtype. Infection with HSV-1 reporter viruses expressing EGFP from immediate early (IE), early, and late gene promoters indicated that the block to productive infection occurred before IE gene expression. Trichostatin A treatment of quiescently infected neurons induced productive infection preferentially from non-A5+ neurons, demonstrating that the non-permissive neuronal subtype is also non-permissive for reactivation. Thus, HSV-1 is capable of entering the majority of sensory neurons in vitro, productive infection occurs within a subset of these neurons, and this differential distribution of productive infection is determined at or before expression of the viral IE genes.
INTRODUCTION

Herpes simplex virus 1 and 2 (HSV-1, HSV-2) replication at the periphery is accompanied by infection of neuronal axons and subsequent retrograde axonal transport to cell bodies of primary sensory neurons, where infection may follow either a productive or latent pathway. In some neurons, lytic gene expression and progeny virus production is thought to result in cell death, while in other neurons, the productive cycle fails and the virus establishes a latent infection. Factors that determine whether HSV progresses through a productive cycle or establishes latency are not clear. It is suspected that different neuronal subtypes and/or presence or absence of certain host factors may be critical in determining the outcome of infection.

Primary sensory neurons are a diverse population of cells that are classified according to cellular morphology, physiological response properties, and patterns of gene expression. We have previously demonstrated that HSV-1 and HSV-2 preferentially establish latency and express the latency-associated transcript (LAT) in different populations of neurons, identified by the A5 and KH10 markers, within sensory ganglia (21, 28, 52). Although all neuronal populations are thought to be capable of supporting a productive HSV infection, some neuronal populations of the trigeminal ganglion appear to be more permissive for productive infection than others, and permissiveness of neuronal subtypes differs for HSV-1 and HSV-2. Based on in situ hybridization for LAT, the neuronal population identified by monoclonal antibody (mAb) A5 is the principal reservoir for HSV-1 latent infection, in mouse models of both ocular and footpad infection. In contrast, HSV-2 establishes a latent infection in very few A5+ neurons in these same models.

In mouse sensory ganglia, the A5 and KH10 markers identify functionally distinct nociceptive neuronal populations. A5+ neurons are NGF responsive, immunoreactive for calcitonin gene-related peptide (CGRP), and project Aδ and C fibers to laminae I and II (outer) of the dorsal horn (14). KH10+ neurons co-label with the lectin BSL-IB4, and are small diameter,
RET-positive neurons that express the ATP-gated ion channel P2X3 and receptors for GDNF and neurturin (4, 14, 28, 33, 41, 54). They project C-fibers to lamina II (inner) of the dorsal horn (14).

Animal models of infection and latency have been valuable in the study of HSV pathogenesis, but have limitations for studying mechanisms that regulate establishment and maintenance of viral latency. These limitations include the relatively small proportion of ganglionic neurons in which latency is established, the asynchronicity of events, the very small number of neurons that can be induced to reactivate, and the difficulty manipulating the molecular state of infected neurons. In vitro models overcome some of these limitations, allowing for synchronized infection of a large number of neurons, as well as coordinated pharmacological manipulation of these cells, but suffer the drawback of not modeling neuronal populations in adult sensory ganglia. An in vitro model using nerve growth factor (NGF)-differentiated PC12 cells has been detailed but the transformed cells take on some, but not all, of the characteristics of autonomic neurons, not sensory neurons. Furthermore, in vitro models with embryonic or neonatal sensory neurons do not reflect the mature heterogeneous populations of neurons in the adult sensory ganglia. To examine neuronal mechanisms that regulate establishment of HSV latency in specific neuronal subtypes, we developed an in vitro model of HSV infection using dissociated adult murine trigeminal ganglion neurons. This in vitro model closely mimics results previously seen with in vivo mouse models, with HSV causing a productive infection in some neurons and a quiescent infection in others. Using this in vitro model, we determined that A5+ trigeminal ganglion neurons are relatively non-permissive for productive HSV-1 infection compared to other populations of trigeminal ganglion neurons. In this model we also determined that preferential permissiveness for productive infection is regulated at, or before, the level of immediate early viral gene expression.
MATERIALS AND METHODS

