Detection of Herpes Simplex Virus Thymidine Kinase and Latency-Associated Transcript Gene Sequences in Human Herpetic Corneas by Polymerase Chain Reaction Amplification

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Herpes simplex virus (HSV) latency in sensory ganglion neurons is well documented, but the existence of extraneuronal corneal latency is less well defined. To investigate the possibility of extraneuronal latency during ocular HSV infection, corneal specimens from 18 patients with quiescent herpes simplex keratitis (HSK) were obtained at the time of keratoplasty. Polymerase chain reaction (PCR) amplification followed by southern blot hybridization with a radiolabeled oligonucleotide probe was done to detect the presence of HSV-1 genome in these human corneal samples. Two pairs of oligonucleotides from the region of the HSV thymidine kinase (TK) gene and the latency-associated transcript (LAT) gene were used as primers in the PCR amplification. The DNA sequences from either the TK or the LAT gene were identified in 15 of 18 HSK corneas (83%). These results demonstrate that the HSV genome was retained, at least in part, in human corneas during quiescent HSV infection, giving further support to the concept of corneal extraneuronal latency.

It is well documented that herpes simplex virus type 1 (HSV-1) establishes latent infections in neurons and intermittently causes recurrent lesions at peripheral sites, such as the skin and eye. The latency state is characterized by retention of the virus in the host without overt clinical disease. The molecular basis of viral neuronal latency has not been clarified completely, especially with respect to HSV genome function and expression that is involved in establishing and maintaining the latency state. Expression of a HSV gene during neuronal latency has been detected by in situ hybridization analysis; the gene is located in the opposite strand to an HSV immediate early gene ICPO. The transcripts from this gene, termed latency-associated transcripts (LATs), are abundant in the latently infected human, rabbit, and murine trigeminal ganglia. Although the function of these transcripts is unknown, it has been suggested that they may be important for initiating and maintaining viral latency. However, studies using HSV mutant viruses in which the region encoding for the LAT gene was deleted have documented mutant virus replication in trigeminal ganglia with kinetics similar to the parent wild-type virus. These LAT-mutant viruses can establish a latent infection, but their reactivation was delayed significantly. These data indicate that LAT alone is not an absolute requirement for establishing and maintaining neuronal latency, but it may have a significant role in HSV reactivation.

In addition to the LAT gene, the HSV thymidine kinase (TK) gene may be important for establishing neuronal latency. Since HSV TK mutants induce a low prevalence of latent infection in sensory ganglia, such observations have led to the concept that viral TK may be necessary for the establishment of latency. Other evidence demonstrating the inability of TK mutants to replicate in neurons may explain their apparent inability to establish latency. Several other investigations showed the importance of host species differences in the study of TK. In rabbits, TK is not
been difficult to detect directly latent HSV genomes in culture was positive during the active epithelial in- 

trivation of latent HSV-1.13 In mice, it is not re-

quired for establishing latency but is essential for reac-

tivation.14 Further definition of the state of viral gene expression during neuronal latency will help to clarify the mechanism(s) of latency at the molecular level.

Extraneuronal retention of HSV, possibly in a la-

tent state, has been postulated. Both ocular15 and der-

mal16,17 extraneuronal HSV latency was suggested during latent infection by co-culture virus recovery and immunofluorescent staining techniques.18 In addition, molecular hybridization assays19 were used for direct detection of HSV nucleotide sequences in latently infected animal and human herpetic corneas. Further experimental verification of the existence of extraneuronal latency would provide new insights into the possible mechanism(s) of virus/host-induced end-organ damage and an improved therapeutic regimen that might combat and possibly eliminate resultant corneal disease.

