Spontaneous Reactivation of Herpes Simplex Virus Type 1 in Latently Infected Murine Sensory Ganglia

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Careful studies of mouse trigeminal ganglia (TG) latently infected with herpes simplex virus type 1 (HSV-1) indicate the presence of productive cycle viral gene products and persistent immune response, suggesting ongoing spontaneous viral reactivation in these tissues. In the present study we set out to determine whether infectious virus is present in murine TG latently infected with HSV-1 (KOS). At 37 days after ocular inoculation we found a small amount of infectious virus in ca. 6% of latently infected murine TG. Furthermore, the amount of infectious virus that we detected (PFU per viral antigen-positive neuron) was similar to that detected in acutely infected ganglia. We conclude that spontaneous reactivation of infectious HSV-1 occurs in the mouse TG and is likely the principal cause of viral protein expression in these tissues. We next examined the role of latency-associated transcript (LAT) in spontaneous ganglionic reactivation by examining ganglia latently infected with KOS dlLAT1.8, a LAT deletion virus. Through the use of immunocytochemistry we found that KOS dlLAT1.8 had a rate of spontaneous ganglionic reactivation very similar to that of HSV-1 (KOS). Studying spontaneous ganglionic reactivation of HSV in the mouse TG allows a direct study of viral reactivation from latently infected neurons without the potential confounders and complicating downstream events that accompany the study of viral reactivation by explantation or peripheral viral shedding. Since most cases of human viral shedding and reactivation are not associated with a known trigger, spontaneous ganglionic reactivation of HSV-1 may be a better model of human disease than existing models.

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Primary infection with herpes simplex virus type 1 (HSV-1) is characterized by viral replication at the site of inoculation. It is also characterized by retrograde axonal transport of the virus to corresponding primary sensory ganglia, where infection follows two very different pathways. In some neurons there is abundant expression of productive cycle viral genes, resulting in the generation of infectious viral progeny, whereas in other neurons a latent infection is established in which viral DNA is present but productive cycle genes are not expressed and infectious virus is not produced (13, 18, 33, 36, 39).

In humans, HSV-1 intermittently reactivates from latently infected primary sensory neurons. This can lead to peripheral shedding of infectious virus and, under certain conditions, recurrent peripheral disease in the form of a dermatitis, mucositis, or keratitis. Reported triggers for recurrent peripheral viral disease in humans include fever, UV light exposure, dental work, and trigeminal nerve surgery (34, 35). It is assumed that these stimuli lead to reactivation of latent virus with subsequent peripheral disease, but increasing evidence suggests that in most individuals HSV is shed frequently (~35% of the time for HSV-1 and 15% of the time for HSV-2) and in the absence of known triggers (11, 40). This suggests that viral shedding only gives rise to peripheral disease when local immunity, perhaps modulated by fever, UV exposure, or hormonal status, is unable to control viral exposure brought about by recurrent shedding.

A number of animal models of HSV-1 infection have been studied in order to elucidate the mechanisms that regulate HSV latency and reactivation (2). In the rabbit ocular model HSV-1 is shed from the surface of the eye both spontaneously and in response to local or systemic stimuli (10, 15). Although this pattern of viral reactivation closely resembles that in humans, the genetic variability of rabbits as well as their high cost has limited the use of this model. As a consequence, mouse models of HSV latent infection (after corneal or footpad dermal inoculation) are the most widely studied. These models have proven useful for studying stimulus-induced viral reactivation and shedding, but spontaneous shedding of virus in the periphery of a latently infected mouse occurs so rarely that it is impractical to study. In studies in which spontaneous shedding of HSV was detected on the murine ocular surface, the rate of shedding was less than once per 100 days (30, 38, 41).

Some investigators have taken advantage of HSV-1 infection in the mouse to study features of viral gene expression during latent infection, independent of spontaneous viral reactivation. However, a number of observations suggest ongoing spontaneous reactivation of infectious HSV-1 in murine sensory ganglia. First, cytokine expression and inflammatory infiltrates persist in these tissues (3, 8, 17). Second, treatment with the viral DNA polymerase inhibitor acyclovir reduces cytokine in these tissues (9). Third, productive cycle genes are transcribed...
and translated at low levels in these tissues (6, 14). Based on careful in situ hybridization studies, Feldman et al. estimated that productive cycle viral genes are expressed in at least one neuron every 10 days in trigeminal ganglia (TG) from mice latently infected with HSV-1 (6). However, in none of these studies did investigators look for the intermittent presence of infectious virus in latently infected TG.