Neuronal cultures. 6-week old female Swiss Webster mice (Simonsen Labs, Gilroy CA) were euthanized by CO₂, followed by transcardial perfusion with cold, calcium- and magnesium-free (CMF) PBS. Trigeminal ganglia (TG) were removed and incubated at 37°C for 20 minutes in papain (25 mg) (Worthington, Lakewood NJ) reconstituted with 5 ml Neurobasal A medium and collagenase (4 mg/ml) (Sigma) on a rotator, followed by mechanical dissociation of the ganglia by triturating with a 1000 ul pipette. The resultant cell suspension was layered on a 5-step Optiprep (Sigma) gradient. Optiprep was first diluted with 0.8% sodium chloride to make a working solution and then further diluted with Neurobasal A medium to make gradient steps as follows: Optiprep: Neurobasal A medium 150:850 ul, 250:750 ul, 300:700 ul, 350:650 ul, 400:600 ul. The cell suspension was layered on top of the gradient and centrifuged 20 min at 800 x g. The lower end of the centrifuged gradient (~3.5 ml), minus the pellet, was then transferred to a new tube and washed twice with Neurobasal A medium supplemented with 2% B27 Supplement (Invitrogen) and 1% penicillin-streptomycin (PS). Neurons were counted and plated on poly-D-lysine/laminin coated 8-well chamber slides (BD Biosciences) at a density of 3000 neurons per well. Neuronal cultures were maintained with complete neuronal media consisting of Neurobasal A medium supplemented with 2% B27 Supplement, 1% PS, L-glutamine (500 uM), nerve growth factor (NGF, 50 ng/ml), glial-cell derived neurotrophic factor (GDNF, 50 ng/ml), and the mitotic inhibitors fluorodeoxyuridine (40 uM), and aphidicolin (16.6 ug/ml) for the first 3 days. The medium was then replaced with fresh medium without fluorodeoxyuridine and aphidicolin (growth factors were from R&D Systems and other supplements were from Sigma). Neonatal trigeminal ganglia were cultured using identical methods and conditions. Adult and neonatal superior cervical ganglia (SCG) were cultured using the same methods, but without gradient centrifugation.
Viruses. The wild type HSV-1 strains KOS, RE and 17syn+ and the wild type HSV-2 strain 333, as well as all mutant virus strains, were propagated in rabbit skin cells (52). HSV1-VP26-GFP and HSV2-VP26-GFP were generated in Vero cells by co-transfection and homologous recombination of plasmid pK26GFP (kindly provided by Prashant Desai, Johns Hopkins University) with purified viral DNA from either HSV-1 strain 17+ or HSV-2 strain 333 using previously described methods (6). The VP26-GFP viruses express a fusion protein of VP26 and GFP(13). KOS/58, an HSV-1 based virus expressing lacZ under the neurofilament light promoter at the gC locus, and KOS/62, an HSV-1 based virus expressing lacZ inserted between SacII and the second Hpal sites downstream of the LAT promoter, have been previously described (29). Reporter viruses based on the RE HSV-1 strain RE-pICP0-EGFP, RE-pgB-EGFP, and RE-pgC-EGFP express EGFP under the ICP0, gB, and gC promoters inserted at the gC locus, in a manner similar to that detailed previously (12, 37). RE-pgC-EGFP and RE-pICP0-EGFP were described previously (12). To develop RE-pICP4-EGFP, plasmid pK1-2 (a kind gift of Neil Deluca, University of Pittsburgh) was digested with HindIII and BamHI to release the ICP4 promoter, which was then cloned into the polylinker upstream of EGFP in the plasmid gC-EGFP to drive EGFP expression, in a manner similar to that detailed previously (12). To develop RE-pICP27-EGFP virus, the upstream portion of ICP27 from 37bp upstream of the ICP27 ATG to a position approximately 1000 bp further upstream was amplified by PCR, using the primers 5’ GCAGATCTgtcggatatggcctctggtgtggcgca and 5’ GAGTAAGCTTcctacacgaaaattacccgcct (capitalized sequences encode restriction sites for cloning). The resulting fragment was then digested with BgIII and HindIII and cloned into the HindIII BamHI sites in the polylinker of gC-EGFP to drive EGFP expression. Viruses were derived and purified by selection for EGFP-positive plaques. RE-gB-EGFP, in which the gB promoter drives expression of EGFP, was constructed using primers as detailed previously (37), that amplify approximately 500 bp of sequence upstream of the gB coding sequences. This was placed to drive EGFP in the plasmid gC–EGFP plasmid, which was linearized and used to
derive and purify fluorescent virus. All viruses were confirmed for correct insertion by Southern blotting. Viral titers were determined by standard viral plaque assay on Vero cells.

Viral Infections. Neuronal cultures were infected with viruses at various multiplicities of infection (MOI) from 1 to 100 pfu/cell, diluted in Neurobasal A medium. After a one-hour adsorption period, virus was removed and replaced with complete neuronal media (without fluorodeoxyuridine and aphidicolin). For infections lasting longer than 15 hours, pooled human IgG was added to the media to inhibit viral spread through the media after the first productive cycle. IgG was removed for assays of infectious virus and viral reactivation. For immunofluorescent assays, paraformaldehyde (PFA) was added directly to the media of the cultured cells after the designated time periods, to a final concentration of 2% for 5-10 minutes. Fixative was then removed, the cultures were immunostained for designated antigens, and neurons were counted using a Nikon Microphot fluorescent microscope. For infectious virus assays, 150 ul of the media was transferred to a cryotube, cells were scraped and suspended in the remaining 150 ul of media in each well before transferring to a second cryotube, and the samples were frozen at -80°C. Samples were freeze-thawed to release infectious virus, and titers of media and homogenate were determined on Vero cells using a standard plaque assay. All experiments were carried out in parallel with uninfected cultures which served as negative controls.