A recently developed technique, the polymerase chain reaction (PCR).20,21 allows for selective amplification of a given sequence of exogenous or endoge-

nous DNA. The ability to amplify DNA and the sensi-
tivity of the PCR procedure facilitate the detection of rare pathogenic DNA sequences in the presence of vast excesses of host nucleic acids. Historically, it has been difficult to detect directly latent HSV genomes and to analyze gene expression due to the low copy numbers of HSV genes in the latently infected host cells.22 We followed PCR amplification by southern blot hybridization to identify the HSV genome in hu-

man corneas obtained from keratoplasty. Two re-

gions from the HSV genome, the TK gene and the 

LAT gene, were chosen for use in PCR amplification of the human corneal samples.

Materials and Methods

Experimental Samples

Eighteen human corneal specimens were obtained at the time of transplantation (one half to two thirds of each corneal button was available for this study). All samples were obtained from patients with clini-

cally quiescent but a well-documented history of re-

current herpes simplex keratitis (HSK). The diagnosis of recurrent HSK was based on the following findings: (1) slit-lamp examination revealed the presence of one or more recurrent diseases—epithelial infectious ulcers including dendritic or geographic ulcers, stromal interstitial keratitis, or stromal disciform kerati-

tis; (2) clinical history showed the recurring and pro-

gressive nature of the disease; and (3) HSV tear film culture was positive during the active epithelial in-

fectious period in patients with a history of dendritic or geographic ulcers. All the HSK patients were in a clinically quiet stage of HSV infection before trans-

plantation, as evidenced by the following: (1) slit-
lamp examination revealed no active corneal epithelial or stromal disease; (2) immediately before surgery, no patient was receiving antiviral therapy, and most were not receiving topical corticosteroid therapy; and (3) preoperative tear film cultures were negative (Ta-

ble 1).

Seven nonherpetic human corneal specimens were obtained at the time of transplantation. Six were from patients with pseudophakic bullous keratopathy (PBK), and one was from a patient with a traumatic corneal scar. These nonherpetic corneas were used for establishing further the specificity of the PCR in our study. All these patients had no previous history of HSK, and their tear film cultures were negative.

Eleven normal donor corneas obtained from the Lions Eyes of Texas Eye Bank (Houston, TX) served as negative control tissues. Care was taken to select normal corneas from previously healthy individuals (eg, accidental death and clear and healthy corneal tissue as recorded from this eyebank).

DNA Extraction

Corneal specimens from HSK and nonherpetic transplant patients in addition to normal donor but-

tons were minced individually in 0.5 ml of 10 mM Tris HCl, pH 8.0, with 100 mM NaCl and 1 mM ethylenediaminetetraacetic acid (EDTA). Proteinase K (Boehringer Mannheim, Indianapolis, IN) and so-

dium dodecyl sulfate (SDS) were added to the corneal suspen-

sions at a final concentration of 2 mg/ml and 1%, respectively. Minced corneas were digested at 37°C for 12 hr, and total DNA was extracted with phenol and chloroform, precipitated in absolute ethanol, and washed according to standard techniques.23

PCR

Two pairs of oligonucleotide primers were chosen from the regions of the HSV-1 TK24 and LAT25 genes. Considerations in selection of the appropriate primers included primer length, guanine plus cytosine base content (G + C), and intrastrand primer complementarity. The TK 1 and TK 2 primers, selected to flank the TK gene region from nucleotides 541–867, ampli-

fied a 327-base pair (bp) DNA fragment (Table 2). Oligonucleotide probe TK 3 was selected to comple-

ment the 327-bp amplified product between nucleo-

tides 680–710. The LAT 1 and LAT 2 primers, se-

lected to flank the LAT gene region from nucleotides 861–1230, amplified a 370-bp DNA fragment. Oligo-
nucleotide probe LAT 3 was selected to complement the 370-bp product between nucleotides 991-1030. Oligonucleotides (including primers and probes) were synthesized in a DNA synthesizer (model 8750; Milligen/Biosearch, Bedford, MA) and purified separately by polyacrylamide gel electrophoresis before use in the PCR reaction and southern blot hybridization analyses.

