Cultured Vestibular Ganglion Neurons Demonstrate Latent HSV1 Reactivation

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Objectives/Hypothesis: Vestibular neuritis is a common cause of both acute and chronic vestibular dysfunction. Multiple pathologies have been hypothesized to be the causative agent of vestibular neuritis; however, whether herpes simplex type I (HSV1) reactivation occurs within the vestibular ganglion has not been demonstrated previously by experimental evidence. We developed an *in vitro* system to study HSV1 infection of vestibular ganglion neurons (VGNs) using a cell culture model system.

Study Design: basic science study.

Results: Lytic infection of cultured rat VGNs was observed following low viral multiplicity of infection (MOI). Inclusion of acyclovir suppressed lytic replication and allowed latency to be established. Upon removal of acyclovir, latent infection was confirmed with reverse-transcription polymerase chain reaction and by RNA fluorescent *in situ* hybridization for the latency-associated transcript (LAT). A total of 29% cells in latently infected cultures were LAT positive. The lytic ICP27 transcript was not detected by reverse-transcription polymerase chain reaction (RT-PCR). Reactivation of HSV1 occurred at a high frequency in latently infected cultures following treatment with trichostatin A (TSA), a histone deactylase inhibitor.

Conclusions: VGNs can be both lytically and latently infected with HSV1. Furthermore, latently infected VGNs can be induced to reactivate using TSA. This demonstrates that reactivation of latent HSV1 infection in the vestibular ganglion can occur in a cell culture model, and suggests that reactivation of HSV1 infection a plausible etiologic mechanism of vestibular neuritis.

Key Words: Vestibular neuritis, herpes simplex virus type I, viral reactivation, viral latency, latency-associated transcript.

Level of Evidence: basic science study.

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INTRODUCTION

Vestibular neuritis is the second most common cause of unilateral peripheral vestibulopathy. It is characterized clinically by vertigo lasting days accompanied by autonomic symptoms (nausea, vomiting, and diaphoresis), and by the lack of symptoms of hearing loss. Most patients are able to achieve central compensation for this peripheral vestibulopathy after a few weeks; however, some patients have lingering symptoms of imbalance and unsteadiness years later due to incomplete compensation.[1] Both the acute syndrome as well as the chronic manifestations result in significant expenditure of healthcare dollars as well as economic losses from absenteeism and impaired work performance of affected individuals.

Multiple etiologies have been proposed for vestibular neuritis;[2] however, vestibular neuritis is most commonly hypothesized to be the result of infection of the vestibular nerve with a neurotrophic virus, herpes simplex virus type 1 (HSV1).[3] Infection by HSV1 is extremely common, with a 50% to 90% prevalence by age 20 to 40 years in developed countries.[1] Following primary exposure in childhood, the virus lies dormant in sensory ganglia in a latent state until reactivated. A variety of stimuli, including stress, ultraviolet light, fever, and tissue damage can trigger reactivation of virus, leading to clinical manifestations of viral infection.[4]

Vestibular ganglia can be primarily infected with HSV1 in murine models, leading to peripheral vestibulopathy in these animals.[5–7] These models are marked by high animal mortality and difficulties pursuant upon working with an *in vivo* model system in terms of determining cellular triggers and intracellular signaling mechanisms that lead to HSV1 reactivation. Previous work indicated that HSV1 quiescent infection can be established in primary neuronal cell cultures of sensory neurons (dorsal root ganglia and superior cervical

sympathetic ganglia).[8–10] However, it has not been determined whether quiescent infection could be established in VGNs or whether a productive HSV1 infection could later be induced in those neurons. VGNs differ substantially from other peripheral ganglion neurons in terms of function, connectivity, and neurotrophin receptor expression and requirements.[8–10]

In this study we explore the potential of HSV1 to cause both lytic and latent infections in primary VGN cultures. We demonstrate that both types of HSV1 infection occur under our culture conditions, and that reactivation can be triggered in latently infected VGNs. These findings lend increased credence to the hypothesis that HSV1 infection may be the etiologic agent of vestibular neuritis.

METHODS

Herpesvirus Stock Preparation

HSV1 Patton strain was modified by adding a green fluorescent protein (GFP) tag to the U_S11 late protein (HSV1-GFP).[11] The U_S11 protein is not required for productive viral growth in culture, and fusion of the GFP coding sequence in frame with the initial ATG of the U_S11 protein does not affect viral replication in cultured neurons.[12]

For production of virus, Vero cells (a monkey kidney epithelial cell line used commonly in HSV1 experiments) were cultivated for 3 days in Dulbecco's Modified Eagle Media (DMEM: Invitrogen, Carlsbad, CA) with 1% calf serum (Invitrogen). On day in vitro (DIV) 4, the cultured cells were treated with a low multiplicity of infection (MOI = 0.001) of virus diluted in minimal essential media (MEM; Invitrogen) for 1 hour at 37°C. The diluted virus was removed, and fresh DMEM/1% calf serum was replaced on the cells. Infected cells were incubated for 4 days at 32°C. Cell-free lysates were prepared by freeze thawing. Viral stocks were quantified by plaque assay on Vero cells.