In the present study we set out to determine whether small amounts of infectious virus are produced in latently infected murine TG and whether this occurs at the same frequency that we previously found ganglionic neurons that were positive for productive cycle viral gene transcripts and viral antigen. We further sought to determine whether the rate of spontaneous ganglionic reactivation differs in murine TG latently infected with KOS compared to TG latently infected with the latency-associated transcript (LAT) deletion virus, KOS d/LAT1.8.

MATERIALS AND METHODS

Animals, viruses, and inoculations. Six-week-old female outbred Swiss-Webster mice (Simonsen Labs, Inc., Gilroy, CA) were anesthetized by intraperitoneal pentobarbital, and their eyes were inoculated with 1.5 x 10^6 PFU of KOS HSV-1 as previously described (42). At either 4 or 37 days postinoculation (p.i.), the mice were euthanized by CO2 inhalation, followed by transcardiac perfusion with 0.1 M phosphate buffer with 0.9% NaCl (PBS). TG were dissected in a sterile fashion and stored at -80°C. This protocol was approved by the UCSF committee on animal research. In additional studies CD-1 mice were infected with wild-type KOS or KOS d/LAT1.8 viruses at 2 x 10^6 PFU/dye for 37 days.

Plaque assays. TG were defrosted, homogenized in 1 ml of minimal essential medium (MEM) in sterile glass tissue grinders, sonicated briefly, and suspended in 40 ml of MEM. The entire 40 ml of suspension from each TG was then inoculated onto monolayers of rabbit skin cells (four 10-cm petri dishes per TG homogenate). After a 1-h incubation at 37°C homogenates were poured off the monolayers and replaced with MEM containing 30 ng/ml of human immunoglobulin G (Sigma, St. Louis, MO). Three days later the monolayers were either stained with 1% crystal violet or fixed with 4% paraformaldehyde for immunocytochemistry.

Immunocytochemistry of plaque assays. Fixed monolayers were incubated with 5% normal goat serum in 0.1 M PBS, followed by a 1-h incubation with rabbit anti-HSV 1 antisera (Accurate Chemicals) diluted 1:300 in PBS with 1% normal goat serum in PBS. Monolayers were then incubated with biotinylated goat anti-rabbit antisera (Vector Labs, Burlingame, CA) diluted 1:200 in 1% normal goat serum-PBS, and the endogenous peroxidase activity was quenched by incubation with 3% H2O2. Antibody labeling was then visualized using the VectaStain Elite ABC kit (Vector Labs) according to the manufacturer’s directions.

Sectioning and staining of latently infected tissue. Mice were perfused and TG were dissected, fixed, and frozen as previously described (6). TG were embedded 10 per block in OCT compound (Miles, Inc., Torrance, CA), flash frozen, and serially sectioned at 20 µm. Sections were blocked for 15 min with 5% normal rabbit serum in 0.1 M PBS for 15 min, followed by incubation with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-HSV-1 antibody (Dako, Carpenteria, CA) or FITC-conjugated monoclonal anti-HSV-1 gC antisera (Virusys, Sykesville, MD) and biotinylated mouse monoclonal anti-Neuronal nuclei protein (Chemicon, Inc., Temecula, CA) for 30 min and a wash with 1% normal rabbit serum in 0.1 M PBS. Tissue sections were then incubated with 8 µg of Texas Red avidin DCS (Vector Labs)/ml in 0.1 M PBS for 10 min, washed in PBS, and then incubated for 30 min in PBS containing 50 ng/ml of To-PRO-3 iodide (Molecular Probes, Eugene, OR) before a final wash with PBS.

Confocal microscopy. Stained tissue sections of TG were evaluated with an Axioper 200 M inverted microscope (Zeiss, Inc., Jena, Germany). Confocal laser scanning image analysis was performed with a Zeiss LSM 5-Pa laser module controlled by Release 3.2 image acquisition and analysis software (Zeiss, Inc.). FITC signals were imaged at an excitation wavelength of 488 nm, Texas Red signals were imaged at an excitation wavelength of 543 nm, and TO-PRO-3 iodide signals were imaged at an excitation wavelength of 633 nm. Images were scanned using a single-channel, multitrack configuration.

RESULTS

We previously published data demonstrating HSV antigen-positive neurons in the TG of mice 37 to 47 days after ocular inoculation (6). In the present study we once again found HSV antigen-positive cells in the TG of latently infected mice at 37 days p.i. (Fig. 1). Based on cellular morphology these included HSV antigen-positive neurons (some in various stages of disintegration), as well as white cells and glia. This destruction of HSV-1 antigen-positive neurons with secondary spread of antigen to reactive glia and white blood cells suggests that not only are viral proteins expressed in rare neurons of “latently” infected ganglia but also that this process is associated with cellular destruction and subsequent spread to adjacent cells (glia and white blood cells). Bar, 50 µm.

