Herpes Simplex Virus Type 1 Corneal Infection Results in Periocular Disease by Zosteriform Spread

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In humans and animal models of herpes simplex virus infection, zosteriform skin lesions have been described which result from anterograde spread of the virus following invasion of the nervous system. Such routes of viral spread have not been fully examined following corneal infection, and the possible pathologic consequences of such spread are unknown. To investigate this, recombinant viruses expressing reporter genes were generated to quantify and correlate gene expression with replication in eyes, trigeminal ganglia, and periocular tissue. Reporter activity peaked in eyes 24 h postinfection and rapidly fell to background levels by 48 h despite the continued presence of viral titers. Reporter activity rose in the trigeminal ganglia at 60 h and peaked at 72 h, concomitant with the appearance and persistence of infectious virus. Virus was present in the periocular skin from 24 h despite the lack of significant reporter activity until 84 h postinfection. This detection of reporter activity was followed by the onset of periocular disease on day 4. Corneal infection with a thymidine kinase-deleted reporter virus displayed a similar profile of reporter activity and viral titer in the eyes, but little or no detectable activity was observed in trigeminal ganglia or periocular tissue. In addition, no periocular disease symptoms were observed. These findings demonstrate that viral infection of periocular tissue and subsequent disease development occurs by zosteriform spread from the cornea to the periocular tissue via the trigeminal ganglion rather than by direct spread from cornea to the periocular skin. Furthermore, clinical evidence is discussed suggesting that a similar mode of spreading and disease occurs in humans following primary ocular infection.

Following infection, herpes simplex virus (HSV) replicates in the epithelium, gains access to axonal terminae, and is retrogradely transported to sensory ganglia. A short period of viral replication within infected sensory ganglia is concomitant with the establishment of a nonproductive latent infection (22). During acute infection, HSV may reemerge from the nervous system and cause disease within the same dermatome at a location distal from the initial site of infection (4, 7, 18, 24, 32). This phenomenon, termed zosteriform spread, has been shown in mouse models to progress from flank to flank, snout to eye, mouth to eye, and neck to pinna. Spread requires an intact nerve supply and has been likened to viral reactivation in that the virus must traverse the nervous system to cause disease at a distal site (26–28). Zosteriform models have been used to determine the effects of antivirals, vaccines, or the immune response on disease (2, 8, 11, 15, 17, 21, 27, 28, 34).

The mouse eye model of HSV infection has provided much information regarding eye diseases such as stromal keratitis (13, 31). Little is known, however, about the progression to and cause of periocular diseases, such as blepharitis and conjunctivitis, despite their high prevalence in HSV-infected individuals (19). Primary corneal infection of mice with HSV-1 results in severe corneal and periocular disease (20, 32). Abundant progeny virus is observed in the eyes and periocular skin throughout acute infection, but due to the close anatomical proximity of these tissues the primary site of viral replication is unknown. Blepharoconjunctivitis, periocular hair loss, and ulcerative lesions become apparent 4 days postinfection and progress in severity until day 15, after which they resolve (29). Interestingly, recombinant viruses that replicate in the cornea but not in the nervous system fail to cause periocular disease (5). We hypothesize, therefore, that periocular disease occurs by viral spread from the cornea to the periocular tissue via the trigeminal ganglia rather than by direct spread from cornea to the skin.

In this study, recombinant reporter viruses were constructed which express luciferase or β-galactosidase under the control of immediate-early (IE) or early (E) gene promoters. These viruses were used to monitor and quantify viral gene expression and replication in the mouse ocular model. Reporter gene activity peaked in the eyes at 24 h, in trigeminal ganglia at 72 h, and in periocular tissue at 84 to 96 h. Periocular disease followed shortly thereafter. A thymidine kinase-deleted reporter virus expressed high levels of reporter activity in eyes, but no reporter gene activity was detectable in the trigeminal ganglia or periocular tissue. This demonstrates a requirement for viral replication within the nervous system for delivery to the periocular tissue and demonstrates that zosteriform spread from the cornea to the eyelids leads to periocular disease. In addition, we discuss clinical evidence that a similar progression results in human periocular disease.

MATERIALS AND METHODS

Cells and virus. African green monkey kidney (Vero) cells were propagated as previously described (23) and were used for the in vitro growth and reporter
assays of all viruses. Growth curve experiments were performed at the appropriate multiplicity of infection (MOI) on 24-well plates seeded 18 to 20 h previously with 10^5 Vero cells/well. All virus stocks were propagated on Vero cells as previously described (23). All viruses were constructed in the context of the KOS strain of HSV-1. The thymidine kinase null mutant, dl8.36tk, referred to in this study as KOS6bΔtk, has been previously described (16).

