Herpes Simplex Virus Type 1 Transcription Is Not Detectable in Quiescent Human Stromal Keratitis by In Situ Hybridization

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Purpose. The purpose of this study was to determine whether herpes simplex virus (HSV) transcripts are present in the corneas of patients with chronic herpetic stromal keratitis.

Methods. Corneal buttons from patients with a history of stromal keratitis, but no ongoing active disease, together with positive and negative control tissues, were analyzed by in situ hybridization using single-stranded RNA probes for all three classes of viral lytic cycle transcripts as well as for the latency-associated transcripts (LATs). Tissues also were screened for presence of HSV genomic DNA using the polymerase chain reaction (PCR).

Results. HSV DNA was detected in 7 of 13 quiescent corneas by PCR, but no viral transcripts were detected in any of these corneas by in situ hybridization.

Conclusions. At the level of detection afforded by in situ hybridization, HSV persistent in scarred human corneas after stromal keratitis appears to be transcriptionally dormant. This contrasts with the situation in neurons of latently infected sensory ganglia, in which LATs are present at high levels. Invest Ophthalmol Vis Sci. 1993; 34:285-292.

Herpes simplex virus (HSV) establishes latency in ganglionic neurons during primary infection at peripheral sites. HSV latency has been defined classically as an asymptomatic period during which infectious virus cannot be recovered from fresh homogenized tissues, but can be induced to reactivate by various stimuli such as explantation and maintenance in organ culture. HSV has been recovered by explantation and in vitro cultivation from a small percentage of corneas from humans, rabbits, and mice long after primary infection, suggesting that HSV may establish and maintain latency in the cornea. These methods, however, may not distinguish between a low-level persistent infection and true latency.

Recent studies by several laboratories have shown that latency in neuronal tissue is not a transcriptionally dormant period, as had once been thought. Viral transcripts from a region of DNA overlapping the gene encoding the infected cell polypeptide 0 (ICP0) but transcribed in the opposite direction have been detected in latently infected neurons. These latency-associated transcripts (LATs) provide molecular markers for latently infected cells. In two previous studies, viral DNA has been detected in corneal tissue from patients with quiescent HSV keratitis by polymerase...
chain reaction (PCR), but in one study its condition and transcriptional status was undetermined, whereas in the other there was conflicting evidence of both lytic cycle and LAT being produced in certain specimens when RNA PCR was employed.

LATs reportedly have been detected by RNA PCR in corneas of latently infected rabbits, but not in those of latently infected mice. To further characterize the nature of HSV persistence in human cornea, we used the technique of in situ hybridization to study the expression of several classes of HSV genes in corneas from patients undergoing penetrating keratoplasty for corneal scarring after HSV keratitis. LATs have been detected in neuronal tissue, and thus their presence in human corneas in the absence of other HSV transcripts may indicate a state of restricted gene expression similar to that seen in neurons. Immunocytochemical staining was used to identify HSV-specific antigens in the tissues. Additionally, to demonstrate the presence of HSV DNA in these corneas, PCR was used to amplify viral genomes regardless of their transcriptional status.

**MATERIALS AND METHODS**

**Processed Human Corneal Tissue**

Full-thickness corneal buttons were obtained at the time of penetrating keratoplasty from 18 eyes with herpetic stromal keratitis (Table 1), 3 with luetic interstitial keratitis, 3 with corneal scarring from trauma or chemical injury, and 3 with keratoconus. Of the 18 herpetic eyes, topical steroids were used up to the time of keratoplasty in 9 and antivirals in 4. Positive cultures for HSV-1 were obtained at some time during the course of keratitis in all 18 patients. Keratoplasty was performed an average of 17.1 yr after the initial occurrence, 1.9 yr after the last known epithelial occurrence, and 1.1 yr after the last stromal involvement. All corneal buttons were fixed in neutral buffered formalin and embedded in paraffin.