Immunofluorescence. Infected cultures were immunostained with IgM mAb A5 (Developmental Studies Hybridoma Bank) followed by incubation with FITC- or rhodamine-labeled anti-IgM secondary antibodies (Santa Cruz). Neurons were initially differentiated from satellite glial cells (SGCs) by NeuN antibody staining (Santa Cruz), and subsequently by morphology. GFP expression by VP26-GFP-expressing viruses was not immunohistochemically amplified. Since neuronal cells are slightly autofluorescent in the GFP spectral range, neurons in infected cultures were compared to neurons in uninfected cultures for determining the presence of GFP expression. EGFP expression by IE, E, and L gene reporter viruses was
amplified by incubation with rabbit anti-GFP antibody (Santa Cruz) followed by FITC-labeled anti-rabbit IgG (Santa Cruz). X-gal staining or anti-β-galactosidase rabbit IgG primary antibody (Abcam) with FITC-labeled anti-rabbit IgG (Abcam) was used to visualize β-galactosidase. Immunolabeled neuronal cultures were evaluated by fluorescence microscopy.

Results
Cultured adult murine trigeminal neurons mimic in vivo neuron characteristics. In previous studies, we used combined fluorescent in situ hybridization (FISH) for LAT in conjunction with immunofluorescence (IF) for neuronal markers to demonstrate that HSV-1 preferentially establishes latent infection of murine sensory ganglia in A5-positive neurons and HSV-2 preferentially establishes latent infection in KH10-positive neurons after ocular or footpad infection of mice (21, 28). One mechanism that could account for these findings is that different types of sensory neurons were differentially permissive for productive infection with HSV-1 and HSV-2. To test this hypothesis directly, we developed an in vitro system for studying direct viral infection of dissociated adult murine trigeminal ganglion neurons, thus minimizing confounding roles of the immune system and the variability of the efficiency of viral delivery to the axons in vivo. Cultures of adult murine trigeminal neurons, generated as described in the methods, contained an average of 9.3% A5+ neurons and 13.8% KH10+ neurons, similar to the proportions of these neurons in tissue sections of murine trigeminal ganglia (Figure 1) (21, 28, 52). In the adult neuron cultures, the A5 marker co-localized with immunoreactivity for calcitonin gene-related peptide (CGRP) but not for staining with the lectin BSL-IB4. In contrast, 98.6% of the KH10-positive neurons co-stained with the lectin BSL-IB4 and only 3.4% expressed CGRP (data not shown). These results demonstrate that our in vitro cultures maintain neuronal heterogeneity and some of the well-established in vivo features of adult murine trigeminal neurons that we have previously reported (28).
Since neonatal neuron cultures are routinely used by others for studies of HSV infection, we also cultured and evaluated neonatal trigeminal neurons (postnatal day 1) for the A5 and KH10 markers, using the same methods as for the adult trigeminal ganglia. After equivalent time in vitro, less than 1% of the cultured neonatal trigeminal ganglion neurons were positive for either the A5 (0.38%) or the KH10 (0.29%) markers (Figure 1). Cultures of adult and neonatal superior cervical ganglia (SCG) were also negative for both the A5 and KH10 markers, using the same conditions (data not shown). This suggests the A5 and KH10 sensory neuronal markers are largely adult specific markers.

**Adult trigeminal sensory neurons are non-permissive for productive infection with HSV-1 and HSV-2.** In previous in vivo studies, we observed that many ganglionic neurons that projected to the site of inoculation (eye or footpad) failed to become productively infected. Some neurons simply did not become infected, while others were apparently infected but exhibited little or no expression of productive cycle viral genes. To determine the percentage of neurons that are productively infected when exposed to virus in vitro, cultured dissociated adult trigeminal neurons were infected with HSV1-VP26-GFP or HSV2-VP26-GFP, which express the virion nucleocapsid protein VP26 as a GFP fusion protein, and incorporate VP26-GFP into the nucleocapsid during active viral replication and assembly. During late gene expression (10-12 hours post-infection), we observed VP26-GFP localizing to distinct regions of the nucleus in a punctuate pattern corresponding to capsid assembly compartments, as previously described (13). Later in infection, VP26-GFP displayed a more diffuse nuclear fluorescence with the appearance of fluorescence at the cell membrane, correlating with maturing infectious viral progeny detected by standard plaque assays. We used detection of GFP during infection with HSV1-VP26-GFP and HSV2-VP26-GFP as an indicator of productive viral infection.