Plasmids and generation of recombinant viruses. A reporter plasmid, pDlux, was generated with both firefly and Renilla luciferase genes in a divergent orientation from a single multidonating site. The plasmid was constructed by NheI/SalI digestion of pGL3-Basic and pRL-null plasmids (Promega, Madison, Wis.) followed by isolation and ligation of regions containing the firefly and Renilla genes. A BamHI/NcoI 822-bp fragment encoding the origin of replication S (oriS) and flanking ICP4 and ICP22/47 promoters was isolated and blunt-end ligated into the NheI site pDlux to yield pDlux/oriS. The Dlux/oriS cassette and the previously characterized pDIIp cassette (10) encoding β-galactosidase under the regulation of the ICP6 promoter were cloned into the BglII site at position 106750 of plasmid pULIC to yield pULIC/Dlux/oriS and pULIC6 (D. J. Davido, D. A. Leib, and P. A. Schaffer, submitted for publication). pULIC-based clones were linearized with PstI and cotransfected with KOS infectious DNA. Resulting viruses were selected based on their ability to express either β-galactosidase or luciferase. Putative recombinant viruses were plaque purified three times, and identity was confirmed via Southern blot hybridization (data not shown).

Growth curve and gene expression analysis. Vero cells were seeded at 10^4 cells/well in 24-well plates. Eighteen to twenty hours postseeding, cells were infected with virus at an MOI of 0.1 or 5. After adsorption for 1 h at 37°C, virus was aspirated, cells were washed, and 1 ml of medium was replaced. Medium was removed at indicated hours postinfection, frozen, thawed, and titered on Vero cells. At the time of harvest, cells were lysed in 100 μl of Passive Lysis Buffer (Promega), frozen, thawed, and assayed for firefly and Renilla luciferase activity using the Dual Luciferase Assay kit (Promega) or for β-galactosidase activity using previously published methods (25). Cycloheximide reversal experiments were performed as described previously (30) with minor alterations. Briefly, pretreatment of cells with 100 μg of cycloheximide per ml for 1 h was followed by infection as above at an MOI of 5 in the presence of drug. Eight hours postinfection, cells were washed and medium was replaced with 10 μg of actinomycin D per ml. Twelve hours postinfection cells were harvested in 100 μl of Passive Lysis Buffer, and luciferase of β-galactosidase activity was measured.

Animal procedures. Outbred CD-1 female mice (body weight, 21 to 25 g; Charles River Breeding Laboratories, Inc., Kingston, N.Y.) were anesthetized with ketamine and xylazine, their corneas were bilaterally scarified, and they were inoculated with 2 × 10^6 PFU of virus in a volume of 5 μl as previously described (23). Tear film material was assayed for virus as previously described (23). Whole eyeballs, trigeminal ganglia, and 6-mm biopsy punches of periocular tissue were removed, placed in dry tubes containing 1-mm diameter beads, and frozen. Upon harvest, 100 μl of reporter buffer (50 mM HEPES [pH 7.5], 1 mM EDTA, 1% gelatin, 5% glycerol, 5 mM dithiothreitol) was added, and tissue was homogenized via beadbeating (Mini-Beadbeater-8; Biospec Products, Bartlesville, Okla.) and sonication. Ten microliters of homogenate was removed, added to 990 μl of medium, frozen, and thawed, and titers were determined on Vero cells. Twenty microliters of the same lysate was assayed for firefly and Renilla luciferase activity or β-galactosidase activity. Data were analyzed throughout using Student’s t-test.

Periocular disease was measured in a masked fashion on a semiquantitative scale as previously described (29). To measure growth in isolated periocular skin, mice were corneally infected with virus and sacrificed 12 h later. Periocular skin was harvested as described above and explanted in culture. Supernatants were harvested and titers were determined every 12 h postinfect.

RESULTS

In vitro replication and reporter gene expression kinetics. To validate the growth of the viruses used in this study, KOS, KOS6b, KOS6bΔtk, and KOS/Dlux/oriS were examined in Vero cells in both single- and multiple-step growth curves (Fig. 1A through D and 2A through C). Monolayers were infected at MOIs of 0.1 (data not shown) and 5 PFU/cell, and at various times postinfection supernatants were titered for infectious virus and cell monolayers were harvested to measure luciferase or β-galactosidase activity. The kinetics of viral growth and egress were similar among all viruses tested (Fig. 2A through C). In addition, reporter gene activity was detectable early in infection and rose concomitantly and proportionally with the detection of viral progeny.

In order to validate the expression kinetics of the recombi-
nant viruses, monolayers were infected in the presence or absence of cycloheximide (Fig. 3). All recombinant viruses exhibited expression of luciferase or β-galactosidase 12 h postinfection in the absence of cycloheximide. Infection in the presence of cycloheximide resulted in high levels of luciferase activity, but β-galactosidase activity was reduced to background levels. These data demonstrate that the luciferases in KOS/Dlux/oriS are regulated with IE kinetics, whereas β-galactosidase expressed by KOS6β and KOS6βΔtk is regulated as an E gene.

