Persistence of Herpes Simplex Virus Type 1 DNA in Chronic Conjunctival and Eyelid Lesions of Mice

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Herpes simplex virus type 1 (HSV-1) causes chronic blepharitis and conjunctivitis as well as keratitis in humans. The pathogenesis of these inflammatory ocular and dermal lesions is not well understood. We have examined the persistence of HSV-1 DNA and its relationship to inflammatory lesions in the conjunctiva and eyelid skin of mice which were inoculated with HSV-1 by the corneal route. Viral DNA was detected by in situ PCR in the conjunctiva and eyelid tissue of infected mice at 5, 11, 23, and 37 days postinfection (p.i.). This DNA was localized in the epithelial cells of the conjunctiva and hair follicles and in the epidermal cells of the eyelid skin. Viral proteins were not detected in the conjunctiva or the eyelid skin after 5 days p.i., even though histopathological lesions were found at 23 and 37 days p.i. in both tissues. The DNA-containing cells were adjacent to sites of inflammation in the chronic lesions in both the conjunctiva and the eyelid skin. A similar temporal and spatial relationship between HSV-1 DNA and inflammatory lesions has been previously reported for the cornea. Our data suggest that the lesions in the cornea, conjunctiva, and eyelid skin progress similarly. Further studies are required to determine whether the long-term presence of HSV-1 is involved in the mechanism by which these chronic inflammatory lesions develop. The presence of HSV-1 DNA in these extraocular tissues for extended periods may constitute persistent viral infection of nonneuronal cells.

MATERIALS AND METHODS

Animal infections. HSV-1 strain F was grown in Vero cells and virus stocks were prepared as previously described (25). Groups of 7- to 9-week-old, female BALB/c mice were anesthetized with methoxyflurane, and each cornea was...
Scratched 10 times with a 26-gauge needle. Each animal received either 5 μl of minimum essential medium containing fetal calf serum (mock-inoculated mice) or 5 μl of medium containing 10⁷ PFU of HSV-1 strain F on each cornea. Groups of mice were killed at 5, 11, 23, and 37 days p.i., and tissues were harvested for analysis by in situ PCR, immunohistochemistry, or viral culture. In one group of mice, the eyes with the attached periocular skin were removed from each mouse, fixed in formalin, and embedded in paraffin. In a second group, the eyelid skin and conjunctiva were dissected free from other ocular tissues under a dissecting microscope and immediately frozen at −70°C for viral culture. All animals used in this study were maintained and handled in accordance with the Association for Research in Vision and Ophthalmology statement for the use of animals in ophthalmic and vision research, and all experimental procedures were approved by the University of Missouri’s Animal Care and Use Committee.

Scoring of clinical lesions. Eyes were examined at ×3 or ×10 magnification with a focal light source before inoculation and at 1, 3, 5, 11, 18, 23, 29, and 37 days p.i. Conjunctival hyperemia, conjunctival swelling, and blepharitis were graded from 1 (least severe) to 4 (most severe) with previously published scoring systems with minor modifications (13, 30). Eyes without detectable lesions were scored as 0. Each clinical sign was graded by specific criteria. Conjunctival hyperemia was scored as follows: 1, pale pink conjunctiva; 2, dark pink conjunctiva; 3, red conjunctiva; and 4, frank hemorrhage. Conjunctival swelling was scored as follows: 1, swollen conjunctiva observed only after eversion of the eyelids or partial prolapse of the globe; 2, conjunctiva visible without eyelid eversion or partial prolapse of globe but obscuring less than 25% of the cornea; 3, conjunctiva obscuring 25 to 75%; and 4, conjunctiva obscuring more than 75% of the cornea. Blepharitis was scored as follows: 1, noticeably puffy eyelids; 2, puffy eyelids with moderate crusting; 3, eyelid swollen half shut with severe crusting; and 4, eyelid crusted and totally shut. Mean disease scores (MDS) for conjunctivitis or blepharitis were calculated for each group of mice on each day of observation. For conjunctivitis, the MDS was calculated from summed conjunctival swelling and hyperemia scores. Four HSV-1-infected mice and three mock-inoculated mice were examined throughout the experiment.