Cultured neurons were found to be relatively resistant to productive infection and GFP expression with both HSV-1 and HSV-2. At an MOI of 10, a maximum of only 22% of neurons
in cultures infected with HSV1-VP26-GFP and 16% of neurons in cultures infected with HSV2-VP26-GFP showed evidence of productive infection, as assayed by expression of GFP. Even at an MOI of 100, only 45% of all of the neurons in the culture became productively infected with HSV2-VP26-GFP or HSV1-VP26-GFP (Figure 2). HSV1-VP26-GFP productive infection was similar at 10 and 15 hours post infection (hpi) but HSV2-VP26-GFP demonstrated slightly slower kinetics, peaking at 15 hpi. To verify that these results were indicative of HSV productive infection rather than an idiosyncrasy of our VP26-GFP constructs, we repeated these studies using wild type HSV-1 (strains KOS and 17+) and HSV-2 (strain 333), followed by detection of expressed productive cycle viral gene products with polyclonal anti-HSV antisera, and once again, found a significant fraction of the neurons failed to be productively infected (data not shown). At an MOI of 10, wild type HSV-1 was detected in 17-32% of cultured neurons and HSV-2 was detected in 17-20% of the cultured neurons, similar to that observed with the viruses expressing VP26-GFP. Standard plaque assays verified production of comparable quantities of infectious virus in neuronal cultures following inoculation with either wild type or HSV VP26-GFP viruses, with infectious virus detected as early as 10 hours post-inoculation and increasing to reach a peak at 48 hours post-inoculation. At the peak, mean viral titers were 136,000 pfu/ml for KOS, 161,600 pfu/ml for 17+, 152,200 pfu/ml for HSV1-VP26-GFP, and 140,400 pfu/ml for HSV2-VP26-GFP (data not shown). We thus conclude that a subpopulation of the cultured neurons was unable to support productive replication by either HSV-1 or HSV-2.

Based on our previous in vivo findings, in which HSV-1 preferentially established latent infection and expressed LAT in A5+ neurons, we hypothesized that A5+ neurons in vitro would be relatively non-permissive for productive infection with HSV-1, compared to the other neurons in the ganglion. To test this, we infected neuronal cultures with HSV1-VP26-GFP and evaluated GFP expression in neurons that co-labeled with mAb A5 (Figure 3). Even at an MOI of 100, less than 4% of A5+ neurons were positive for productive infection with HSV1-VP26-GFP. At an MOI of 10, only about 1% of A5+ neurons scored positive for productive viral infection. In
comparison, a relatively large percentage of non-A5+ neurons became productively infected under the same conditions (56.1%, 45.9% and 25.4% of non-A5+ neurons at MOIs of 100, 30, and 10, respectively) (Figure 3A). Furthermore, productively infected non-A5+ neurons were frequently found immediately adjacent to A5+ neurons in which there was no evidence of VP26-GFP expression, despite a high multiplicity of infection (Figure 3B). Similar results were observed when we infected cultures with wild type HSV-1 (KOS and 17+) and visualized productive infection by assaying for HSV antigen expression with polyclonal antisera (data not shown). Although it is possible that some A5+ neurons may undergo a low level of productive HSV-1 viral infection, below the threshold of our assays, productive infection was not detected by either GFP expression in cultures infected with HSV1-VP26-GFP, or by viral antigen expression in cultures infected with wildtype viruses. When we infected adult neuronal cultures with HSV2-VP26-GFP, in contrast to our observations with HSV-1, HSV2-VP26-GFP productively infected both A5+ and non-A5+ neurons to a similar extent (Figure 3A) and GFP expression was frequently found in A5+ neurons (Figure 3B). This result indicates that non-permissiveness of A5+ neurons for productive infection is specific for HSV-1 and not HSV in general.

Non-permissiveness of A5+ neurons for HSV-1 productive infection is not due to preferential viral entry. To determine if the absence of HSV-1 productive infection in A5+ neurons was due to a failure of HSV-1 to enter these neurons, we assayed neuronal cultures that had been infected with KOS/58, which expresses β-galactosidase (β-gal) under the neurofilament light (NFL) promoter at the gC locus (23, 32). We predicted that this virus should express β-gal in all infected neurons, whether productively or quiescently infected, since the neurofilament light promoter is constitutively expressed in all sensory neurons. As detected by dual immunofluorescence for β-gal and the A5 neuronal marker, β-gal expression was detected...
in 86-90% of all cultured neurons following infection with KOS/58 at MOIs of 10, 30 and 100 (Figure 4). Furthermore, β-gal expression was detected equivalently in A5+ and non-A5+ neurons, regardless of the MOI of the inocula. These results indicate that the limited productive infection in A5+ neurons that we observed with HSV1-VP26-GFP or wild type HSV-1 strains KOS or 17+ were a consequence of a relatively non-permissive nature of these neurons for productive infection rather than restricted viral entry and DNA release.

Regulation of permissiveness for productive infection occurs at or before the level of immediate early gene expression. Productive cycle gene expression during HSV infection occurs in a characteristic temporal cascade. Upon entry, viral tegument protein VP16 forms a complex with cellular Oct-1 and HCF-1 to initiate expression of immediate early (IE) genes and the production of IE proteins, which then sequentially initiate early gene expression, DNA replication, and late gene expression and viral assembly. To determine the level at which productive HSV-1 infection was blocked in A5+ neurons, we examined the expression of productive cycle viral genes using several HSV-1 recombinants that express the fluorescent reporter protein EGFP under the control of immediate early (IE), early (E), and late (L) gene promoters inserted at the gC locus of HSV-1. At a MOI of 30, the peak IE, E, and L gene promoter activity occurred at 6, 8, and 10 hpi, respectively. Immediate early gene promoter activity was assessed using the reporter virus RE-pICP0-EGFP (12), which expresses EGFP under the ICP0 promoter; early gene promoter activity was assessed using the RE-pgB-EGFP virus expressing EGFP under the gB promoter similar to that detailed previously (37); and late gene promoter activity was assessed using the reporter virus RE-pgC-EGFP, which expresses EGFP under the gC promoter (12). As summarized in Figure 5A, we found ICP0 promoter activity (as reported by EGFP expression) in 31.8% of the non-A5+ neurons but only 2.1% of the A5+ neurons. Likewise, early gene promoter activity was found in 30.6% of the non-A5+ neurons and 2.4% of the A5+ neurons, and late gene promoter activity was found in 32.8% of
the non-A5+ neurons and 3.2% of the A5+ neurons. These data strongly suggest that the block
to HSV-1 productive infection in A5+ neurons occurs at or before the level of viral IE gene
expression.