Genomic DNA (1 μg) from herpetic, nonherpetic, and normal corneas was heated individually at 94°C for 5 min to inactivate any remaining proteases and then subjected to PCR amplification in 100 μl of single concentrated reaction buffer (10 mM Tris HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, and 0.1% gelatin). The reaction buffer also contained 2.5 units of Taq polymerase (Cetus, Norwalk, CT), 200 μmol of each deoxynucleotide triphosphates (dNTP), and 0.5 μmol of each primer. Thirty independent repeated cycles of the PCR reaction were done at 94°C for 1 min (denaturation), 55°C for 2 min (annealing), and 72°C for 3 min (extension) in a Perkin-Elmer Cetus Thermal Cycler (model PCR 1000; Norwalk, CT).²⁶

Analysis of Amplified Products

After PCR amplification, 10-μl aliquots from each reaction mixture underwent electrophoresis through a 1% Seakem/3% NuSieve agarose gel (FMC, Rockland, ME) in single-concentrated 90 mM Tris, 64.5 mM boric acid, with 2.5 mM EDTA, pH 8.3, for 2 hr at 5 V/cm. The agarose gel was then denatured for 40 min in a solution of 0.5 M NaOH and 1.5 M NaCl. The DNA was transferred overnight

Table 2. Sequence of synthetic oligonucleotide primers and probes

<table>
<thead>
<tr>
<th>Primer</th>
<th>Location and length (bp) of amplified product or probe*</th>
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<tbody>
<tr>
<td>TK 1 5'-ATCGTCTACCTACCCGAGCCATGACTTAC</td>
<td>541–867</td>
</tr>
<tr>
<td>TK 2 5'-GCCGTCGCCCCGCTATTGCTGCCCCGATTCGTA</td>
<td>327</td>
</tr>
<tr>
<td>LAT 1 5'-GTGATTCTCTGGCTGACCGCATTCTTCTT</td>
<td>861–1230</td>
</tr>
<tr>
<td>LAT 2 5'-TGTTGGGCAGGCTCTGGTGTTAACCACAGA</td>
<td>370</td>
</tr>
<tr>
<td>Probe</td>
<td>680–710</td>
</tr>
<tr>
<td>LAT 3 5'-CCAGATACAAATTGCGATCGCTTATGCCGCTT</td>
<td>991–1031</td>
</tr>
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* TK, reference 24; LAT, reference 25.
to a Hybond-nylon filter (Amersham, Arlington Heights, IL) in a solution of tenfold concentrated SSC (single-concentrated SSC contains 0.15 M NaCl and 0.013 M sodium citrate, pH 7.0). The filter was cross-linked by ultraviolet light for 5 min to immobilize the DNA.

To detect HSV DNA amplification products, the filter was prehybridized in a solution containing fivefold concentrated SSPE (single-concentrated SSPE contains 0.18 M NaCl and 10 mM sodium phosphate, pH 7.4, with 1 mM EDTA), fivefold concentrated Denhardt’s solution (single-concentrated Denhardt’s contains 0.02% polyvinylpyrrolidone, 0.02% Ficoll, and 0.02% bovine serum albumin), 0.5% SDS, and 0.02 mg/ml salmon sperm DNA at 60°C for at least 2 hr. Oligonucleotide probes were labeled with γ-32P-deoxyadenosine triphosphate using T4 polynucleotide kinase. Unincorporated nucleotides were removed with a NACS PREPAC column (BRL, Gaithersburg, MD). After the addition of labeled oligonucleotide (0.1 pmol/ml), the blots were hybridized in the prehybridization solution at 60°C overnight. They were washed subsequently four times for 5 min each in single-concentrated SSC at room temperature and twice for 15 min each at 50°C. The blots were autoradiographed on Kodak X-Omat film (Rochester, NY) for 1.5 hr to 3 days.