**Control Tissue**

Paraffin-embedded, formalin-fixed control tissues for in situ hybridization and immunocytochemical staining included HSV latently infected human trigeminal ganglia, HSV-infected human brain from a patient who died of HSV encephalitis, HSV acutely infected rabbit cornea (killed 4 days after corneal scarification and inoculation with 10⁴ plaque-forming units [PFU] of HSV type 1 [McKrae strain]), uninfected rabbit cornea, and uninfected human brain tissue. Infected (10 PFU/cell) and mock-infected VERO cells were harvested 48 hr later, cytospun onto silanized slides, and postfixed with 4% paraformaldehyde for 10 min at room temperature. As an additional positive control, HSV culture-positive corneal tissue was harvested from mice that had been latently infected with HSV and reactivated by exposure to ultraviolet (UV) light, as follows. Briefly, mice were given human serum containing anti-HSV antibodies (ED₅₀ = 1/640) 24 hr before scarification and inoculation of the right cornea with 10⁶ PFU of HSV-1 McKrae strain. Twenty-eight days after inoculation, selected mice were exposed to 250 mj/cm² of UV-B (peak 302 nm) to the

| Table 1. Clinical Features of the Chronic Herpetic Stromal Keratitis Cases |
|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| Cornea Number | No. of Years Since Initial Occurrence | Time Since Last Epithelial Occurrence | Time Since Last Stromal Involvement | Steroids | Anti-virals |
| 1 | 16 | 6 yr | 6 yr | + | – | – |
| 2 | 6 | 3 yr | 3 yr | + | – | – |
| 3 | 16 | ND | 1 yr | + | – | + |
| 4 | 32 | 4 yr | 4 mo | + | Trifluridine | + |
| 5 | 4 | 1 yr | 1 yr | + | – | – |
| 6 | 30 | 1 yr | ND | + | Ara-A | + |
| 7 | 2 | 2 yr | 2 mo | + | Trifluridine | – |
| 8 | 11 | ND | 1 yr | + | – | + |
| 9 | ND | ND | 1 yr | + | Trifluridine | + |
| 10 | 6 | 1 yr | None | – | – | – |
| 11 | 63 | 3 mo | 3 mo | – | – | + |
| 12 | 2 | ND | 1 mo | – | – | – |
| 13 | ND | 1 mo | 1 mo | – | ND | ND |
| 14 | ND | ND | ND | – | ND | ND |
| 15 | ND | ND | ND | – | ND | – |
| 16 | ND | ND | ND | – | ND | ND |
| 17 | ND | ND | ND | – | ND | ND |
| 18 | ND | ND | ND | – | ND | ND |

ND, not determined.
cornea. Three days after irradiation, mice were killed; their eyes were formalin fixed and paraffin embedded for routine processing and immunocytochemical studies.

For PCR studies, an additional positive control was a human corneal button removed from a patient with active herpetic keratitis. Additional negative controls were six corneal buttons obtained from cadavers with no history of herpetic eye disease. Animals used to provide control tissues were treated in accordance with the ARVO Resolution on the Use of Animals in Research.

Immunocytochemical Staining

Sections of formalin-fixed tissues were deparaffinized by immersion in xylene and a series of graded alcohols. Tissues were incubated for 20 min in a solution of hydrogen peroxide in methanol solution to quench any endogenous peroxidase. Rabbit anti-herpes simplex type 1 IgG and normal rabbit immunoglobulin (Accurate Chemical Co., Westbury, NJ) provided the respective primary positive and negative control sera. The avidin-biotin complex technique was used (Vectastain, Burlingame, CA).

RNA Probes

Four single-stranded, 35S-labeled RNA probes were used to detect transcription from specific regions of the HSV genome. The four probes were transcribed from fragments of the HSV-1 genome cloned in the plasmid pGEM3Z (Promega Biotec, Inc., Madison, WI). This plasmid contains a multiple cloning site flanked by T7 and Sp6 RNA polymerase promoters, enabling single-stranded RNA probes to be generated from either strand of a cloned DNA fragment by using the appropriate RNA polymerase.

One RNA probe was generated from the 8.6-kilobase pair EcoRI-I fragment of the HSV-1 genome and was complementary for early and late transcripts derived from this region. An RNA probe generated from the 10.4-kilobase pair HSV-1 EcoRI-F fragment was used to detect transcripts coding for the early proteins ICP8 and glycoprotein B. The third and fourth probes were generated from opposite strands of the same 3-kilobase pair SpI restriction fragment of the viral genome. One probe was complementary to the immediate-early transcript encoding ICP0, whereas the probe derived from the opposite strand could be used to detect LATs. The use of these four probes thus permitted detection of transcripts from all three classes characteristic of productive viral infection as well as those associated with latency. Because of the large size of these RNA probes as initially transcribed, they were shortened to approximately 500 bases in length by hydrolysis at 40°C in 100 mmol/l sodium carbonate pH 10.0 before use in hybridization studies.