In situ PCR. Paraffin-embedded sections of eyes and periocular skin from HSV-1-infected or control mice were deparaffinized with xylene and ethanol, and HSV-1 DNA localization was evaluated by in situ PCR as previously described (15, 27). After the slides were heated to 82°C for 2 min, the reaction mixture (also at 82°C) was added, and in situ PCR was performed for 15 cycles of 1 min each at 96°C, 1 min at 59°C, and 1 min at 72°C in a thermocycling oven. The reaction mixture contained 10 μM each of dATP, dCTP, and dGTP, 3.5 μM dTTP, 6.5 μM digoxigenin-dUTP, 10% glycerol, 10% salmon sperm DNA, 2.5 mM MgCl₂, and 4 U of native Taq polymerase (Stoffel fragment), 10 mM Tris·HCl (pH 8.3), 10 mM KCl, and 0.25 μM of each primer. Oligonucleotide primers (forward: 5’-TACCGAGACTATCTCAGACCACTG-3’, reverse: 5’-GCGTTGTTGTTACCTAGC-3’) (21, 29) were used to amplify a 130-bp fragment from the thymidine kinase gene of HSV-1. The slides were washed three times at room temperature in a mixture of 10 mM Tris·HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, and 0.001% gelatin for 5 min each time, three times in 2× SSC–50% formamide (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 37°C for 10 min each time, and twice at room temperature in 2× SSC for 15 min each time. The in situ PCR product was visualized by using alkaline phosphatase-conjugated antidigoxigenin antibody and an enzyme color reaction as described in the literature accompanying the DIG nucleic acid detection kit (Boehringer Mannheim).

At least two sections from each of the eyes from five mice infected with HSV-1 were tested at each time point. In addition, in several eyes serial sections from the same location were tested for labeling. As negative controls, primers which had been used in earlier studies for the in situ PCR detection of a 92-bp fragment of the feline herpesvirus type 1 thymidine kinase gene (5’-TTGCTTGATAGTGGCGGGTCC-3’ and 5’-TGCTGGTGGTATCTAAGGCGG-3’) (21, 29) were substituted for the HSV-1 primers in selected sections obtained from adjacent to or near sections which had been labeled for HSV-1 DNA. Additionally, in each group of slides sections of eyes from mock-inoculated mice were always tested with the HSV-1 primers.

Histopathology and immunocytochemistry. Sections from the eyes that were assayed by in situ PCR were also evaluated for evidence of lesions by light-microscopic evaluation of hematoxylin- and eosin-stained sections. Other sections from the same eyes were deparaffinized and assayed for the presence of viral antigen by the avidin-biotin-peroxidase method (Vector) with HSV-1 antiserum. As negative controls, uninfected mouse eyes were reacted with the HSV-1 antiserum and infected eyes were reacted with control rabbit serum in the same assays.

Viral culture. Sections of eyelid skin and conjunctiva which had been dissected free from other ocular tissues were homogenized, and each homogenate was assayed for HSV-1 on Vero cells overlaid with methylcellulose in a standard plaque assay (26). Culture results were expressed as the number of eyes from which virus could be cultured from combined eyelid skin and conjunctiva. Tissues from 10 eyes were cultured at each time point except at 11 days p.i., when only 6 eyes from mice infected with HSV-1 were assayed.

RESULTS

Clinical lesions in mice infected with HSV-1. All HSV-1 strain F inoculated mice that were examined developed clinical evidence of moderate to severe conjunctivitis and blepharitis. Conjunctivitis was evident in all mice by 3 days p.i. The MDS for conjunctivitis was at its maximum level at 11 and 18 days p.i. and remained at >0 for the duration of the experiment (Fig. 1A). Clinical evidence of blepharitis was first detected at 3 days p.i. The MDS was greatest at 11 days p.i. and remained at >0 until the termination of the study (Fig. 1B). One mock-inoculated mouse developed mild conjunctival swelling (score = 1) in one eye at 1 day p.i. No other clinical evidence of conjunctivitis or blepharitis was detected in any of the other mock-inoculated mice examined during the experiment.