VGN Purification and Cell Culture

Following administration of anesthesia, superior and inferior vestibular ganglia were harvested via modified middle fossa approach from postnatal Sprague-Dawley rat pups of postnatal age 5 to 6 days. The ganglia were treated with 0.0125% trypsin and 0.01% collagenase (Sigma, St. Louis, MO) for 15 minutes at 37°C. The reaction was stopped by adding 1/10 volume of fetal bovine serum (FBS). The vestibular ganglia were triturated using a P1000 pipette tip. This generated cultures containing neurons, Schwann cells, and fibroblasts. Cells were filtered through a 70-micron cell culture filter (BD Falcon, Bedford, MA) and plated on 96-well cell culture plates coated with polyornithine (0.1 mg/cc; Sigma) and laminin (20 μg/cc; Sigma) at a concentration of 9 to 18 neurons/microliter (630–1,260 neurons per well) at 37°C/5% CO_2. Cell culture media was Neurobasal media (NBM; Invitrogen) supplemented with B27 additives (Invitrogen) with 5% FBS (Invitrogen), 20 μM Z-VAD-FMK (Z-VAD: Calbiochem, La Jolla, CA), and ofloxacin (0.0003%: Daiichi Pharmaceutical Corp., Montvale, NJ). Cell cultures were treated with 10 μM 5-fluoro-2′-deoxyuridine (5-FU: Sigma) and 10 μM aphidicolin (Calbiochem) for 2 days to kill rapidly dividing cells. FBS and aphidicolin were removed from the cell culture media at DIV 2. Following treatment with 5-FU, aphidicolin, and serum starvation, nonneuronal cells remaining in the cultures remain viable but do not divide.

Induction of Primary Lytic HSV1 Infection

At DIV 4, VGN cultures were infected with HSV1-GFP. Using a titer experimentally determined using Vero cells, the virus was diluted to an MOI = 1 (by count of neuronal cells) in minimal media. The relative number of neuronal cells compared with other cells in the culture is 1 neuron to every 10 to 20 nonneuronal cells, lowering the approximated MOI relative to the total number of cells in the cultures. Additionally, previous studies have shown that HSV1 stocks titered on Vero cells have at least 10-fold decreased titer on neuronal cultures (unpublished observations). Thus, the effective MOI on these cultures is roughly 0.01. This was confirmed visually by evaluating GFP expression on lytically infected VGN cultures. The cell culture media was removed from the well and quickly replaced with the diluted herpesvirus. Cells were exposed to the virus at 37°C for 1.5 hours. Diluted virus was removed and replaced with fresh cell culture media (B27/Neurobasal media with 20 μM ZVAD, 5% FBS, and ofloxacin). At least two wells of each 96-well plate were treated in this manner as a positive infection control to determine the residual activity of the herpesvirus stock during the latency experiments described below.

Induction of Quiescent HSV1 Infection

Cultures were treated at DIV 2 with 100 μM acyclovir (ACV; Calbiochem) following serum starvation and removal of aphidicolin as described in the preceding section. At DIV 4, these neurons were infected with the same dilutions of HSV1-GFP virus as the primary infection control neurons. However, following removal of diluted herpesvirus after the 1.5-hour incubation, the fresh cell culture media used for these neurons included 100 μM ACV in addition to the other components (B27/Neurobasal media with 20 μM ZVAD, 5% FBS, and ofloxacin). The cultures were observed daily for GFP+ cells, which appear at a baseline in approximately 5% to 6% of wells. Wells that demonstrated GFP+ cells prior to removal of ACV were not used to determine the rates of viral reactivation.

Reactivation of Quiescent HSV1 Infection in VGNs

Following quiescent HSV1 infection and maintenance in culture for 4 days, cultures were inspected for presence of viral reactivation using fluorescence microscopy. Wells containing GFP-positive cells were not used for reactivation experiments. Culture media was changed to B27/Neurobasal media with 20 μMZVAD, 5% FBS, 1:1,000 dilution of ofloxacin, and 1 μM trichostatin A (TSA; Sigma). TSA-containing media was left on cultures for 30 minutes at 37°C, then replaced with media without TSA (B27/Neurobasal media with 20 μM ZVAD, 5% FBS, ofloxacin). Cultures were then checked daily for appearance of GFP-positive (GFP+) cells using fluorescence microscopy. Reactivation was scored by number of positive wells (e.g., number of wells containing GFP+ cells). A time course for these experiments is shown in Figure 1. Results from 24 different experiments were gathered and compared. Every experiment was performed on a different date with neurons dissected from different litters of animals. Each experiment consisted of an average of 5.7 wells of a 96-well plate treated with 1 μM TSA as well as an average of 5.7 wells of baseline controls (media changed but no TSA added). One experiment was excluded from final analysis because percent reactivation in baseline control wells was greater than two standard deviations from the mean.