Results

Purified HSV DNA was obtained from in vitro infected human embryonic lung cells (American Type Culture Collection [ATCC], Rockville, MD) by standard procedures and used to determine the sensitivity of the PCR amplification technique. Purified HSV DNA was serially diluted to 10^3, 10^2, 10^1, and 1 gene copy and then mixed separately with 1 μg of normal human corneal DNA. The mixture was amplified through 30 independent PCR cycles. The amplification results from these reconstruction mixtures showed that the assay was sensitive to the level of one copy of HSV DNA (Figs. 1A-B, Lanes 2-5; Figs. 1C-D, Lanes 2-5). The location of the amplification products (bands) from TK 1 and TK 2 primers in ethidium bromide-stained gels confirmed that these bands were 327 bp and corresponded to expected PCR amplification products (Fig. 1A, Lanes 2-5). Hybridization with labeled probe TK 3 verified further that these bands were the anticipated 327-bp amplification products (Fig. 1B, Lanes 2-5). The location of the amplification products (bands) from LAT 1 and LAT 2 primers in ethidium bromide-stained gels demonstrated that these bands were 370 bp and corresponded to the anticipated PCR amplification products (Fig. 1C, Lanes 2-5). Hybridization with labeled probe LAT 3 further verified that these bands were the 370-bp amplification products (Fig. 1D, Lanes 2-5).

Negative control PCR amplifications were done along with each positive and experimental PCR am-
mplification. No amplification signal of HSV DNA sequences was detected in the human donor corneas (Figs. 1A–B, Lanes 6–10, representing normal corneas from donors H1–5, respectively; Figs. 1C–D, Lanes 6–10, representing normal corneas from donors H5–9, respectively; Fig. 2, Lanes 9 and 10, representing normal corneas from donors H10 and H11, respectively). No amplification signal of HSV DNA sequences was detected in nonherpetic human corneas (Figs. 3A–B, Lanes 4–10, and Figs. 3C–D, Lanes 5–10), representing PBK and traumatized corneas. Reconstruction, negative control, and nonherpetic cornea experiments showed that the two pairs of primers selected from the TK and LAT regions ensured specific PCR amplification signal generation.

Experimental use of TK primers resulted in amplification of a 327-bp TK DNA sequence in 15 of the 18 (83%) herpetic corneas (Figs. 2A–C, Lanes 3–8). Use of the LAT primers resulted in amplification of a 370-bp LAT DNA sequence in 15 of the 18 herpetic corneas (Figs. 2D–F, Lanes 3–8). Lanes 3–8 in Panels A and D represent HSK patients 1–6; Lanes 3–8 in Panels B and E represent HSK patients 7–12; and Lanes 3–8 in Panels C and F represent HSK patients 13–18 (Table 2). These amplification results were demonstrated both in the gel ethidium bromide-stained gel (data not shown) and in southern blot hybridization with labeled oligonucleotide probes complementary to the amplified products. Figure 2, Panels A–C, demonstrated hybridization results with probe TK 3, and Panels D–F, with probe LAT 3. The TK sequences were not detected in corneas from HSK patients 2, 14, and 15 (Panel A, Lane 4; Panel C, Lane 4 and 5); LAT sequences were not detected in corneas from HSK patients 7, 14, and 17 (Panel E, Lane 3; Panel F, Lanes 4 and 7). In only one corneal sample, HSK patient 14, there were no TK or LAT sequences identified by the PCR amplification procedure.

Discussion

The potential for retention of the HSV genome in extraneuronal tissues, possibly in a latent state, has attracted research attention in recent years. Using