In Situ Hybridization

The protocol used was a modification of that described by Haase et al.20 and Gendelman et al.21 Slides were deparaffinized and digested with proteinase K (Sigma Chemical, St. Louis, MO; 100 µg/ml) at 37°C for 20 min. Prehybridization was performed at 45°C for 2 hr in 50% deionized formamide, 0.6 mol/l sodium chloride, 10 mmol/l Tris-Cl, 1 mmol/l EDTA, 0.02% Ficoll, 0.02% polyvinylpyrrolidone-360, 500 µg/ml yeast tRNA, 10 mmol/l dichiothreitol, and 1 mg/ml acetylated bovine serum albumin. The hybridization solution included all the prehybridization reagents, as well as 10% dextran sulfate, and 106 cpm/12 µl of the appropriate RNA probe. Hybridizations were carried out overnight at 50°C. Slides were washed at 60°C,20 dipped in NTB3 autoradiographic emulsion (Eastman Kodak, Rochester, NY) diluted 1:1 with 0.6 mol/l ammonium acetate, and exposed for 4 days at 4°C. Slides were developed in D-19 (Eastman Kodak) and stained with hematoxylin.

Extraction of DNA from Corneas

Corneal tissue samples were incubated for 1 hr in 0.02% collagenase solution at 37°C. They were then ground in glass homogenizers containing PBS at 4°C. Homogenates were concentrated by centrifugation and resuspended in extraction buffer (10 mmol/l Tris, 10 mmol/l EDTA, pH 8.0, 0.6% sodium dodecyl sulfate).22 Proteinase K was added to 0.5 mg/ml and the suspension was incubated at 37°C for 2 hr. The preparation was then extracted twice with a 1:1 mixture of phenol:chloroform and the organic phase discarded. The DNA was precipitated from the aqueous phase by adding an equal volume of isopropanol and collected by centrifugation. It was washed with ethanol, lyophilized, resuspended in water and stored at −20°C before use in PCR amplification.

Polymerase Chain Reaction

Pairs of oligonucleotide primers were synthesized complementary to opposite strands of DNA within two viral genes, the immediate-early gene encoding ICP0, and the early gene encoding thymidine kinase (TK). Sequences of the primers were as follows:

For the ICP0 sequence:

5'-TTCGGTCTCCGGCCCTCGAGACT-3' and
5'-AACTCGTTGGGTGTCTGATTGA-3'

For the TK sequence:

5'-ATACCGAGCATATGCAGACCT-3' and
5'-TTATGGCAGTCATGCTGAGG-3'

In addition to the two sets of HSV-specific primers, a pair of primers, PC03 and PC04, comple-
Synthesized. Sequences of these primers are as follows:

PC03: 5'-ACACAACGTGCTCCTAGCC-3'
PC04: 5'-CAACCTCTCCACGTGCC-3'

One-microgram aliquots of DNA purified from tissues were amplified by PCR in a reaction mixture containing 10 mmol/l Tris-Cl, pH 8.3, 50 mmol/l KCl, 1.5 mmol/l MgCl₂, 0.001% (w/v) gelatin, 200 µmol/l each of dATP, dCTP, dGTP, and dTTP, and 1 µmol/l of each oligonucleotide primer in a total volume of 50 µl; 2.5 U of taq DNA polymerase were used for each reaction. For all amplifications, the following cycle profile was used: 94°C for 2.5 min (denaturation), 56°C for 2 min (annealing), and 72°C for 3 min (polymerization). This cycle was repeated 40 times, and then the amplification products were analyzed by polyacrylamide gel electrophoresis alongside DNA size markers, followed by ethidium bromide staining. Predicted sizes of the amplification products were as follows: 144 base pairs (bp) from the HSV-1 ICP0 sequence, 110 bp from the HSV-1 TK sequence, and 110 bp from the human β-globin sequence.

Hybridization of PCR Products

After visualizing PCR products on polyacrylamide gels, additional aliquots were subjected to electrophoresis on 2% agarose gels. The DNA was then transferred to Hybond-N nylon membrane filters (Amer sham Corporation, Arlington Heights, IL) by the standard Southern blot method.