Histopathological lesions and viral antigen distribution in mice infected with HSV-1. Histologic lesions in the conjunctiva and eyelid skin were present in all eyes at all times p.i. and were located in the follicular epithelium, epidermis, conjunctival epithelium, and subjacent tissues. At 5 days p.i., inflammatory infiltrates were comprised predominantly of macrophages and neutrophils (Fig. 2B and results not shown). Necrosis of the conjunctival, epidermal, and follicular epithelial cells and edema of the dermis and conjunctival substantia...
propria were also common. Beginning at 11 days p.i. and continuing through 37 days p.i., there were multifocal areas of moderate to severe inflammation in the conjunctival and follicular epithelium, the epidermis, and the subjacent dermis and substantia propria. At 23 and 37 days p.i., the inflammation was of a chronic active nature and the infiltrate consisted predominantly of macrophages, lymphocytes, and neutrophils (Fig. 3B and 4B and E). Hyperplasia of the epidermis, follicular epithelium, and conjunctival epithelium was also present in some sections obtained at later times p.i.

At 5 days p.i., viral antigens were detected in the conjunctiva in 5 of 10 eyes (Fig. 2D and 5A) and in the eyelid skin in 8 of 10 eyes (Fig. 5B). The areas of the conjunctiva and eyelid skin in which viral antigens were detected corresponded to but were not confined to the areas with histologic lesions. Viral antigen was not detected in conjunctiva or eyelid skin after 5 days p.i. in any of the samples (Fig. 5).

Localization of HSV-1 DNA. HSV-1 DNA was localized predominantly within epithelial cell nuclei of the conjunctiva, epidermis, and hair follicles of the eyelid skin; its location was related to acute and chronic inflammatory lesions. HSV-1 DNA was detected by in situ PCR in the conjunctiva and eyelid skin of virus-infected mice at all times p.i. (Fig. 5). Viral DNA was detected in the conjunctivae of 9 of 10 eyes examined at 5 days p.i., 7 of the total of 10 eyes examined at 11 and 23 days p.i., and all 10 eyes examined at 37 days p.i. (Fig. 2A, 3A, and 5A). Conjunctival samples from uninfected control mice were negative for viral DNA (Fig. 2E and 3C). HSV-1-infected conjunctiva was negative when tested by in situ PCR with primers specific for feline herpesvirus type 1 (Fig. 2C). Viral DNA was detected in the eyelid skin of 10, 8, 7, and 10, respectively, of the 10 eyes examined at 5, 11, 23, and 37 days p.i. each (Fig. 4A and D and 5B). Skin from uninfected control mice was negative for viral DNA by in situ PCR (Fig. 4C). HSV-1 DNA was localized predominantly in the nuclei of conjunctival and follicular epithelial cells, as well as in the nuclei of epidermal cells of the eyelid skin (Fig. 2A, 3A, and 4A and D). In both the conjunctiva and skin, the DNA-containing cells were found in regions adjacent to sites of acute (Fig. 2A and B) or chronic (Fig. 3A and B and 4A, B, D, and E) inflammation.

Apparent absence of infectious virus in conjunctiva and skin of chronically infected mice. At 5 days p.i., HSV-1 was cultured from homogenates of combined skin and conjunctiva from all 10 eyes (five of five mice). Virus cultures were negative for skin and conjunctiva taken from all eyes at 11, 23, and 37 days following infection with HSV-1.
DISCUSSION

In this study, we have demonstrated that HSV-1 infection results in chronic inflammatory lesions in the conjunctiva and eyelid skin and that viral DNA persists in these tissues in a mouse model. The HSV-1 DNA was found in epithelial cells of the conjunctiva and hair follicles and in epidermal cells. It was usually located in or near inflammatory lesions. In chronically infected mice HSV DNA has been previously detected in cell types other than latently infected neurons. HSV-1 DNA has been detected in corneal epithelial cells up to 4 months p.i. (27), and HSV-2 DNA has been shown to remain for a long time in the astrocytes in the brain (14). Replicating virus has been recovered from skin and other tissues of SCID mice chronically infected with a VP16-negative mutant of HSV-1 (36). In this study we have detected HSV-1 DNA in two other nonneuronal cell types in chronically infected mice.

