Tissue-Specific Accumulation of Latency-Associated Transcripts in Herpes Virus–Infected Rabbits

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PURPOSE. Herpes simplex virus (HSV) DNA persists in the corneas of patients and animals with a history of herpetic keratitis. The purpose of this study was to detect viral transcripts in the corneas of latently infected rabbits with a history of herpetic keratitis to determine whether the viral DNA represents latent virus, characterized by the restricted transcription of HSV genes and accumulation of the stable latency-associated transcripts (LATs), as occurs in neurons.

METHODS. Rabbits were injected in the subalveolar mucosa with HSV strain RE. After 30 days, corneas were infected by intrastromal injection of HSV. Corneal disease was evaluated, and 7 to 378 days after infection, the rabbits were killed. DNA and RNA were isolated from corneas and trigeminal ganglia and amplified by PCR using gene-specific primers.

RESULTS. Herpetic keratitis developed in all rabbits. All corneas of these immune rabbits contained viral DNA as many as 120 days after infection and then the frequency decreased over the next 260 days. Overall, viral DNA was detected in all ganglia and in 57% of corneas. All latently infected ganglia but no corneas contained LATs. Transcripts of the early viral gene for thymidine kinase were detected in 25 of 30 ganglia and 10 of 17 corneas. Transcripts for the late viral glycoprotein C were not detected in either tissue.

CONCLUSIONS. These data document that after HSV keratitis, viral DNA persists in corneas in the absence of stable LATs and with restricted expression of other viral genes. (Invest Ophthalmol Vis Sci. 1998;39:1847–1853)
in the promoter-enhancer. Transient transfection studies of LAT promoter-enhancer function have shown upregulation of gene expression in a neuronal cell line and in neural crest-derived human cornea stromal cells, but not in mesodermally derived fibroblasts. However, studies of LAT expression and accumulation in corneas of humans and in animal models have produced conflicting data. In latently infected rabbits after a primary corneal infection, LATs were detected using RT-PCR in 2 of 22 corneas taken between 41 and 147 days after infection. Both LAT-positive corneas were from the same animal. The animals had been infected with a strain of virus, McKrae, which spontaneously reactivates at a frequency of approximately 10%. Thus, it is tempting to speculate that the LATs detected in this case were present in corneas undergoing reactivation. In latently infected mice in which primary infections were established by the corneal route, LATs were detected by in situ hybridization in 25% of pooled corneal samples, whereas only 3% of those samples contained detectable ICp0 transcripts. Therefore, studies in two animal models suggest that LAT expression may exist in some corneas during latency.

Human corneas from recipients of penetrating keratoplasty who had a history of herpetic keratitis but were not shedding virus at the time of surgery, frequently contain HSV DNA. In one study of such corneas, viral DNA was detected in 7 of 13 corneas by PCR, but no evidence was found of viral transcripts by in situ hybridization. A second study of viral transcripts in corneas of presumably latently infected humans found eight of eight corneas contained LATs, whereas only two of the eight possessed transcripts of the late viral gene for glycoprotein C (gC). Results in the latter study strongly suggested that virus latent in corneas may be in a form similar to that characteristically found in neurons.

Thus, it remains to be determined whether latent infections with all the characteristics of neuronal latency can be established in cell types other than neurons. Our previous studies and the work of others have clearly established that viral DNA persists in the corneas of latently infected rabbits that have had an episode of herpetic keratitis. Furthermore, we have documented that HSV RE strain can be recovered from 10% of rabbit corneal cell cultures established as many as 118 days after infection, conditions that indicate that latency was established in the corneas. The studies reported here document an absence of detectable LAT expression in these corneas. Thus, the latent virus, as it exists in latently infected corneas is in a form that does not accumulate LATs and that represses but does not absolutely block the expression of all other viral genes.