To determine whether expression of immediate early gene promoters other than ICP0
were similarly restricted in A5+ neurons, we also evaluated expression of the ICP27 and ICP4
promoters in HSV-1 constructs made similarly to that of RE-pICP0-EGFP, but in which the
ICP27 and ICP4 promoters were used to express EGFP at the gC locus of HSV-1 strain RE. As
summarized in Figure 5B, ICP27 promoter activity was seen in 21.3% of the non-A5+ neurons
but only 1.5% of A5+ neurons, and ICP4 promoter activity was found in 29.4% of non-A5+ and
4.5% of A5+ neurons. Figure 5C illustrates the typical appearance of dual stained neurons in
these studies. We conclude that A5 positive neurons are highly refractory to HSV-1 gene
expression.

LAT promoter activity is present in neurons quiescently infected with HSV in vitro and
quiescent HSV can be reactivated. Absence of lytic gene expression, expression of LAT, and
the ability to reactivate from a quiescent state are the hallmarks of latent HSV infection. We next
sought to determine whether these were features of quiescent HSV-1 infection of adult sensory
neurons in vitro. Adult trigeminal ganglion neuronal cultures were infected with KOS/58,
KOS/62, or HSV1-VP26-GFP (Figure 6). After infection with HSV1-VP26-GFP, GFP expression
and infectious virus increased for 48 hours and then decreased steadily until Day 5, at which
time no infectious virus could be detected in the media and minimal GFP expression was
observed in the cultures. These observations are consistent with a recent report showing that
HSV lytic activity is essentially complete before Day 5 post infection in cultured porcine
trigeminal neurons(11). Five days after infection with KOS/58, β-gal expression from the
neurofilament light promoter was detected in 90.5% of the cultured neurons, demonstrating that
nearly all of the surviving neurons were infected with HSV-1 (Figure 6A and 6B). However, no
infectious KOS/58 virus was detected by plaque assay in the medium at this time point and only a mean of 75 plaque forming units (pfu) were detected in the cell homogenate from each culture well. We detected LAT promoter activity in 33.4% of the surviving cultured neurons five days following infection with KOS/62, as detected by β-gal expression under the LAT promoter at the LAT locus (Figure 6A and 6B). These findings correlate well with reports of in vivo latency where LAT expression can be detected in about one third of latently infected neurons by in situ hybridization for LAT compared with viral DNA assayed by in situ PCR or PCR following laser capture microdissection of individual neurons (8, 30). Five days after infection with HSV1-VP26-GFP, we observed GFP from the VP26-GFP fusion protein in only 2.5% of surviving neurons, demonstrating that the vast majority of infected neurons had no evidence of productive infection at that time point, as assessed by GFP expression (Figure 6A). Furthermore, when VP26-GFP expression was observed, it occurred in discrete focal plaques (with an average of 1-2 plaques per culture well of approximately 2000 neurons), involving one or more neurons and suggestive of focal spontaneous reactivation with local spread. Thus, at five days post infection, the vast majority of cultured adult trigeminal ganglion neurons were quiescently infected with HSV-1 and approximately one third of the infected neurons demonstrated LAT promoter activity. However, it should be noted that LAT reporter activity in KOS/62 may underestimate the abundant accumulation of LAT in wildtype HSV-1, since there is partial disruption of the LAT enhancer in KOS/62 (27).

To test for HSV reactivation competence from quiescently infected neurons in vitro, we infected cultures with the HSV1-VP26-GFP virus and five days later, cultures that showed little or no GFP expression were treated with trichostatin A (TSA), a histone deacetylase inhibitor that has been used previously to reactivate quiescent virus in differentiated PC12 cells (10, 31). TSA has also been shown to activate viral gene expression from quiescent viral genomes in cultured neonatal DRGs (1). As shown in Figure 6D, we detected GFP activity from the HSV1-VP26-GFP virus in 2.5% of all neurons in control cultures 24 hours after treating with fresh medium.
that did not contain TSA on Day 5 post-inoculation. No VP26-GFP expression was detected in
A5-positive neurons in these control cultures. In contrast, in cultures that received fresh media
containing TSA, GFP expression was observed in 13.6% of the total neurons in 24 hours after
treatment. However, GFP expression was detected primarily in non-A5+ neurons after TSA
treatment (Figure 6D), suggesting that the A5+ neurons are less permissive for reactivation than
the non-A5+ neurons, which correlates with our observation that A5+ neurons are less
permissive for productive infection during the initial infection with HSV-1. Figure 6E illustrates a
typical VP26-GFP positive neuron after TSA treatment, displaying strong nuclear GFP
expression. To verify that we were observing productive infection rather than just VP26 gene
activation after TSA treatment, neuronal cultures quiescently infected with 10 MOI of HSV1-
VP26-GFP were treated with TSA five days after infection and virus production was assessed
24 hours later. We found a mean viral titer of 1560 pfu/well in homogenates of TSA-treated
cultures as compared to a mean viral titer of 84 pfu/well from control cultures. Similar results
were observed following TSA treatment of neuronal cultures quiescently infected with wildtype
HSV-1 strain 17+. In these cultures we observed a mean increase of 1062 pfu/well in infectious
virus as compared to controls that did not receive TSA. Therefore, treatment with TSA induced
quiescent virus to reactivate and produce viral progeny. In total, these findings strongly suggest
that neurons labeled with the A5 marker are highly refractory to HSV-1 lytic cycle gene
expression and productive infection, during both initial infection and during reactivation.