Further experiments are required to determine whether the presence of HSV-1 DNA is mechanistically associated with progression of chronic inflammatory lesions in the conjunctiva and eyelid skin. The observations presented in this report are important for understanding the mechanism by which HSV-1 causes chronic lesions of the eyelid skin and conjunctiva as well as the cornea. In a study in humans (20), eyelid or conjunctival disease was present in 54% of patients examined for their first episode of ocular herpes simplex. Although the mechanism by which HSV-1 induces chronic inflammatory lesions in extraocular tissues is poorly understood, it may be similar to the mechanism involved in chronic corneal inflammatory lesions.

It has been previously shown that herpetic stromal keratitis in the mouse model is an immune system-mediated, chronic inflammatory lesion mediated primarily by CD4+ T lymphocytes (12). Experiments were initiated to determine whether the inflammatory lesions of the eyelid and conjunctiva which we observed in mice infected with HSV-1 were the result of an immunopathological response, as has been previously shown for the corneal lesions. We attempted to determine whether CB17 SCID mice, which do not possess T or B lymphocytes, could develop the chronic inflammatory lesions after infection with HSV-1 strain F. The data from these experiments were not definitive. SCID mice inoculated with 10⁵ PFU of HSV-1 per eye died by day 11 (9 of 11 mice) or day 12 (2 of 11 mice) p.i., thus precluding an analysis of the chronic inflammation. Control mice and SCID mice inoculated with 10⁴ PFU of HSV-1 strain F per eye survived, but none developed lesions. In all of the other experiments presented in this report, mice received 10⁷ PFU of HSV-1 strain F per eye. Further experiments with other strains of HSV-1 are necessary to examine whether chronic lesions of conjunctiva and eyelid skin develop in SCID mice after infection.

The continued presence of HSV-1 appears to be related to the progression of chronic inflammatory lesions within the cornea (5, 27). An unanswered question is whether an autoantigen found only in the cornea is involved in the progression of stromal keratitis. Some recent data suggest the existence of a corneal autoantigen (4, 38). It has also been suggested that the mechanisms by which HSV-1 induces inflammation in the cor-
neai and in the skin differ (16, 17). We speculate that the continued presence of HSV-1 may be central to the progression of lesions in the cornea, conjunctiva, and eyelid skin. Further studies comparing the mechanisms of lesion progression in the conjunctiva, eyelid skin, and cornea should help to better clarify the pathogenesis of inflammation in the cornea as well as the eyelid skin and conjunctiva. Initial experiments have not demonstrated the presence of HSV-1 mRNA in chronic lesions of the eyelid and conjunctiva. Further experiments to examine whether HSV-1 RNA is present in these chronic lesions are in progress. It is possible that persistent infection of these tissues by HSV-1 could result in the presence of low levels of viral antigen which cannot be detected by immunocytochemistry but which are recognized by the immune system.

FIG. 4. In situ PCR labeling of HSV-1 DNA in sections of chronically infected eyelid skin. (A) In situ PCR labeling of HSV-1 DNA in follicular epithelial cells at 37 days p.i. with HSV-1. The epithelial cell indicated by an arrow is shown at higher magnification in the inset. (B) Hematoxylin- and eosin-stained section in the same series obtained from near the section shown in panel A. Note the inflammatory cell infiltration of follicular epithelium and surrounding dermis. (C) In situ PCR testing of a section of eyelid skin including both epidermal and follicular epithelial cells from a mock-inoculated mouse at 11 days p.i.; note the absence of labeling. (D) In situ PCR labeling of HSV-1 DNA in epidermal cells of eyelid skin at 37 days p.i. with HSV-1. (E) Hematoxylin- and eosin-stained section in the same series obtained from near the section shown in panel D. Note the inflammatory cell infiltration in the epidermis and dermis. Bar, 19.6 μm in the main photomicrographs and 11.5 μm in the inset.
and therefore result in immunopathological lesions. The presence of HSV-1 DNA in epithelial cells of these tissues for protracted periods following inoculation may be an example of persistent infection of nonneuronal cells by HSV-1.

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