Fig. 2. Southern blot hybridization of amplified HSV DNA sequences from 18 herpetic corneal specimens. One microgram of DNA extracted from each corneal specimen was amplified by PCR either with TK 1 and TK 2 primers (A, B, and C) or LAT 1 and LAT 2 primers (D, E, and F). Ten-microliter aliquots of the amplified products were electrophoresed through a 1% Seakem/3% NuSieve agarose gel, transferred to a Hybond-nylon filter, and hybridized with a [32P]-labeled oligonucleotide probe (A, B, and C: TK 3; D, E, and F: LAT 3). Lane 1, 1 μg of DNA from the φX174 phage digested with Hae III, was used as the size standard. Lane 2, purified HSV DNA: 10 copies (D and E) and 1 copy (A, B, C, and F). Lanes 3–8 in A and D show DNA hybridization results from HSK patient nos. 1–6; lanes 3–8 in B and E are from HSK patient nos. 7–12, and lanes 3–8 in C and F are from HSK patient nos. 13–18. Lanes 9 and 10 in all panels represent normal corneal DNA from donor corneas H 10 and H 11.
whole-cell co-culture techniques, Easty et al reported recovery of HSV from keratoplasty specimens of patients with chronic HSK. To obtain these results, however, corneal co-culture time was extended from 4–11 days; yet the rate of virus recovery from the HSK specimen remained relatively low (29.4%). Another study isolated HSV from experimentally infected rabbit corneas during latency after prolonged whole-cell co-cultivation, and others recovered HSV from cornes (anterior segment) of latently infected inbred mice (HSV was not recovered from retinal and posterior segment cultures). Their data suggest that HSV latency may be established in corneal cells. Using in situ nucleic acid hybridization, Sabbaga et al demonstrated the presence of the HSV genome in corneal epithelium, stromal keratocytes, and corneal endothelium in rabbit corneas latently infected with HSV. In another study using slot-blot hybridization, the HSV genome was detected in 50% of the HSK corneal specimens. We used PCR amplification, and the detection rate of HSV sequences was increased over other hybridization methods. Both TK and LAT gene sequences were identified singly or in combination in 83% of the corneal specimens. In only one patient specimen were neither TK nor LAT sequences detected. Selective amplification of HSV genes by the PCR technique makes this assay especially suitable for detecting low copy numbers of latent virus genes in a low number of infected host cells (eg, only 1% of the neurons in a trigeminal ganglion, approximately 1500 neurons, become latently infected after resolution of primary gingivostomatitis). From data generated in our reconstruction experiment (Fig. 1), our PCR assay technique could detect as low as one copy of the HSV genome mixed in 1 μg of normal human corneal DNA (approximately 10^9 host cells). Although PCR is a sensitive technique for detecting genes, it does not provide the same information as co-cultivation, which demonstrates the presence of the entire viral genome and the ability of the latent viral genome to reactivate. The PCR techniques are not restricted to detection of the viral genome alone. A HSV-RNA PCR amplification analysis after reverse transcriptase template formation can provide more information about gene expression and function during latency, specifically the mechanism(s) of HSV latency at the molecular level.

The presence of HSV DNA sequences was demonstrated in 17 of 18 corneas from HSK patients of different age groups and various types of disease (TK or LAT amplification, or both; Table 1) in our study. Detection of HSV genes in these corneal samples was not related either to patient age or the clinical manifestation of ocular HSV. Patient age ranged from 17–84 yr (average, 59 yr), with a range of duration of HSK disease from 5 months to 77 yr.

The PCR amplification results from most of the HSK corneal samples consistently demonstrated TK and LAT sequence detection (repeat data not shown). However, DNA sequences from only one region (TK or LAT) were detected in HSK patients 2, 7, 15, and 17. Neither TK nor LAT sequences were detected in HSK patient 14. The explanation of these results is
unclear. It is possible that our failure to detect TK or LAT sequences was an artifact of the processes of DNA extraction, transfer, or hybridization. The state of the HSV genome during latency is also unclear. As evidenced by a release of infectious virus after extrapolation in vitro, most investigators agree that latently infected ganglia must contain a complete HSV genome. Marker rescue studies, in which human ganglia were infected with mutants carrying specific markers, suggested that defective viral genomes may reside in ganglionic cells. Another report, investigating cloning of HSV-1 terminal fragments from DNA extracts of trigeminal ganglia harboring latent virus, suggested that most parts of the latent genome undergo rearrangement (the nature of which was not determined in this study). However, these reports focus on the trigeminal ganglia but do not support the hypothesis that a defective or rearranged latent gene may exist in the cornea.