Reverse-Transcription Polymerase Chain Reaction (RT-PCR) for Viral Transcripts

The three groups of cultures VGNs (noninfected, lytically infected, and quiescently infected) were established as described
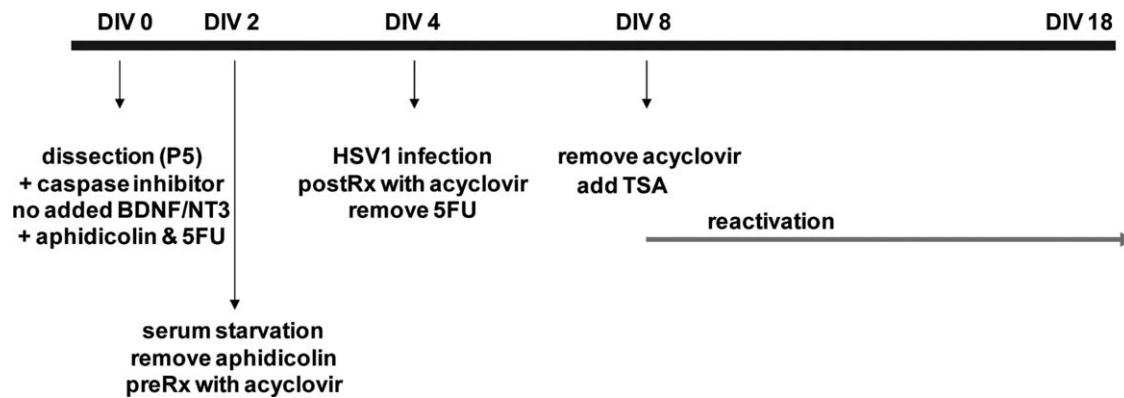
Fig. 1. Time course of viral reactivation experiments. Following harvest of VGNs from rats at postnatal age 5, cultures are grown in conditions to kill rapidly dividing cells, enriching them for neurons. Cultures are pretreated (preRx) with acyclovir (ACV) and then infected with HSV1. Treatment with ACV is continued for 4 additional days (postRx). Following withdrawal of ACV, cultures are treated with trichostatin A (TSA) to induce viral reactivation. Cultures are monitored with fluorescent microscopy for the expression of green fluorescent protein (GFP).

above. At DIV 19, cultures were inspected for the presence of GFP+ cells prior to RNA extraction. Total RNA was extracted from at least five wells/condition/sample (two to three samples per condition) using RNeasy microspin columns (Qiagen) per the manufacturer's instructions. Reverse transcription and polymerase chain reaction of the latency associated transcript (LAT) was performed using OneStep RT-PCR reagents (Qiagen, Chatsworth, CA) per the manufacturer's instructions and repeated two to three times per sample. LAT, GAPDH, and ICP27 primer sequences are listed in Camarena et al.[10] Reverse transcription was followed by amplification of target sequences. For LAT, the sequence was amplified using 35 cycles of 95°C for 30 seconds, 62.5°C 1 minute, then 72°C 1 minute, followed by a 10-minute extension at 72°C. For the GAPDH and ICP27 sequences, the amplification program was 95°C for 30 seconds, then 60°C 1 min, then 68°C 1 minute for 35 cycles, with a final 10-minute extension at 68°C. RT-PCR products were visualized on agarose gels following electrophoresis.

LAT In Situ Hybridization

VGNs were plated onto eight-well permanox plates (Nunc Nalgene, Naperville, IL) and latently infected with HSV1 as described above. Prior to *in situ* analysis, cultures were carefully inspected for GFP positivity. If positive, the cultures were not used for these experiments. At DIV19, 14 days following infection and 10 days following withdrawal of ACV, cultures were rinsed with HBSS (Gibco, Gaithersburg, MD), and then fixed in RNAse-free 4% paraformaldehyde (EMS). Cells were rinsed in 2 mM $MgCl_2$ in PBS and equilibrated in 1× SSC for 10 minutes. A total of 80 ng single-stranded Salmon Sperm DNA (Gibco) and 10 μg yeast tRNA (Invitrogen) in 80% formamide/1× SSC were heated for 5 minutes at 90°C and then chilled on ice 3 minutes. An equal volume of hybridization buffer (10% dextran sulfate [Sigma]/40 μg/mL bovine serum albumin (BSA) [Roche, Indianapolis, IL])/20 mM ribonucleoside vanadyl complex (Sigma)/10 mM sodium phosphate buffer pH 7.0/2× SSC) was added, and the dish was incubated at 42°C for 1 hour. A total of 20 ng of DNA oligonucleotide was mixed with carrier (Salmon Sperm DNA/*Escherichia coli* tRNA, above mix: 1 ng probe). Four DNA oligomers (5 ng each) were used for each probe. Probes used for LAT detection were based on the HSV1 (strain 17) sequence (GenBank accession # NC_001806).[13] Each probe was single-stranded DNA and contained a 5′ fluorescein molecule (Invitrogen). Probe sequences are listed in Camarena et al., 2010.[10] The positive control probe was a single-stranded DNA poly(T)$_{25}$ with a 5′ fluorescein molecule (Invitrogen). Negative controls were cultures of VGNs that had not been infected with HSV1, but were otherwise treated identically to latently infected cultures. Probes were diluted 1:1 with 80% formamide/1× SSC per well, heated at 90°C for 5 minutes, then place on ice for 3 minutes. Equal volumes of hybridization buffer was added to the probe mix per well. Probes were added and the plate incubated at 42°C for 5 hours. Wells were washed with warmed 40% formamide/1× SSC at 42°C for 20 minutes, then with 1× SSC.

Immunofluorescence

Slides were equilibrated in 50 mMTris-HCl pH 7.4/150 mM NaCl (TBS50) buffer 5 minutes, and then cells permeabilized with 0.25% Triton X-100/TBS50 for 5 minutes. Slides were equilibrated with 200 mMTris-HCl pH 7.4/ 0.1 mM Glycine for 5 minutes and blocked with 2% BSA (Heat Shock Fraction V, Roche) in TBS50 (IF buffer) for 20 minutes. Primary antibodies (anti-FITC-antibody, 488 conjugated [Invitrogen;1:300 in IF buffer] and anti-NF200 antibody [Sigma; 1:2000 in IF buffer]) were added to wells at room temperature for 1 hour. Slides were then washed in TBS50 three times. Secondary antibody (Invitrogen; 1:500) was added for 30 minutes at room temperature in the dark. Slides were washed three times with TBS 50, once with PBS, and treated with Hoechst (Invitrogen; 1:5,000 in PBS) for 5 minutes, and mounted with Mowiol (Sigma).