**METHODS**

**Rabbit Model of Herpetic Keratitis**

The rabbit model of herpetic keratitis used in this study was similar to other models in which animals possess an immune response to HSV before initial ocular infection. Animals were anesthetized with an intramuscular injection of 25 mg/kg ketamine HCl (Fort Dodge Laboratories, Fort Dodge, IA), 5 mg/kg xylazine-HCl (Phoenix Pharmaceuticals, St. Joseph, MO), and two drops of 0.5% proparacaine HCl (Alcon Laboratories, Hamacao, PR) at the site of injection. Primary infection was produced by injection of 10⁵ plaque-forming units (PFU) of HSV type 1 RE strain in the alveolar mucosa at the inferior margin near the midline of the mandible. Injection of virus resulted in a subclinical infection. Neutralizing antibody was detected in the serum of more than 90% of animals by 14 days after infection. Shedding of virus at the ocular surface was assessed by rinsing the ocular surface and then placing the rinse (total, 0.1 ml) onto Vero cells, which were subsequently examined for viral cytopathic effect. No virus was detected nor were there any signs of herpetic eye disease observed 30 days after infection of the alveolar mucosa.

Viruses (10⁵ PFU of RE strain) was then injected into the corneal stroma to produce herpetic keratitis. Corneal epithelial disease was evaluated by a 0 to +4 scoring system in which the area of fluorescein stain was estimated on slit lamp examination. Corneal stromal disease was evaluated by measuring corneal thickness using a pachymeter. Ocular disease was characterized by the appearance of dendrites followed by stromal edema, disciform and/or interstitial keratitis, and spontaneous resolution of disease with residual corneal infiltrate (Fig. 1). Significant differences (P ≤ 0.001) among lesion scores were determined by the nonparametric Mann-Whitney test.

The two important advantages of this model are: Keratitis was produced in an immune animal as it usually is in humans, and no deaths attributable to HSV infection occurred during the course of the studies. All protocols were approved by the
college’s Animal Care Committee and conformed to guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Detection of Viral Nucleic Acids

At various times after infection, animals were killed (1.0 ml/5 kg intravenous anesthesia (Beuthanasia-D; Schering-Plough, Kenilworth, NJ) and the corneas and trigeminal ganglia (TG) harvested by dissection and immediately placed in separate vials in liquid nitrogen. Frozen tissues were pulverized in a liquid nitrogen-cooled tissue pulverizer and then placed in a guanidinium isothiocyanate–based reagent (Tri Reagent, MCR, Cincinnati, OH) for the simultaneous extraction of DNA and total RNA. The tissue–reagent mixture was homogenized on ice using a tissue homogenizer (model 2000; Omni International, Waterbury, CT) equipped with disposable tips. All isolated RNA samples were treated with RNase-free DNase (Fast Protein Liquid Chromatography purified; Pharmacia Biotech, Piscataway, NJ) to eliminate DNA contamination. The yield of DNA per cornea was 29.8 ± 9.8 μg (n = 8) and per TG was 31.3 ± 12.9 μg (n = 8). The yield of RNA per cornea was 33.1 ± 8.2 μg (n = 8) and per TG was 40.8 ± 14.6 μg (n = 8). DNA was quantitated by standard curve methodology using fluorescein probe (PicoGreen; Molecular Probes, Eugene, OR) with excitation 490 nm and emission 550 nm. RNA was quantitated by standard curves using another probe (SYBR Green II, Molecular Probes) with excitation 490 nm, emission 550 nm.