For each pair of PCR primers used, an oligonucleotide probe also was synthesized and was complementary to the predicted amplified product. The probe sequences were as follows:

- For ICP0: 5'-ATGTCTGGGTGTTTCCCTGCATCTCCCGG-3'
- For TK: 5'-ATGTCTGGGTGTTTCCCTGCATCTCCCGG-3'

These probes were end-labeled with 32P and membrane filters were hybridized at 37°C overnight with the appropriate probe. Autoradiography was performed by exposing filters for 3 days at -80°C.

RESULTS

Multiple sections from varying tissue levels of every case of chronic herpes simplex stromal keratitis were analyzed by in situ hybridization and immunocytochemistry. All corneas showed histologic evidence of herpetic stromal keratitis, including pannus formation, fragmentation of Bowman’s membrane, scarring, and stromal infiltrates comprising polymorphonuclear cells, plasma cells, lymphocytes, macrophages, and rare eosinophils. HSV antigens were not detected in any tissue sections from either herpetic (Fig. 1A) or nonherpetic corneal buttons. Control human brain (Fig. 1B) and uninfected VERO cells displayed no HSV antigen staining. In contrast, human brain tissue from a patient who died of active herpes simplex encephalitis (Fig. 1C), mouse corneas with HSV keratitis following UV light-induced reactivation (Fig. 1D), and HSV-infected VERO cells (Fig. 1E) showed strong positive antigen staining.

No HSV RNA sequences were detected by in situ hybridization to the four HSV riboprobes in sections from either the herpetic or nonherpetic full-thickness corneal buttons (Fig. 2A). No hybridization signals were obtained in uninfected rabbit cornea or uninfected VERO cells. In contrast, latently infected human trigeminal ganglia showed strong hybridization signals in a small percentage of neurons exclusively with the LAT probe (Fig. 2B). These signals were observed primarily over the nuclei of neurons. Acutely infected rabbit cornea and HSV-infected VERO cells (Fig. 2C) gave strong positive hybridization signals with all the HSV riboprobes except the LAT probe, which gave a moderately positive signal.

After sections had been cut from each of the paraffin-embedded herpetic corneal buttons, the remaining tissue was deparaffinized and the DNA extracted and purified. As negative control material, DNA also was extracted from six corneal buttons removed from cadavers with no history of herpetic eye disease. For positive controls, DNA was prepared from a complete corneal button removed from a patient with active herpetic keratitis and from HSV-1–infected VERO cells.

A 1-µg aliquot of each DNA sample was initially amplified using the oligonucleotide primers directed to β-globin sequences to confirm the presence of amplifiable DNA. This was present in all the controls and in 13 of the preparations from the quiescent herpetic corneas.

Aliquots of these globin-positive preparations were then amplified using primers directed at sequences of the HSV-1 gene encoding ICP0, and the resulting amplification products were visualized by polycrylamide gel electrophoresis (Fig. 3).

PCR amplification of DNA preparations from quiescent herpetic corneas generated strong bands of the correct size in three cases (tracks 6, 8, and 15) and fainter bands of the correct size in four other cases (tracks 4, 10, 12, and 13). DNA samples corresponding to tracks 1 through 9 also were amplified using primers directed at the viral TK gene. Again, strong bands of the correct size were generated in specimens...
corresponding to tracks 6 and 8 on the ICP0 gel and a weaker band in that corresponding to track 4 (data not shown). With both sets of primers, no amplification products of the correct size were generated from the six negative controls, results from two of which are shown in Figure 3, or from blank target DNA-free controls run in parallel.

Southern blot hybridization of the amplification products with the appropriate probes followed by autoradiography yielded strong signals with intense bands of the correct size in three samples (ie, tracks 6, 8, and 15) and weaker signals with the corresponding bands in the other lanes (ie, tracks 4, 10, 12, and 13). There was no hybridization to negative controls. In summary, 7 of 13 quiescent herpetic corneas were positive for HSV-1 sequences.

**DISCUSSION**

Evidence for persistence of HSV in human cornea includes the isolation of virus by organ culture from 10 of 34 (29.4%) corneal buttons from patients with her-
FIGURE 2. There is no hybridization of the LAT or ICP0 probe on (A) human herpetic corneal button. (B) Hybridization with the LAT probe was observed in neuronal nuclei of a latently infected human trigeminal ganglia. (C) HSV-infected VERO cells. (Original magnifications: A, X400; B, X400; C, X400.)