Viral Titer Determination

Media was removed from lytically infected VGN cultures at day 4 following infection with HSV1 (DIV 8). Media was removed from latently infected VGN cultures at day 7 following infection and day 3 following removal of ACV (DIV11) for unreactivated cultures and at day 3 following application of TSA (DIV11) for treated cultures. Media was serially diluted and placed on confluent Vero cultures for 2 hours at 37°C. Subsequently, the viral media was removed and replaced with fresh Vero media. Plates were incubated at 37°C for 48 hours, then fixed with 10% trichloroacetic acid and stained with crystal violet.

RESULTS

Lytic infection of VGNs with HSV1

VGNs were harvested from the superior vestibular ganglia of postnatal day 5 rats and grown in culture in
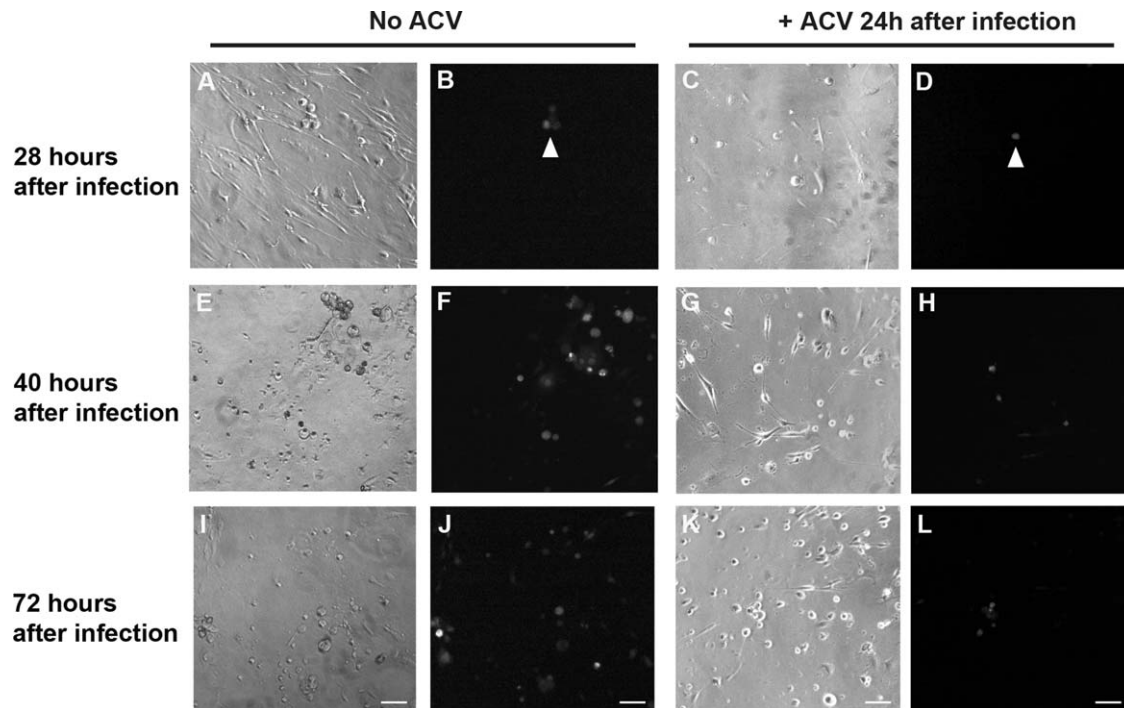
Fig. 2. VGNs lytically infected with HSV1. VGN cultures following lytic infection with HSV1/U$_S$11-GFP are shown in phase contrast microscopy (A, E, I) and fluorescent (B, F, J) photomicrographs, demonstrating progression of lytic infection throughout cultures. Following first appearance of green fluorescent protein (GFP)-positive cells, spread of infection can be halted through application of acyclovir (ACV) (C, D, G, H, K, L). Arrowheads indicate faintly GFP positivity in single cells during early infection. Size bar = 50 microns.

the presence of ZVAD, a general caspase inhibitor. At day *in vitro* 5 (DIV 5), the cells were infected with HSV1. Characteristic micrographs of these infections are shown in Figure 2. For these studies, an enhanced GFP-tagged HSV1 (HSV1-GFP) virus was used.[11] U$_S$11 is transcribed late during the viral lytic cycle, and so expression of the chimeric protein allows rapid identification of cells that have progressed to this advanced point in the productive growth cycle of the virus.[12]

Infection of VGNs with HSV1 yields a productive, lytic infection even in the presence of general apoptosis inhibitor ZVAD as shown in Figure 2. GFP+ cells can be detected with fluorescent microscopy as early as 16 to 18 hours following incubation of cells with the virus. At the MOI used in these studies, not every cell in the well is initially GFP positive, but all eventually express GFP over the course of 4 to 5 days. Typically neurons are the first cells which express GFP in each well, with other cells (Schwann cells and fibroblasts) becoming GFP+ as the infection progresses (Fig. 2A and B). Infection spread primarily to adjacent cells (Fig. 2E, F, I, and J), although occasionally spread appears to be mediated along neurites to more distant areas in the culture (data not shown). Cytopathic effects of viral infection are detected by light microscopy 24 hours after GFP positivity is noted. Cells became smaller and developed pyknotic nuclei prior to cell death (Fig. 2A, E, and I). Within lytically infected cultures, spread of infection was halted by application of 100 µM ACV 24 hours following initial infection (Fig. 2 C, D, G, H, K, and L). Thus, cultured VGNs are capable of supporting lytic replication of HSV1.