Viral DNA was detected by PCR using viral-specific primers. PCRs contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂ (2.1 mM for LAT amplification), 200 μM of each of the four deoxyribonucleoside triphosphates, 0.4 μM primers, 1.5 U DNA polymerase (AmpMasa; Perkin–Elmer, Branchburg, NJ), and 1 μg to 5 μg DNA. The sequences of the gene-specific primers have been published: latency-associated transcript (LAT:1,2), a 195-bp product; thymidine kinase (TK:1,2,3,4), a 274-bp product; glycophosphatidylinositol (GPI)-anchored protein (gC:1,2), a 115-bp product; and infected-cell protein 4 (ICP4:1,2), a 101-bp product. Cellular DNA was detected using primers specific for the rabbit interleukin-1 (IL-1) gene, which generated a 215-bp product. These primers (IL-1:2,3) were of our design based on a sequence of the rabbit IL-1 gene provided by Dainain Pharmaceutical, Osaka, Japan. The sequences were (2) 5'-GAGCGCAACCCA-CAGGTCGTT-3' and (3) 5'-GAGGGCTACGACATGCTCA-3'. All assays were conducted by a hot-start procedure using Ampli- wax (Perkin–Elmer). The cycling procedure (model 480 thermocycler, Perkin–Elmer) was as follows: 1 cycle: 94°C/5 min-65°C/1 min-72°C/1 min; 40 cycles: 94°C/1 min-65°C/1 min-72°C/1 min; and 1 cycle: 94°C/1 min-65°C/1 min-72°C/5 min; then, hold at 4°C.

Viral RNA was detected using RT-PCR. The RT reaction contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM MgCl₂, 1 mM deoxyribonucleoside triphosphate (0.25 mM each), 1 U/ml RTase inhibitor (Perkin–Elmer), 2.5 U/μl MuLV reverse transcriptase (Perkin–Elmer), 1 μM 3′-specific PCR primer (cited earlier), and approximately 1 μg RNA. Reactions were performed at 42°C for 30 minutes. Then, 1 μl of the RT reaction, containing the cDNA, was amplified using the PCR conditions cited earlier. Cellular RNA was subjected to reverse transcription and then amplified using primers specific for glyceraldehyde 3-phosphate dehydrogenase (GAPDH:1,2), generating a 194-bp product. Control RT-PCR reactions were routinely conducted in the presence and absence of reverse transcriptase to ensure that the PCR product was generated from RNA and not DNA.

DNA products of PCR were separated on agarose gels and transferred to ZetaProbe GT (Bio-Rad, Hercules, CA) by a reverse Southern procedure, in alkaline buffer. The viral sequences were detected by hybridization to viral-specific oligonucleotide probes using a commercial kit (QuikHyb; Stratagene, La Jolla, CA). The sequences of these probes were those in the publications cited for the primer sequences. Oligonucleotide probes were end-labeled with γ[32P]-adenosine triphosphate (New England BioLabs, Beverly, MA). Preparation of Standard for Determining the Sensitivity of Detection of LATs

To establish the sensitivity of LAT detection, a standard LAT RNA preparation was made. Herpes simplex virus (strain RE) was purified by differential centrifugation and the DNA extracted from purified virions and amplified, using LAT:1,2 primers to generate a 195-bp product. This product was cloned into the lacZ gene of the pCR2.1 cloning vector (Invitrogen, Carlsbad, CA), transformed into Escherichia coli TOP10F’ cells (Invitrogen, Carlsbad, CA), and lacZ’ colonies were screened using PCR to detect the LAT insert. The sequence of the insert was confirmed using cycle sequencing by the dye-labeled terminator method using the DNA polymerase FS (AmpliTaq; Perkin–Elmer), and an ABI310 Genetic Analyzer. A stock of purified plasmid was prepared (Wizard Maxi Prep DNA purification system; Promega, Madison, WI) and linearized by digestion with SpeI, and the DNA was purified by proteinase K treatment followed by phenol–chloroform extraction and ethanol precipitation. The resultant linear DNA product was used as a source of template for in vitro transcription by T7 RNA polymerase (RiboMax; Promega). A 500-base RNA product containing the LAT RNA segment amplified by our primers was synthesized. The LAT-containing RNA was isolated by phenol–chloroform–isoamyl alcohol extraction. The aqueous phase was passed through G-50 spin columns and the RNA precipitated with ethanol. The purity of the RNA was analyzed on 1.5% agarose gels stained with SYBR Green II and the quantity of RNA contained in the resultant standard solution was assessed by quantitative RNA assay, using SYBR Green II. The sequence of the 195-bp PCR product generated by this standard LAT RT-PCR reaction was confirmed using the dye-labeled termination method previously described. Serial dilutions of this standard RNA preparation were then used to determine the sensitivity of our RT-PCR system using standard curve methodology.