FIG. 1. Immunohistochemical staining of TG 37 days postinoculation. Four examples of HSV-1 antigen staining in “latently” infected tissue. (A) HSV-1 antigen-positive neuron. (B) HSV-1 antigen and apoptotic cellular material surrounded by inflammatory cells. (C and D) HSV-1 antigen and cellular debris in foci of inflammation. These images suggest that not only are viral proteins expressed in rare neurons of “latently” infected ganglia but also that this process is associated with cellular destruction and subsequent spread to adjacent cells (glia and white blood cells). Bar, 50 µm.
infected TG at any point in time; this is very similar to the frequency with which we found HSV antigen-positive neurons in the TG of mice 6 to 7 weeks after ocular inoculation with the same virus (6).

The small number of plaques generated by “positive” homogenates of latently infected TG led us to investigate the relative sensitivity of this assay for the detection of virus generated by productively infected ganglionic neurons. Four days after the ocular inoculation with HSV-1, homogenates of 18 acutely infected TG were individually evaluated by standard viral plaque assay, and tissue sections of eight acutely infected TG were evaluated by immunohistochemical staining for HSV antigen-positive neurons. An estimate of the total number of HSV antigen-positive neurons in a TG was determined by counting the total number of HSV antigen-positive neuronal profiles in every fourth tissue section, a technique that we have used before to avoid double counting the same labeled neuron in consecutive tissue sections. Taking this approach, we detected an average of 3,025 plaques (range, 97 to 8,560) and 700 HSV antigen-positive per acutely infected TG, or 4.3 viral plaques for every productively infected neuron. Similar results were obtained when monoclonal antisera specific for a late viral gene (gC) was used in lieu of polyclonal anti-HSV-1 antisera (653 antigen-positive neurons). Although this approach yields only an estimate of the average amount of infectious virus detected per antigen-positive neuron at a single time point during acute infection, the results that we obtained are surprisingly similar to both (i) the amount of virus that we detected in “positive” TG at 37 days p.i. and (ii) the amount of infectious virus found in latently infected murine ganglia undergoing induced reactivation (20, 29, 30).

In a previous study (6) we were very strict with our criteria for identifying neurons that expressed productive cycle genes in latently infected TG. In order to be scored as “positive,” these cells were required to both be positive for viral mRNA or antigen in consecutive tissue sections and have the classic morphology of a ganglionic neuron. However, the altered morphology of reactivating neurons, as well as the inevitable loss and damage of sequential frozen tissue sections, may have led to a significant underestimate of the number of such neurons in latently infected TG. In an attempt to address this issue, we once again assayed latently infected TG for productively infected neurons, but this time in order to minimize the chance of undercounting productively infected neurons due to tissue loss or ambiguous neuronal morphology, we used confocal microscopy to assay 40-μm serial tissue sections costained with polyclonal antisera to both HSV and the neuron-specific marker neuN (19). Taking this approach, we identified five HSV antigen-positive neurons in 50 latently infected TG (Fig. 4). No ganglion contained more than one HSV antigen-positive neuron. This result correlates closely with the frequency with which we find infectious virus in latently infected ganglia and is very similar to that which we previously reported (6). It is therefore unlikely that our previous study underestimated the rate of spontaneous reactivation of HSV-1 in the mouse TG.

One feature of LAT deletion viruses is that they demonstrate reduced frequency or delayed kinetics of spontaneous and induced viral reactivation (reviewed in reference 2). We next sought to determine whether a LAT deletion virus has a similarly reduced frequency of spontaneous reactivation. To accomplish this, the corneas of CD-1 mice were inoculated

FIG. 2. Crystal violet staining of viral plaques from TG homogenates 4 and 37 days after ocular inoculation with HSV-1. The inset is a magnified view of the single plaque from the “latently” infected mouse at 37 days p.i.

FIG. 3. Immunostaining of viral plaques from TG homogenates 4 and 37 days after ocular inoculation with HSV-1. The inset is a magnified view of the single HSV-1 antigen-positive plaque from the “latently” infected mouse at 37 days p.i.