Quiescent Infection of VGNs with HSV1

To induce a latent or quiescent infection in the cultured VGNs, cultures were treated with ACV for at least 2 days prior to incubation with HSV1 at DIV 5. Acyclovir is a guanosine analogue that acts as a substrate for the viral DNA polymerase as well as a chain terminator, thus inhibiting viral DNA synthesis.[14] Following incubation with HSV1, the VGN cultures were treated with ACV for 4 days to prevent lytic infection (Fig. 1). A few wells showed GFP fluorescence despite treatment with ACV during these 4 days of postinfection ACV treatment (DIV 5–9). They were considered failures of primary suppression of lytic infection under these culture conditions. At DIV 9 (day 5 following latent infection with HSV1), ACV was removed from cell culture media. Under these conditions, baseline viral reactivation (as measured by presence of GFP+ cells by fluorescent microscopy) would occur in 15% of wells, typically on days 2–5 following ACV removal (DIV 11–14) (Fig. 3). Typically GFP would be detected in one or only a few neurons and then spread throughout the well. Rarely, the initial site of GFP positivity would be exclusively in a nonneuronal cell (fibroblast or Schwann cell) (data not shown). Sometimes early stages of spread would be restricted to VGNs that were connected via neurites, similar to that seen rarely with lytic infections.

RNA was collected from quiescently infected wells that showed no evidence of GFP+ cells by DIV 19 (day 14 following HSV1 infection and day 9 following removal of ACV from latently infected wells). Reverse-transcription polymerase chain reaction (RT-PCR) demonstrated

0h after ACV removal

20h after ACV removal + TSA treatment

44h after ACV removal + TSA treatment
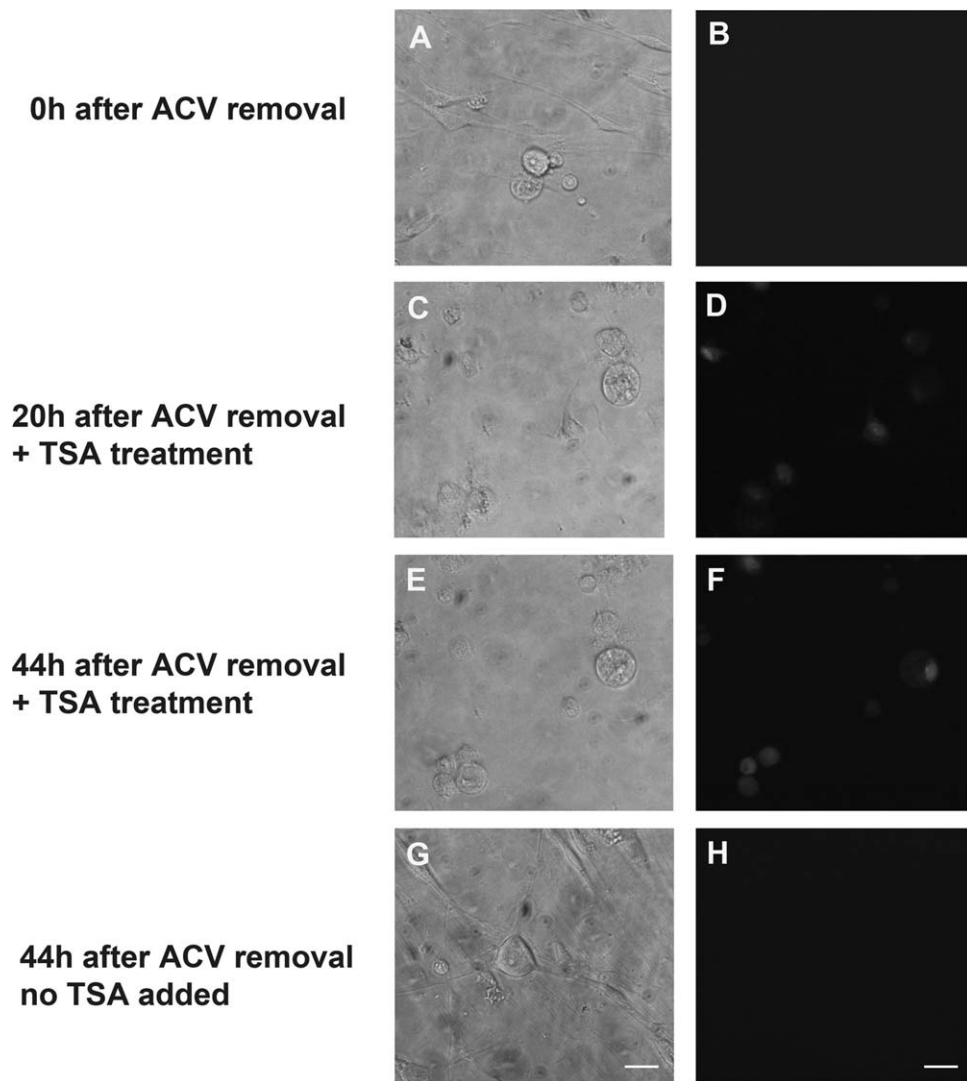
44h after ACV removal no TSA added

Fig. 3. Latent HSV1 infection and reactivation in cultured VGNs. Following establishment of latent HSV1 infection, acyclovir (ACV) is removed from cultures (phase contrast light microscopy [A], fluorescent microscopy [B]). Following application of trichostatin A (TSA), cytopathic viral effects and green fluorescent protein (GFP)-positive cells can be detected as early as 20 hours following treatment (C, D). These changes become more apparent and increasingly widespread as time progresses (E, F). The majority of untreated cultures will not show similar changes (G, H). Size bar = 20 microns.

the presence of LAT in quiescently infected cells (Fig. 4, lane 3). In these quiescently infected cells, there was no evidence of the lytic cycle ICP27 RNA (marker an immediate early mRNA) by RT-PCR. As expected, both ICP27 and LAT transcripts were present in lytically infected cultures (Fig. 4, lane 2). GAPDH transcripts provided a measure of RNA recovered from each sample.