RNA at concentrations as low as 0.1 fg was detectable in our assay, but 0.01 fg was not (Fig. 2). The molecular weight of the 300-nucleotide transcript was 300 nucleotides × 339 g/mol nucleotide = 101,700 g/mol transcript; 0.1 fg (10−15 g) RNA = 101,700 g/mol = 9.8328 × 10−22 mol detectable; 9.8328 × 10−22 mol × 6.02 × 1023 molecules/mol = 592 molecules. Therefore, the minimum detectable number of transcripts was between 592 and 59 copies of LAT RNA in the presence of a fixed amount (1 μg) of rabbit corneal RNA.
RESULTS

Detection of Viral DNA and Transcripts during Acute Herpetic Keratitis

To establish that our PCR and RT-PCR methods could detect viral DNA and transcripts in tissue extracts, we harvested corneas and TG from rabbits 7 days after intrastromal infection. Fluorescent staining of the corneas on the day before death showed dendrites indicative of acute disease. Cultures of corneal rinses on the day of harvest showed shedding of infectious virus at the ocular surface. Viral DNA was detected in the corneas and TG by PCR using primers for the viral TK (Table 1). Amplification of nucleic acids from corneas or TG of uninfected rabbits using the viral-specific primers resulted in no detectable product, indicating that our primers did not amplify cellular sequences. Cellular DNA from infected and uninfected tissues was amplifiable with primers from the viral IL-1 gene.

Reverse transcription-polymerase chain reaction analysis of RNA isolated from acutely infected corneas and TG documented that transcripts from LAT and TK genes could be detected (Table 1). Transcripts from ICP4 and gC regions of the genome were also occasionally detected (data not shown). Transcripts of the cellular glyceraldehyde 3-phosphate dehydrogenase gene were detectable in all RNA preparations. Control reactions containing RNA isolated from uninfected tissues or samples without RT produced no PCR products of the appropriate size that hybridized to HSV-specific probes.

Detection of Viral DNA and Transcripts in Corneas and TG of Latently Infected Rabbits

Rabbits injected by the alveolar mucosal route seroconverted, containing neutralizing anti-HSV antibodies in their serum. Intrastromal injection of HSV into the corneas of these rabbits caused the development of significant stromal edema 5 to 20 days after infection (Fig. 1). Corneas returned to normal thickness by 30 days after corneal infection. Cultures of the ocular surface showed that virus was not shed before death. Animals were killed at various times between 54 days (1.8 months) and 378 days (12.6 months) after infection of the corneas.

Viral DNA was detected in 100% (30 of 30) of TG analyzed during the first year after corneal infection using primers for TK (Table 1). Detection of TK DNA using nested primer pairs was our most sensitive assay, detecting as few as three genome equivalents of virus. When samples were grouped by the time after infection, the frequency of detection of DNA in TG did not decline with time (Table 1). Reverse transcription-polymerase chain reaction detected the presence of LATs (Table 1), but not transcripts from gC (data not shown) in 100% of the TG. Ethidium bromide-stained gels of RT-PCR reactions and Southern blots of these gels probed with 32P-labeled LAT-specific probe showed LAT product from ganglia (Fig. 3). RNA transcribed from the TK gene was detected in 23 of 30 latently infected TG, consistent with recent reports from other laboratories (Table 1).16,17 These data documented that latent infec-tions existed in the TG of all rabbits.

At earlier times (120 days or less) after infection, 100% of corneas of latently infected rabbits contained viral DNA (Table 1). When animals were grouped by time after corneal infection, the frequency of detection of viral DNA in the cornea declined significantly after 4 months, after which viral DNA was detected in approximately 50% of the corneas of latently infected rabbits (5 of 16) using our most sensitive primer pair (Table 1). The frequency of detection of viral DNA with less sensitive primer pairs was similarly reduced.