DISCUSSION

In previous studies, we demonstrated that HSV-1 preferentially establishes latency in
neurons recognized by mAb A5, while HSV-2 preferentially establishes latent infection in
neurons recognized by mAb KH10 (21, 28). In the studies described in the current manuscript,
we report that dissociated adult trigeminal neurons are relatively non-permissive for productive
infection with HSV. At an MOI of 10, only about 20% of the cultured neurons became
productively infected; even at an MOI of 100, only 45% of the cultured neurons supported productive viral infection. Data from studies with KOS/58 indicate that this was not a consequence of limited viral entry or uncoating of the genome. Furthermore, we show that neuronal subtypes were not equally permissive for productive infection; when we specifically evaluated the ability of A5+ neurons to support a productive infection, less than 5% were permissive at an MOI of 100, an inoculum presumably much greater than biologically relevant levels. Forty percent of the non-A5+ population also did not support productive infection of HSV-1 at this high MOI, indicating that other neuronal populations are also refractory to productive infection. These results correlate well with our previous in vivo reports indicating that HSV-1 preferentially establishes latent infection in A5+ neurons, with approximately 50% of HSV-1 latent sites located in A5+ neurons, although only about 11% of the neurons in the trigeminal ganglion are A5+ (52). This would suggest that HSV-1 enters a quiescent or latent state in A5+ neurons because these neurons cannot support productive infection of HSV-1. In contrast, our work demonstrates that A5+ neurons and non-A5+ neurons support productive infection of HSV-2 equivalently, suggesting that the restriction by A5+ neurons for productive infection is specific for HSV-1. This result also correlates with our previous finding that HSV-2 latent infection is infrequently found in A5+ neurons (28). Thus, HSV-1 and HSV-2 productive infection is regulated differently in different types of neurons in the trigeminal ganglion.

The neuronal composition of the trigeminal and dorsal root ganglia is heterogeneous, and undergoes significant change during embryogenesis, as well during the first several weeks of life. During embryonic development, approximately 80% of rodent ganglionic neurons require NGF for survival. Postnatally, a population of small neurons that is selectively labeled by the lectin IB4 and identical to the KH10+ population, stops expressing TrkA (the high affinity NGF receptor) and starts expressing Ret and the GDNF receptor alpha (GFRα), switching their dependence from NGF to GDNF (3, 33). This postnatal reduction in the percentage of TrkA+ neurons occurs gradually over a period of 3 weeks (P1-P21), coinciding with the critical period...
during which alteration of neurotrophins can permanently alter the physiology of neonatal dorsal root and trigeminal ganglion neurons (19, 25). Down-regulation of TrkA by IB4-binding neurons results in differences in expression profiles and signaling cascades in response to certain classes of stimuli (33, 39, 53). Between P1 and P6, the neuronal expression of somatostatin, FRAP, P2X3, and oligosaccharide conjugates (which include lactoseries carbohydrates recognized by the A5 and KH10 mAbs), changes dramatically in response to GDNF (18, 48). These differences are significant since A5+ neurons, which are non-permissive for productive infection of HSV-1 \textit{in vitro} and maintain the latent HSV reservoir \textit{in vivo}, are mostly TrkA positive, while the IB4/KH10+ neurons, which support productive infection with HSV-1, express the GDNF receptor. Neonatal and adult sensory neurons also differ in their response to injury. IB4-binding neurons are selectively vulnerable to neonatal axotomy (49) and adult neurons are more resistant than neonatal neurons to apoptotic stimuli, including PI3K inhibition and NGF-withdrawal (47). Finally, although adult ganglionic neurons vary in size (both \textit{in vitro} and \textit{in vivo}), cultured neonatal neurons are homogeneous in size (19). Thus, neonatal cultures may lack specific neuronal populations as well as important regulatory signaling cascades and functional properties critical to the regulation of HSV infection in the fully developed nervous system.