Viral nucleic acid also has been detected in the corneas of herpetic keratitis patients by PCR and in I of 32 corneal epithelial samples from normal-appearing eyes in an autopsy study. In one study, viral DNA sequences encoding TK and LAT were detected by PCR in 17 of 18 specimens from patients with quiescent HSV keratitis. DNA from both regions was detected in only 13 of the 18 specimens, however, suggesting the presence of defective viral genomes in at least 4 of the corneas. Unlike in the current study, no in situ hybridization was performed to determine the transcriptional status of the genome. Another investigation demonstrated the presence of 3 HSV sequences in each of 10 corneas from patients with quiescent HSV keratitis, as well as in 5 of 10 corneas from patients with no history of this condition. By RNA PCR, LAT sequences were detected in mRNA prepared from 8 of the 10 herpetic corneas (the other two preparations were shown to be contaminated with DNA) and 3 of the second group of corneas. Two of the corneas from HSV keratitis patients, however, also apparently contained glycoprotein C mRNA characteristic of productive infection. Without in situ hybridization studies or quantitative analysis, it is not possible to determine the level of expression or the cellular distribution of LATs in these corneas. In latently infected neurons, LATs are known to be expressed at levels comparable to lytic cycle late transcripts; if equivalent amounts of LAT were present in quiescent corneas it should have been possible to detect them by in situ hybridization in the current study.

Our studies using PCR revealed the persistence of HSV genomes in 7 of 13 of the previously infected corneas. This proportion is similar to that capable of reactivating virus on explant cocultivation. In each case in our study, DNA had been extracted from the tissue remaining after sections had been cut for in situ hybridization, so for many specimens there was a only a small fragment of the cornea left for PCR analysis. If only a distinct area of the cornea contained latently infected cells, this area may have been sectioned before the DNA was extracted for PCR analysis, resulting in a negative PCR result. The proportion of whole corneas that actually contained viral DNA may therefore have been closer to that found in the earlier investigations. We did not detect viral RNA transcription by in situ hybridization in any of the 18 cases of herpetic stromal keratitis. In accord with the in situ hybridization data, we also found no evidence of HSV-specific antigen expression in the cornea. In situ hybridization using antisense RNA probes can detect as few as 10 to 20 copies of an mRNA species per cell against the background level, and all the transcripts for which the corneal tissues were probed in our study are known to be expressed at much higher levels in infected cells. It is therefore unlikely that our inability to detect viral transcription was due to lack of sensitivity of the in situ hybridization technique.

One limitation inherent to our in situ hybridiza-
HSV-1 in Human Corneas

FIGURE 3. Polyacrylamide gels showing amplification products generated using primers directed at ICP0 sequences. DNA samples were prepared from an acutely infected herpetic human cornea (track 1), uninfected human corneas (tracks 2 and 3), quiescent herpetic human corneas (tracks 4-16), and HSV-1-infected VERO cells (track 17). The ICP0-specific band is 144 bp in size.

Other investigators using inbred mice have detected persistently infected corneas with the same frequency (12%-14%) using a cocultivation technique and with in situ hybridization for LATs. LAT detection by in situ hybridization was limited, however, to 0.008% to 0.28% of dissociated corneal cells from latently infected mice after collagenase digestion. In contrast, others have failed to identify LATs in corneas of latently infected mice using the sensitive PCR, although it was detected in 9 of 22 corneas from latently infected rabbits. Because we had only purified DNA from the remaining portion of each cornea sample, we were not in a position to look for LATs by PCR.

It is possible that the time course, pattern, or abundance of viral gene expression in persistently infected nonneuronal cells could differ from that of latently infected neurons. Certainly, the concentration of LATs in neurons is very high, being estimated to be between 10^4 to 10^5 copies per cell. Recent reports, however, suggest that the LAT promoter may be neuron specific, in which case the levels of LATs in nonneuronal cells of a latently infected cornea will be much lower and may elude detection by in situ hybridization. This would account for our ability to detect LATs in human trigeminal ganglia, but not in scarred human corneas after herpetic keratitis. The presence of genome in the absence of demonstrable virus-specific transcription suggests that HSV persistence in naturally infected human corneas is distinct from that occurring in neurons.

Key Words
cornea, herpes simplex keratitis, herpes simplex virus, in situ hybridization, latency associated transcripts.

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