FIG. 4. Confocal images of a spontaneously reactivating neuron in a latently infected mouse trigeminal ganglion. (A) Nuclear staining with TO-PRO-3-iodide. (B) Neuronal specific staining with anti-NeuN antisera. (C) Staining of productively infected neuron with HSV-1 specific antisera. (D) Merged image. Bar, 100 μm.
with either HSV-1 KOS or KOS dlLAT1.8, a well-studied LAT deletion virus that establishes latency as efficiently as wild-type KOS (16). At 37 days p.i. the TG were fixed, removed, sectioned, and stained with polyclonal anti-HSV antisera as previously described (6). CD-1 mice were specifically chosen for the present study in order to match the experimental conditions previously used by Leib et al. (16) in their study of KOS dlLAT1.8. Analysis of the stained tissue sections in a masked fashion revealed evidence of spontaneous reactivation of both KOS and KOS dlLAT1.8 (Fig. 5). Furthermore, the rate of spontaneous reactivation was similar for the two viruses; six antigen-positive neurons in 38 TG latently infected with KOS dlLAT1.8 and six antigen-positive neurons in 42 TG latently infected with KOS. Thus, in contrast to the role that LAT has been reported to play in facilitating induced viral reactivation, we find no evidence that in the mouse LAT is required for efficient spontaneous reactivation as assayed by immunocytochemistry of the TG.

**DISCUSSION**

Intermittent viral reactivation from latency is a key feature of human infection with HSV-1 and is responsible for most of the morbidity caused by this virus, as well its epidemic spread. For this reason a significant research effort has focused on the mechanisms responsible for the regulation of HSV-1 latency in neurons, primarily in mouse models of viral infection. An unfortunate drawback to studying this process in the mouse has been the apparent lack of spontaneous viral reactivation and shedding. We recently published data describing productive cycle viral gene transcripts and viral protein in rare neurons of latently infected murine ganglia, thus providing evidence for spontaneous molecular reactivation of latent HSV-1 (6). In the present study we confirm and expand upon this observation, providing strong evidence for spontaneous reactivation of infectious HSV-1 in latently infected murine TG.

The amount of infectious virus that we detected in latently infected TG was at the lower limit of the sensitivity of our viral plaque assay but is unlikely to represent contamination. Not only did we fail to detect infectious virus in control ganglia from uninfected and sham-inoculated mice but the frequency that we observed infectious virus in latently infected TG closely matched the frequency with which we detected viral antigen-positive neurons in these ganglia. Furthermore, the amount of infectious virus that we detected in “positive” latently infected TG (2 PFU) correlated closely with the number of PFU generated per HSV-antigen positive neuron in both (i) acutely infected murine ganglia and (ii) latently infected murine ganglia undergoing induced reactivation (20, 29, 30). Taken together, these data indicate that infectious virus is present in latently infected murine TG and is most likely the result of spontaneous reactivation.

The relatively low level of infectious virus that we detected in “positive” ganglia may have several different explanations. First, ganglionic neurons may simply generate very little infectious virus in their cell bodies since viral capsid and membrane proteins are axonally transported out of the cell bodies separately (32) and thus not as an infectious unit. Second, a rapid immune response may severely limit the amount of time that reactivated infectious virus can persist in the ganglion, thereby decreasing the amount of infectious virus found at a single experimental time point. Third, ganglionic homogenates may contain substances that bind, neutralize, or inactivate infectious virus. Regardless of the explanation, since we were operating at the lower limit of sensitivity of our assay, it is likely that we underestimated both the percentage of latently infected ganglia that harbor infectious virus and the amount of infectious virus generated by spontaneous reactivation in these ganglia.

A key feature of spontaneous reactivation of HSV-1 in our model is that it is not a rare event. The data presented in our present and our prior study suggest a rate of spontaneous reactivation of at least once every 10 days, which in our model represents at least 15% of all latently infected TG neurons over the average 2-year life span of the mouse. This is similar to the rate of spontaneous shedding of HSV-1 onto the ocular surface of rabbits (22) and more frequent than the average rate of spontaneous shedding of HSV DNA in the human eye, mouth, or genital tract (11, 40). Furthermore, the rate of spontaneous viral reactivation in our model is only about half of that reported for hyperthermia-induced reactivation of HSV-1 (KOS) from the mouse TG (27, 28).

The data and conclusions presented in the present study help to reconcile a number of previously published observations about latent infection of murine ganglia with HSV-1. These include reports of (i) rare spontaneous shedding of virus onto the ocular surface of latently infected mice (30, 38, 41), (ii) persistent inflammatory cell infiltrates (3, 8, 17, 31) and cytokine expression (3, 8) in latently infected murine TG, (iii) PCR-detectable immediate-early and early viral gene transcripts in latently infected TG (12, 14), (iv) rare HSV antigen-positive neurons in latently infected ganglia (6, 26), (v) reduced cytokine expression in latently infected TG under conditions that limit viral DNA replication (4, 9), and (vi) rare viral PFU in latently infected ganglia serving as negative controls for studies of HSV reactivation (25, 26). All of these observations can be explained by spontaneous reactivation of latent virus from TG neurons.