During productive infection, HSV undergoes tightly regulated temporal expression of productive cycle genes. Assuming that acute infection of neuronal cultures follows a similar cycle, our work strongly suggests the block to HSV-1 productive infection in A5-positive neurons occurs at or before the level of viral IE gene expression. It is clear that many cellular factors regulate the expression of HSV-1 immediate early genes, including Oct1, Oct2 and HCF, and it is likely that specific cellular factors present in the biochemically distinct neuronal populations of the sensory ganglia play a role in initiating or repressing HSV immediate early genes, which in turn regulate productive vs. latent infection within specific neuronal cell types. Mechanistically, the most widely accepted view of latency establishment is a failure of IE gene activation (16, 17, 22), which we observed in the A5+ neurons in the TG cultures after infection with HSV-1.
Chromatin modulation, regulatory microRNAs, or cellular localization of specific neuronal factors are proposed mechanisms that could differentially regulate IE gene transcription in different types of sensory neurons (7, 23). Chromatin structure modulation plays an important role in transcriptional regulation in neurons (36, 38) and proposed mechanisms of HSV chromatin modulation include host cell factor-1 (HCF-1)-mediated recruitment of lysine-specific demethylase-1 (LSD-1) to viral immediate early promoters (26, 34) and HSV IE promoter repression by Nab2 (42), early growth response gene 1 (Egr-1) (2) or the REST/NRSF/CoREST complex (15, 35). Micro RNAs also play an important role in the transcriptional regulation of neurons (24, 44) and have been proposed to regulate productive HSV infection by silencing the expression of ICP0 (43, 45) and/or ICP4 (40, 45).

In the current study we also demonstrated that HSV-1 established a quiescent infection with LAT expression in cultured adult murine trigeminal ganglion neurons and that the quiescent infection, like latent infection in vivo, represented a reactivation competent state. Of interest, only about one third of the quiescently infected neurons had detectable expression from the viral LAT promoter. These data are consistent with previously reported studies in which approximately one third of the infected cells express LAT at latent time points (8, 30). We have previously shown that the LAT region, specifically exon 1, appears to regulate the neuron type-specific establishment of latency (5), and it is well known that a number of different host cell factors, including Oct-1, HCF-1, Sp1, CREB, GRB-2, EGR-1, AP2 and AP1, regulate gene expression from this region of the viral genome (reviewed in Millhouse 2000(32)). We were able to reactivate HSV-1 from its quiescent state by treating the cultures with TSA, a histone deacetylase inhibitor. However, HSV-1 did not reactivate equivalently from all types of neurons in response to the TSA treatment. A5+ neurons, which were non-permissive for productive infection at earlier time points, were also largely non-permissive for reactivation induced by the histone deacetylase inhibitor, indicating that either A5+ neurons may be completely non-permissive for HSV-1 replication and reactivation in general, or that mechanisms other than
those involving histone deactylases are responsible for inducing reactivation in A5+ neurons. Our studies did not, however, address the possibility that the reactivating cell population derives from a proportion of neurons that survived initial lytic infection or if this population initially established quiescence immediately upon infection. Since LAT is not equally expressed in all latently infected neurons and the virus does not equally reactivate from all neuronal populations, dissociated adult trigeminal neurons that maintain neuronal heterogeneity in culture will likely be useful for determining the specific mechanisms regulating LAT expression and the role it plays in establishment of latency and reactivation from a latent state.

Although previous in vitro models of HSV neuronal infection have been valuable for studying several aspects of HSV pathogenesis, most are limited or restricted in their use for investigating the mechanisms regulating preferential establishment of latency in different neuronal subtypes. We consider that our in vitro model of HSV infection detailed here using dissociated adult murine trigeminal neuron cultures is superior in many respects. These cultures maintain neuronal heterogeneity with proportions of A5+ and KH10+ neurons nearly identical to those found in vivo. Furthermore, HSV infection of these neuronal cultures leads to a heterogeneous outcome, with productive infection in some neurons and a quiescent infection in others, without the use of acyclovir to suppress lytic growth. Thus, the system that we have developed models a number of in vivo characteristics of HSV infection not modeled by PC12 cells, sympathetic neurons, or embryonic/neonatal sensory neurons. However, it is important to point out that the neuronal culture system that we have described for studying HSV infection is not without its limitations. First, we found that we were unable to maintain quiescently infected neurons for extended periods of time at higher MOI, as has been reported for embryonic cultures (50, 51) or differentiated PC12 cells (9). However, unlike these prior studies, we did not attempt to use acyclovir to induce or maintain a quiescent state. Second, by five days post-inoculation, the vast majority of infected neurons no longer expressed productive cycle genes, similar to previous reports of in vitro infection of neonatal DRGs and TGs (1, 11). However, we found
substantial well-to-well variability, and GFP-positive plaque-like formations occurred periodically after Day 5 post-inoculation. The relatively low frequency, and clustering, of GFP positive neurons at these later time points in cultures previously negative for GFP expression, likely represents spontaneous reactivation events, similar to those that occur in vivo. These spontaneous reactivations appeared to originate from an individual GFP-positive neuron and spread to adjacent neurons, with spread presumably limited by the pooled immunoglobulin in the culture medium. Third, we observed that satellite glial cells (SGCs) became infected early in infection and transformed into phagocytic cells, significantly reducing neuronal cell survival in cultures infected at high MOIs. While the focus of this report is not the SGCs, these observations suggest that SGCs play an important role in clearing virus in the ganglia but may also exacerbate neuronal damage in response to viral infection, consistent with previous reports (20, 46). However, resident SGCs could not be removed from the cultures entirely without jeopardizing the health of the neurons.