During latent HSV1 infection, latency-associated transcript (LAT) microRNA is the only viral transcript produced.[15,16] To further characterize latent VGN infection *in vitro*, RNA FISH for the LAT transcript was performed on latently infected VGNs at DIV19, 14 days following infection with HSV1 and 10 days after withdrawal of ACV. Prior to *in situ* analysis, cultures were carefully inspected for GFP positivity, and if positive, were not used for these experiments. Single-stranded DNA probes were generated from the LAT intron sequence[13] and labeled at the 5′ end with fluorescein (Fig. 5). Negative controls included noninfected cells with LAT probes, latently infected and noninfected cells without LAT probes, and both latently infected and noninfected cells without antifluorescein antibodies. In

latently infected neurons, LAT transcripts should accumulate in the nucleus.[17] As expected, LAT transcripts were found in nuclei of latently infected VGNs as shown in Figure 5 (A–D). Analysis of latently infected cultures revealed 29% of cells with LAT transcript present in the nucleus (totaled over four separate experiments and 359 neurons). There were no LAT-positive nuclei in control, uninfected VGNs (0 of 331 neurons and 4 experiments) (Fig. 5 E–H).

Reactivation of HSV1 in Latently Infected VGNs

Baseline viral reactivation (as measured by presence of GFP+ cells by fluorescent microscopy) occurs in 5% of cells, typically on days 2–5 following removal of ACV (DIV 11–14) (Fig. 6A). Reactivation of HSV1 in previously published *in vivo* models and in PC12 cells can occur after treatment with trichostatin A (TSA), a type-I/II histone deacetylase inhibitor. TSA and sodium butyrate have been shown to reactivate lytic HSV1 infection in other systems.[18–20] Application of 1 μM TSA to latently infected cultures at DIV 9 yields viral
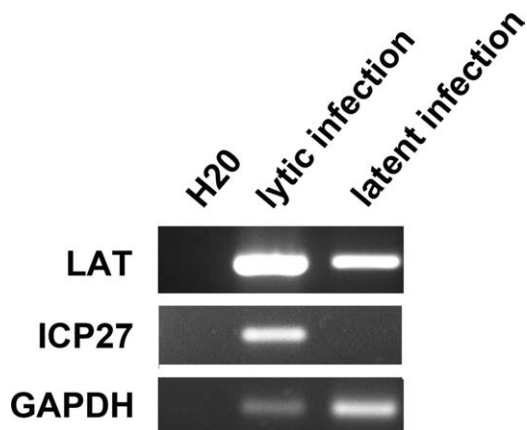
Fig. 4. Reverse-transcription polymerase chain reaction (RT-PCR) of LAT and lytic viral transcripts. Total RNA was obtained from cultured VGNs were treated as noted (lytically infected and quiescently infected). Endpoint RT-PCR was performed for LAT, viral lytic transcripts (ICP27), and intracellular housekeeping transcripts (GAPDH). As expected, the ICP27 and LAT transcripts are present in lytically infected cultures. For latently infected VGNs, LAT is present but ICP27 is not, confirming quiescent infection.

reactivation in 61.7% of wells by DIV15 (or the 10th day after application of TSA) (Fig. 6A). Higher doses of TSA result in cell death that does not allow for viral reactivation. Examination of cultures via light microscopy revealed that HSV1 can only reactivate in healthy VGNs.

Media from infected cultures was removed and the yield of infectious virus was determined by plaque assay (Fig. 6B). Lytic infection resulted in an average of 9.8×10^3 pfu/well. Following removal of ACV, latently infected cultures had no detectable infectious virus unless treated with TSA (average pfu/well $= 6.6 \times 10^3$), thus confirming the results of infection assays scored by GFP-positivity. More importantly, these HSV1 titer results demonstrate that latently infected VGNs do not produce detectable infectious virus unless induced to do so by application of TSA, a known reactivation stimulus.

DISCUSSION

Although many potential etiologies of vestibular neuritis have been suggested, there is a variety of clinical evidence suggesting HSV1 reactivation is at least one of the causative agents of this syndrome. HSV1 is found in 60% of postmortem adult VGNs.[21–24] Many patients with vestibular neuritis report a viral prodrome preceding the acute onset of vertigo,[25] consistent with HSV1 reactivation following an upper respiratory infection. Patients presenting with symptoms of vestibular neuritis also have been found to have multiple cranial neuropathies that are self-limited and not coincident with other known etiologies of these disorders, suggesting localized spread or reactivation of a neurotrophic virus.[26] Clinical courses of patients who have multiple episodes of vestibular neuritis are suggestive of HSV1 infection with multiple reactivation events as the cause of their symptoms.[1]