**Clinical disease in infected animals.** Mice were infected with KOS/Dlux/oriS, KOS6β, or KOS6βΔtk and scored for periocular disease using a masked, semiquantitative scale (Fig. 4). Little or no clinical disease was observed until day 5 despite detectable viral titers within the periocular tissue from 24 to 96 h postinfection. Clinical scores of mice infected with KOS/Dlux/oriS and KOS6β increased from days 5 to 8 and in accordance with previously published studies involving wild-type virus (29). KOS6βΔtk-infected mice, however, displayed no clinical disease at any time scored (Fig. 4).

**In vivo growth kinetics and gene expression.** To determine viral IE and E gene expression patterns during acute infection, mice were infected via the scarified cornea with KOS/Dlux/oriS and KOS6β (Fig. 5 and 6). At various times postinfection mice were sacrificed and titers were measured in whole globes, trigeminal ganglia, periocular skin, and the tear film via corneal swab. Reporter gene expression was also measured in the same samples harvested for viral titration in order to correlate the presence of virus with infectious centers within adjacent tissues.

The patterns of viral replication and reporter gene activity of

![FIG. 2. Single-step growth kinetics and reporter gene activity for KOS (A), KOS6β and KOS6βΔtk (B), and KOS/Dlux/oriS (C). Vero cells were infected at an MOI of 5. Data represent standard errors of the means of three independent experiments. The limit of detection is 10 PFU/ml. RLU, relative light units.](http://jvi.asm.org/)

![FIG. 3. Reporter gene activity under cycloheximide reversal or control conditions. Vero cells were pretreated for 1 h and then were infected at an MOI of 5 in the presence or absence of cycloheximide (100 μg/ml) for 8 h, after which time cells were washed and plated in the presence of actinomycin D (10 μg/ml) for 4 h. Monolayers were harvested and assayed for reporter activity. RLU, relative light units.](http://jvi.asm.org/)

![FIG. 4. Periocular disease scores in mice following corneal scarification and infection with 2 × 10⁶ PFU per eye of KOS/Dlux/oriS, KOS6β, and KOS6βΔtk. Animals were scored as follows: 0, no lesions; 1, minimal eyelid swelling; 2, moderate swelling and crusty ocular discharge; 3, severe swelling, moderate periocular hair loss, and skin lesions; 4, severe swelling with eyes crusted shut, severe periocular hair loss, and skin lesions. Data represent combined averages of at least 10 mice per time point.](http://jvi.asm.org/)
KOSβ were comparable to those of KOS/Dlux/oriS in all tissues tested (Fig. 5 and 6). Titers peaked in the eyes at 24 h and in the trigeminal ganglia at 72 h. Reporter activity of both luciferase and β-galactosidase followed the same trend of peak and kinetics. The relationship between reporter activity and viral replication in the periocular tissue differed from that seen in the eyes and ganglia. The initial high titers observed 24 h postinfection were accompanied by a small amount of reporter activity in the skin, which dropped to background levels after 36 to 72 h. At 84 and 96 h a significant increase of viral titer was accompanied by a precipitous peak of reporter activity coincident with early signs of periocular disease for both KOS6β and KOS/Dlux/oriS. The appearance of viral progeny, reporter activity, and disease symptoms is consistent with zosteriform spread of the virus from the cornea to the periocular tissue via the trigeminal ganglia.

An alternative route considered was direct spread of virus from the cornea to the periocular skin. Previous studies have used nerve transection to define pathways of viral spread in vivo (26, 27). Due to the delicate nature and inherent problems with such surgery on cranial and facial nerves, we utilized a genetic lesion at the thymidine kinase locus within the virus to rule out direct spread. Thymidine kinase mutants of HSV-1 grow to slightly reduced levels in the cornea but are unable to replicate in trigeminal ganglia (6). If direct spread is sufficient for acute infection in the periocular skin and development of disease therein, then a tk null virus will resemble wild-type virus during infection. If, on the other hand, replication in the trigeminal ganglia and anterograde return to the skin are required for disease, the tk null virus will be absent in the skin late during the assay period and, consequently, will be incapable of causing disease.

Patterns of KOS6βΔtk reporter activity and replication were similar to those of KOS6β in the tear film and whole eyes until 24 h, after which both rapidly dropped to background levels (Fig. 6). As expected, little or no replication and reporter activity was observed in the trigeminal ganglia of KOS6βΔtk-infected animals (6). In stark contrast to KOS6β in the periocular tissue, KOS6βΔtk failed to show any increase in levels of gene expression or titer between 72 and 96 h postinfection. In addition, no periocular disease was observed, despite inoculation of 30-fold more KOS6βΔtk than KOS6β. This lack of disease, β-galactosidase activity, and detectable titers strongly supports the hypothesis that replication in trigeminal ganglia and retrograde return to the skin is required for the replication and disease observed in the periocular tissue late in the assay period.