Spontaneous viral reactivation in neurons of the murine TG may prove to be a more accurate model of HSV-1 reactivation...
in humans than other commonly used murine and rabbit models. Although trauma, fever, UV exposure, and hyperthermia have all been reported as triggers of reactivation of HSV-1 from the human TG, the vast majority of episodes of recurrent HSV cannot be tied to a specific trigger (37, 40). Thus, most cases of recurrent human HSV-1 are either spontaneous events or a consequence of the uneventful stresses of day-to-day living, making spontaneous viral reactivation in the mouse an attractive model for human disease.

In considering our results it is important to entertain the possibility that the very small amount of virus that we found in “latently” infected TG might represent residual virus from the initial productive infection of the TG rather than viral reactivation from a latently infected ganglionic neuron. Although an interesting alternative, this hypothesis does not explain our in situ hybridization (6) and immunocytochemical data or the tight correlation between the percentage of ganglia with infectious virus and those with neurons expressing productive cycle viral genes. It also does not explain why the amount of infectious virus that we detected in positive ganglia closely matched the amount of infectious virus generated per neuron during both acute ganglionic infection and induced reactivation (20, 29, 30). Finally, this hypothesis implies that the virus has evolved mechanisms that allow it to persist as an infectious particle in an immune activated ganglion for greater than a month, mechanisms distinct and in addition to the very successful strategy of viral latency and reactivation.

In contrast to the role that LAT has been reported to play in facilitating induced viral reactivation in the mouse and rabbit and spontaneous shedding of virus on the ocular surface of the rabbit (2), our data do not support a role for LAT in facilitating spontaneous reactivation in the mouse. This cannot simply be attributed to the use of a different viral construct, mouse strain, or means of ocular inoculation than previously studied, since under virtually identical conditions Leib et al. (16) reported that the rate of explant reactivation of KOS dl-LAT1.8 from latently infected TG is significantly less than that with either wild-type KOS or an appropriate viral rescuant. It is more likely that the region of the virus deleted in dl-LAT1.8 plays little, if any, role in spontaneous viral reactivation, but this can only be ascertained if one studies this process directly at the site of reactivation rather than at a downstream surrogate event such as ocular viral shedding; an outcome that reflects not only viral reactivation at the neuronal cell body but multiple downstream events. Although it is probably not wise to rely on a single experimental model of human HSV disease, given the logic of direct observation of a spontaneous event (rather than a downstream surrogate event), we feel that it is important to consider the relevance of studying spontaneous reactivation in the mouse TG to gain a broad and accurate understanding of the mechanisms involved in latency and reactivation. This is particularly important in light of our rapidly evolving understanding of the multifunctional nature of the LAT region of the viral genome, which appears to code for transcripts in both the sense and the antisense directions (24), including functions that inhibit apoptosis (7, 23), interfere with interferon expression (21), inhibit transactivation by ICP0 (5), and stimulate the expression of heat shock proteins (1); functions that should all be altered by the deletion in KOS dl-LAT1.8.

It is important to point out that our studies with KOS dl-LAT1.8 were carried out by assaysing latently infected ganglia by immunocytochemistry for HSV antigen-positive neurons rather than for infectious virus. We felt comfortable with this approach since data from the present study as well as that published previously (6) demonstrate a tight correlation between the frequency that antigen-positive neurons and infectious virus are found in latently infected TG. However, in the absence of studies of infectious virus in ganglia infected with KOS dl-LAT1.8 it could be argued that it would be more appropriate to conclude that the region of the virus deleted in KOS dl-LAT1.8 plays little, if any, role in spontaneous molecular reactivation, more restrictive terminology that we have used previously (6).

In conclusion, the data presented above provide clear evidence of frequent spontaneous reactivation of HSV-1 from latently infected neurons of the mouse TG. This process is relatively easy to study and can be carried out in a relatively efficient manner by batching ganglia and assaying relatively thick (35-μm) tissue sections. In theory this process could even be carried out with whole mounts, in a manner similar to that used by Sawtell (26). Studying spontaneous viral reactivation in this manner allows direct analysis of viral reactivation from neurons in vivo, thus avoiding a number of potential experimental confounders inherent to both explant reactivation and induced viral shedding on the ocular surface.

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