Electronystatograms of patients taken 2 days following labial outbreaks of HSV1 showed changes in 35 of 50 patients, and these changes resolved within 7 days.[27] Supporting the concept of HSV1 reactivation causing this syndrome, only 1 of 49 patients with vestibular neuritis had significant anti-HSV1 IgM levels.[28] This low rate of IgM positivity would be consistent with HSV1 reactivation, rather than primary HSV1 infection. One of the most striking pieces of clinical evidence is a case report documenting HSV1-positive cerebrospinal fluid in a patient who had vestibular neuritis followed 3 days later by temporal lobe encephalitis.[29] Additionally, in a small nonrandomized trial of patients with recurrent vertigo due to vestibular neuritis, 75% of patients had "complete or almost complete relief of their vertigo where no additional medications was required" during a
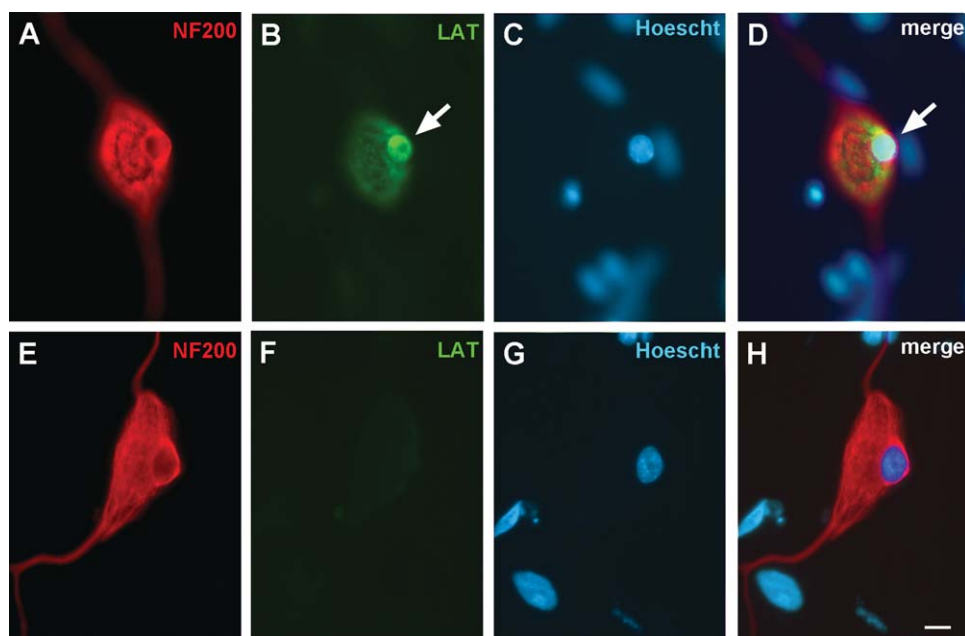

Fig. 5. LAT in situ in latently infected cultured neurons. VGN cultures latently infected with HSV1 were examined for presence of the latency-associated transcript (LAT) using RNA fluorescent in situ hybridization (FISH). LAT is the only HSV1 transcript produced in a latent HSV1 infection, so presence of LAT positivity indicates latently infected cells. All neurons were identified in the cultures as neurofilament 200 (NF200) positive (A). LAT positive neurons had increased signal in nuclei (B) as confirmed by nuclear stain (C). The merged image is shown in panel D. Arrows in the LAT and merged view windows indicate the LAT positive nucleus (B,D). In negative controls, there were no LAT-positive nuclei (E–H). Size bar = 10 microns.

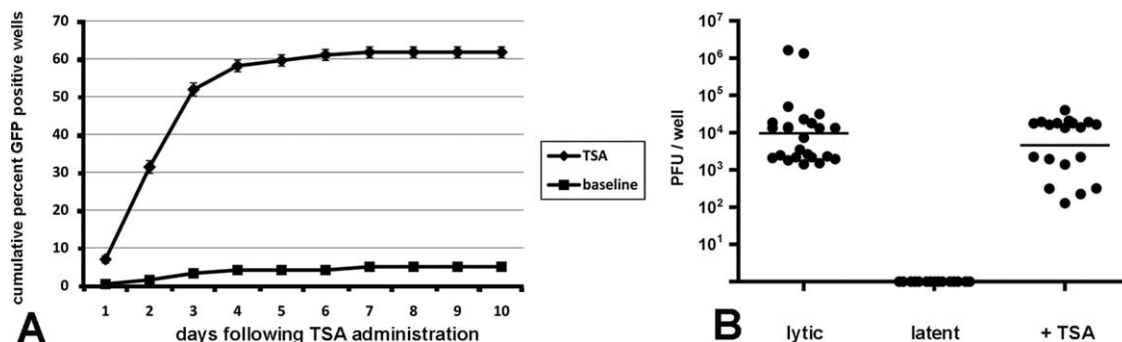Roehm et al.: HSVI Reactivation in Vestibular Ganglion Neurons

Fig. 6. HSV1 reactivation in quiescently infected VGNs. (A) VGN cultures latently infected with HSV1 were cultured in the presence of fresh media with or without trichostatin A (TSA) for 30 minutes, then culture media was replaced again. HSV1 reactivation was measured as a cumulative percentage of GFP+ wells as measured by fluorescence microscopy over 23 separate experiments. Reactivation rates following postacyclovir removal and treatment with 1 μM TSA averaged 61.7% (solid line). Negative control cultures (dotted line) had a HSV1 reactivation rate of 5%. Error bars = standard error of the mean. (B) VGN cultures were either lytically or latently infected with HSV1. Other latently infected cultures were treated with TSA. HSV1 titers of media taken from culture wells were measured by plaque assay on Vero cells.