Of central importance in the current study was the question: are these PCR-amplified gene sequences associated with HSV extraneuronal latency? At least three possibilities can explain detection of HSV sequences retained in our human cornea samples after resolution of active disease: (1) the DNA sequences may be a result of virus reactivation in the ganglion from preoperative anxiety and stress, (2) the DNA sequences could represent defective, nonfunctional virus sequences retained in the cornea after acute or recurrent disease, or (3) the sequences could represent a latent HSV gene. The detection of the HSV genome in HSK corneas is an initial step at the molecular level to approach understanding of the HSV latency mechanisms. Additional analyses, possibly RNA amplification by PCR, are necessary to establish conclusively whether the genes detected in the cornea are functionally active or relate to the HSV latency process. At present we cannot eliminate the first two possibilities, nor can we prove the third.

A recent study demonstrated HSV immediate early gene product (ICP 4, 6) antigens in 25% of the keratectomy specimens from patients with stromal HSK. In these specimens, a granulomatous reaction was found at the level of Descemet's membrane. This investigation increases the possibility that DNA detected by PCR may represent the genes from chronic or recurrent ocular HSV infection.

The PCR amplification involves the use of two oligonucleotide primers flanking the DNA segment to be amplified with repeated cycles of heat denaturation of the DNA, annealing of the primers to their complementary sequences, and extension of the annealed primers with DNA Taq polymerase. The efficiency and specificity of the PCR amplification were determined by several factors. With regard to primer design, ~50% G + C content is required, and the number of complementary bases between the two primers needs to be minimized. In this way, "primer–dimer" formation is reduced, which may affect overall PCR efficiency. The primer–dimer artifact may occur (1) at the molecular weight sum for the two primers (or shorter in the case of 3'-end overlaps), and (2) in the presence of high primer concentration or at low starting template DNA concentrations. During the screening process of primers we used, some did not work as well as others, eg, a few produced more than one amplification signal. The pair of LAT primers selected was obviously weaker than the TK primers as demonstrated by ethidium bromide staining (Figs. 1A, 1C but not in Figs. 1B, 1D, where the exposure time was prolonged), and a weak primer–dimer band was found at the bottom of Figure 1D. Primer–dimer formation may be one explanation for weak amplification signals of the LAT region. Despite these weak signals, the LAT primers generated a single specific band and relatively high ratio of HSV gene amplification in the HSK corneas. The concentrations of the primers, magnesium, Taq polymerase, and dNTP are also important to ensure PCR efficiency and specificity. We optimized these parameters to achieve a single, strong amplification signal. Also, the annealing temperature was increased from 37–55°C, resulting in a significant increase in the PCR amplification efficiency and specificity. After maximizing all these experimental variables, we obtained a high specificity of LAT and TK primers based on the following criteria: (1) a single amplification band in a size that was expected for TK and LAT, (2) lack of an amplification signal in negative-control corneas, (3) lack of an amplification signal in nonherpetic corneas, and (4) testing the specificity of our primers against purified varicella zoster virus (VZV) DNA, which did not elicit positive signals (data not shown). Unfortunately, we do not have human corneal samples from other viral infections for analysis in our PCR assay because these other virally infected corneas are rarely transplanted, except occasionally for VZV. Finally, avoiding false positives is a critical step to ensure fidelity in all PCR reactions. The exquisite sensitivity of the PCR technique means that exogenous DNA contamination of samples may or will give false-positive results, making interpretation of test results difficult. Precautions must be taken such as isolation of PCR preparations and products, use of positive displacement pipettes with disposable tips and plungers (Gibson Microman; Rainin, Woburn, MA), and inclusion of "no DNA" reagent controls and negative sample controls with each set of amplification samples.

Key words: HSV-1, polymerase chain reaction, PCR, extraneuronal latency, herpes simplex keratitis, HSK, latency-associated transcripts, LATs, thymidine kinase, TK
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References