Previous reports have demonstrated that tk null viruses grow

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**FIG. 5.** In vivo growth and reporter gene expression of KOS/Dlux/oriS after ocular infection. Mice were infected via corneal scarification and inoculation of $2 \times 10^6$ PFU per eye. At various times postinfection tissues were harvested and assayed for infectious virus and luciferase activity. Data points represent 12 tissues from two independent experiments with three mice. In periocular skin, both luciferase activity and titer were significantly increased ($P < .05$) at 84 h relative to earlier time points. RLU, relative light units.
to slightly reduced levels in the cornea (6). Consequently, a growth defect of KOS6βΔtk within the periocular tissue could result in a lack of progeny virus and gene expression late in the assay period. To ensure both viruses could replicate to equivalent levels within the periocular tissue, mice were infected with KOS6β or KOS6βΔtk and periocular tissue was harvested and assayed for infectious virus and β-galactosidase activity. Data points represent 12 tissues from two independent experiments with three mice. In KOS6β infections, β-galactosidase activity and titer were significantly increased ($P < 0.05$) at 84 h relative to earlier time points and significantly higher than those of KOS6βΔtk ($P < 0.05$) from 72 h onward.

FIG. 6. In vivo growth and reporter gene expression of KOS6β and KOS6βΔtk after ocular infection. Mice were infected via corneal scarification and inoculation of $2 \times 10^6$ PFU of KOS6β or $8.5 \times 10^7$ PFU of KOS6βΔtk per eye. At various times postinfection tissues were harvested and assayed for infectious virus and β-galactosidase activity. Data points represent 12 tissues from two independent experiments with three mice. In KOS6β infections, β-galactosidase activity and titer were significantly increased ($P < 0.05$) at 84 h relative to earlier time points and significantly higher than those of KOS6βΔtk ($P < 0.05$) from 72 h onward.

FIG. 7. Growth of KOS6β and KOS6βΔtk in periocular tissue explants after ocular infection. Mice were infected via corneal scarification and inoculation of $2 \times 10^6$ PFU of KOS6β or $8.5 \times 10^7$ PFU of KOS6βΔtk per eye. Twelve hours postinfection tissue was harvested and explanted in medium. Supernatants were titers at various times postinfection. Data represent the mean of data from four mice from two independent experiments.

DISCUSSION

Reporter gene viruses have been previously utilized to monitor and elucidate viral promoter regulation (3, 14, 33). Recombinant viruses containing promoter-reporter cassette insertions into genes dispensable for growth (e.g., tk and gC) have been useful for elucidating promoter elements during the lytic life cycle in vitro. Such viruses, however, face limitations with respect to pathogenesis in vivo due to insertions into loci which may be virulence determinants. To address these limitations, we utilized a locus whose disruption did not significantly affect growth in vitro and pathogenesis in vivo (this study and data not shown). This locus was a BglII site (map position...
terized by a geographic corneal ulcer. A diagnosis of primary involvement relative to corneal infection strongly agrees with human ocular HSV disease in which the timing of periocular prevalence in HSV-infected individuals. We detail here a case of that periocular involvement following ocular infection is highly during recurrent episodes (19). This previous study suggests and conjunctival disease during their first episode and 31% of 122 HSV ocularly infected individuals, 54% exhibited eyelid Arch. Ophthalmol.

Yamamoto, Y. Shimomura, S. Kinoshita, and Y. Tano, Letter, vol. 112:1515–1516, 1994). In a case study of 122 HSV ocularly infected individuals, 54% exhibited eyelid and conjunctival disease during their first episode and 31% during recurrent episodes (19). This previous study suggests that periocular involvement following ocular infection is highly prevalent in HSV-infected individuals. We detail here a case of human ocular HSV disease in which the timing of periocular involvement relative to corneal infection strongly agrees with our observations in the mouse ocular model. An 18-year-old male presented to the San Francisco General Hospital Ophthalmology Clinic with an ocular infection which was characterized by a geographic corneal ulcer. A diagnosis of primary HSV geographic epithelial keratitis was made, and he was prescribed topical trifluoridine. He was seen again 5 days later, and upon examination his corneal ulcer had reduced in size by 50%, but he now had periorbital swelling and redness with discrete vesicles, characteristic of HSV, on the lid margin and brow (Fig. 8). The patient was treated with oral acyclovir and had an uneventful recovery. The timing and progression of symptoms in this case of primary ocular infection strongly correlate with the mouse data presented in this study, suggesting a similar mode of viral spread. This suggests parallel patterns of zosteriform spread within mice and humans and that treatment of primary infection with systemic, rather than topical, antivirals may serve to better control the spread of infection.

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