6–20-month period following a 3-week treatment course of oral ACV.[30]

Human temporal bone studies have shown atrophy of the vestibular nerve and labyrinth, consistent with postinfectious changes, in patients who have suffered from vestibular complaints.[31–33] One study also found decreased synaptic density in the ipsilateral vestibular nucleus in addition to the findings of atrophy of the vestibular labyrinth and VGN on the same side that had previously shown absent ENG responses.[32] Suzuki et al.[22] found the HSV1 latency-associated transcript (LAT) in five of eight human VGN by reverse transcription-polymerase chain reaction (RT-PCR), showing that HSV1 can establish a latent infection in VGN.[22] Overall, these studies support the role of HSV1 infection reactivation in vestibular ganglia in some cases of human vestibular neuritis.

Infection by HSV1 is extremely common, with 80% to 90% prevalence by age 20 to 40 years in developed countries. Primary exposure typically occurs in childhood and is asymptomatic. Following initial exposure, the virus reaches sensory ganglia via retrograde neuronal transport. There the virus multiplies in a small number of cells and remains in a latent state for the remainder of the life of the host cell. Stress, ultraviolet light, fever, and tissue damage, among other nociceptive stimuli, can also trigger reactivation of virus, leading to clinical manifestations of reactivated viral infection, including herpes labialis.[4] Incidence of vestibular neuritis plateaus at 40 to 50 years of age, many years after HSV1 seroconversion.[34] Thus, reactivation of HSV1, rather than primary infection with HSV1, better fits the peak age prevalence of vestibular neuritis.

Signs of vestibular neuritis have been generated in animal models of HSV1 infection, including head deviation and nystagmus to the side inoculated with HSV1, and corresponding localization of histopathologic changes and HSV1 antigens to ipsilateral VGN. In a murine model generated by postauricular inoculation with HSV1, mice showed degeneration of VGN cells and HSV1 antigens in the ipsilateral VGN.[5] Similarly, in a rat model developed using middle ear injection of HSV1, VGN were positive for HSV1 DNA by PCR in actively infected animals and HSV1 DNA and LAT (by RT-PCR) in latently infected animals.[6,7] These studies demonstrate that lytic HSV1 infection can mimic some of the clinical manifestations of vestibular neuritis. Similar effects would be seen following reactivation of latent HSV1 infection in VGNs. However, reactivation of latent HSV1 infection of VGN in *in vivo* model systems has not been reported.

There are only two previously published models of *in vitro* HSV1 lytic and quiescent infection in primary neuronal cell cultures. These are in dorsal root ganglia (DRG)[8] and cervical sympathetic ganglia (CSG).[9,10] Within these cultures, withdrawal of nerve growth factor (NGF) ends quiescence and leads to viral reactivation.[8–10] Other stimuli that lead to viral reactivation include forskolin, which increases cyclic adenosine monophosphate levels (cAMP)[35] and could potentially be related to effects of NGF withdrawal on neurons. Although these *in vitro* models have their own merits, they do not duplicate characteristics of the vestibular ganglion. VGNs have different biology, intracellular pathways, Trk receptor expression and connectivity.

We have demonstrated that both lytic and quiescent infection of primary VGN cultures is possible. Additionally, we have been able to reactivate HSV1 within quiescently infected VGN cultures using TSA, a histone deactylase inhibitor known to reactive HSV1. In other systems, TSA has been shown to reactivate lytic HSV1 infection in other systems by deacetylating viral DNA and allowing transcription of transcripts other than LAT.[18] Thus, our findings lend credence to the hypothesis that HSV1 reactivation may be the mechanism of vestibular neuritis. The application of our results to understanding of human disease is potentially limited by the fact that these studies have been performed on cultured rat vestibular ganglion neurons. However, vestibular neuritis is a syndrome that can only be indirectly studied in live patients and animals because it affects structures (the vestibular ganglia) that are deeply

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imbedded in the temporal bone. Additionally, previous *in vivo* animal studies of acute infection in vestibular ganglia have been limited by high animal mortality. Thus, the information derived from VGN cell culture greatly enhances understanding of the pathobiology of vestibular neuritis. Ultimately, this cell culture system can be utilized to answer specific questions about the reactivation of HSV1 in VGNs.

The presence of the general caspase inhibitor Z-VAD in the cell culture media does not interfere with establishment of lytic viral infection. In fact, Z-VAD may serve a similar role to that of HSV1 proteins U_S3 and ICP27, which are involved in blocking apoptosis.[36,37] The expression of these HSV1 proteins and mRNAs allow viral replication to continue and completion of the viral lifecycle in cells that otherwise would die due to apoptosis triggered by viral infection. Reactivation of HSV1 in the presence of this antiapoptotic agent, as well as absence of evidence of viral reactivation (GFP fluorescence) in cells displaying evidence of death by light microscopy, suggests that cell death is not a trigger a priori for HSV1 reactivation.

CONCLUSIONS

In vitro establishment of persistent, nonreplicating HSV1 infection that resemble the latent state observed *in vivo* has been reported in the literature since 1987.[9] However, this study represents the first report using VGNs, which have significantly different tyrosine kinase receptor complement, connectivity, and numerous other differences compared with previously studied neuronal systems. We have developed techniques to establish a quiescent HSV1 infection in cultured VGNs that resembles latency in human neurons *in vivo*. These cultures stably maintain HSV1 until they are induced to reactivate. We propose that this system can be used to study the mechanism of HSV1 reactivation in VGNs, a process that may underlie some cases of vestibular neuritis.

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