In summary, we have shown that in cultured dissociated adult sensory neurons, the A5+ neurons are relatively non-permissive for productive infection with HSV-1, a finding that correlates with selective establishment of HSV-1 latency of A5+ neurons in vivo. Using the model culture system described above, we are now poised to efficiently dissect the mechanisms that regulate preferential productive or latent infection in specific types of neurons.

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FIGURE LEGENDS

Figure 1. A5+ and KH10+ neuron distribution in uninfected neuron cultures. A) Percentage of A5+ and KH10+ adult murine trigeminal neurons *in vivo* (tissue sections), dissociated adult trigeminal neurons in cultures *in vitro*, and dissociated neonatal trigeminal neurons in cultures *in vitro*. B) Representative fluorescent microscopy images of cultured adult trigeminal neurons positive for the A5 (top) and KH10 (bottom) markers using monoclonal antibodies and a rhodamine-labeled secondary antibody.

Figure 2. Percentage of neurons expressing GFP following infection with HSV1-VP26-GFP and HSV2-VP26-GFP. Neuronal cultures were infected with HSV1-VP26-GFP or HSV2-VP26-GFP (MOI 100, 30, 10) for 10, 15, or 24 hours and assayed for GFP expression as a marker of productive infection. Both viral constructs express a VP26-GFP fusion protein during active viral assembly, permitting GFP visualization during productive infection. Results reflect data from six separate experiments for each virus for the 10 and 15 hour time points and two experiments for each virus for the 24 hour time point. Between 2636 and 12027 neurons were assayed for each time point.

Figure 3. Percentage of A5+ and non-A5+ neurons expressing GFP following infection with HSV1-VP26-GFP and HSV2-VP26-GFP. A) Neuronal cultures were infected with HSV1-VP26-GFP or HSV2-VP26-GFP. A5+ and non-A5+ neurons were evaluated for GFP expression as a marker of productive infection at 10 hours post-infection. Results reflect data from two separate experiments for each MOI. A minimum of 3520 total neurons were assayed for each MOI. B) Fluorescent microscope images of A5+ neurons (red) with HSV1-VP26-GFP productive infection (green). Inserts are higher magnification images of A5+ neurons. HSV1-VP26-GFP productive infection, indicated by GFP expression, was frequently found in non-A5+ neurons adjacent to non-productively infected A5+ neurons while HSV2-VP26-GFP was found in A5+ neurons as well as non-A5+ neurons.
Figure 4. Percentage of neurons expressing β-galactosidase following infection with KOS/58. β-gal expression, driven by the neurofilament promoter at the HSV-1 gC locus, was detected in A5+ and non-A5+ neurons using immunofluorescence with mAb A5 and an anti-β-gal polyclonal antibody. Experiments were carried out in duplicate and a minimum of 3726 neurons were assayed for each MOI.

Figure 5. Viral gene expression in A5+ and Non-A5+ neurons. Neuronal cultures were infected at an MOI of 30 with viral constructs expressing EGFP under immediate early (ICP0), early (gB), and late gene (gC) promoters, inserted at the gC locus. A5+ and non-A5+ neurons were evaluated for EGFP expression. A) RE-pICP0-EGFP for 6 hours, RE-pgB-EGFP for 8 hours and RE-pgC-EGFP for 10 hours. Results reflect data from five separate experiments with ~4100 to 9300 neurons assayed for each virus studied. B) RE-pICP27-EGFP and RE-pICP4-EGFP for 6 hours. Results reflect data from three separate experiments with ~4100-4200 neurons assayed for each virus studied. C) Fluorescent microscope images of A5+ neurons (red) with EGFP expression (green) after infection with RE-pICP0-EGFP (top), RE-pgB-EGFP (middle) or RE-pgC-EGFP (bottom), as well as corresponding brightfield images.

Figure 6. Viral gene expression from KOS/58, KOS/62 and HSV1-VP26-GFP. A) Neuronal cultures were infected with KOS/58, KOS/62 or HSV1-VP26-GFP (MOI 10) and evaluated for β-gal or GFP expression five days post-inoculation. B) β-gal+ neurons (blue) 5 days post infection with KOS/58 (β-gal from neurofilament light promoter). C) β-gal+ neurons (blue) 5 days post infection with KOS/62 (β-gal from LAT promoter). D) Five days after infection with HSV1-VP26-GFP (MOI 10), cultures were treated with TSA or control medium containing no TSA, and A5+ and non-A5+ neurons were evaluated for GFP expression 24 hours later. E) GFP expression in a cultured neuron 24 hours after TSA treatment of an HSV1-VP26-GFP quiescently-infected culture. Results reflect data collected from two or more separate experiments with a minimum of 1503 total neurons assayed for each virus.
REFERENCES


Figure 1
Figure 3

![Bar chart and images showing A5+ HSV1-VP26-GFP Brightfield and A5+ HSV2-VP26-GFP Brightfield AB]