Reverse transcription-polymerase chain reaction analysis of viral RNA indicated that in contrast to acutely infected

| TABLE 1. Summary of HSV Infection, Disease, and Detection of Nucleic Acid |
|-----------------------------|------------------|----------------|------------------|-------------------|
| Injection Site             | Proportion of   | Months after  | Proportion Containing DNA or RNA |
| Alveolar                  | Corneas         | Disease       | Corneas          | Latent           | Tk               |
| Mucosa*                   | Acute           | Stromal       | DNA              | RNA               | RNA              |
| Rabbit injected by         | 0/6             | 0/6           | 0/6              | 0/6               | 0/6              |
| RE 30 days before corneal  | 8/8             | 8/8           | 8/8              | 8/8               | 8/8              |
| +                           | 8/8             | 8/8           | 8/8              | 8/8               | 8/8              |
| +                           | 5/6             | 6/6           | 5/6              | 5/6               | 5/6              |

* Rabbits injected by subalveolar mucosal injection of 105 PFU HSV RE 30 days before corneal infection. All except un.injected control animals developed HSV-neutralizing antibody of 1:20 or more.

HSV, herpes simplex virus; LAT, latency-associated transcript; TK, thymidine kinase; TG, trigeminal ganglia; PFU, plaque-forming units.
products of reverse transcription-polymerase chain reaction. This level of sensitivity would detect one latently infected neuron. Lanes 2, 3, 6, 9, and 12, products of amplification of RNA from latently infected corneas; lanes 4, 5, 8, and 9, products of amplification of RNA from latently infected trigeminal ganglia; and lane 12, amplification product from an authentic LAT RNA standard.

DISCUSSION

Our data clearly show that LATs did not accumulate to detectable levels in corneas of latently infected rabbits that possessed HSV DNA expressed detectable LATs (Table 1). Using an in vitro transcribed LAT RNA standard, the minimum amount of the LAT-containing RNA detectable was 0.1 fg in 1 μg of corneal RNA, corresponding to 592 molecules of LAT (Fig. 2). This level of sensitivity would detect one latently infected neuron. Transcripts from the viral TK region could be detected in 10 of 17 corneas containing HSV DNA (Table 1). The frequency of detection of TK transcripts among corneas containing HSV DNA did not decrease with time after infection. Transcripts of ICP4 were detected in only 2 of 14 corneas possessing HSV DNA. In no case did our second most sensitive primer pair gC detect transcripts in the corneas of latently infected rabbits containing viral DNA.

Tissue-Specific Accumulation of LATs

Corneas, all of which contained viral DNA and expressed LATs, none of the corneas of latently infected rabbits that possessed HSV DNA expressed detectable LATs (Table 1). Using an in vitro transcribed LAT RNA standard, the minimum amount of the LAT-containing RNA detectable was 0.1 fg in 1 μg of corneal RNA, corresponding to 592 molecules of LAT (Fig. 2). This level of sensitivity would detect one latently infected neuron. Transcripts from the viral TK region could be detected in 10 of 17 corneas containing HSV DNA (Table 1). The frequency of detection of TK transcripts among corneas containing HSV DNA did not decrease with time after infection. Transcripts of ICP4 were detected in only 2 of 14 corneas possessing HSV DNA. In no case did our second most sensitive primer pair gC detect transcripts in the corneas of latently infected rabbits containing viral DNA.

Previous investigators have attempted to detect LATs in human corneas. Laycock et al. indicated that LATs were not detected by in situ hybridization in corneas of patients with a history of herpetic keratitis, even though viral DNA could be detected in 7 of 13 samples by PCR. In contrast, Kaye et al. found that 10 of 10 corneas of patients with a history of keratitis contained viral DNA detectable by PCR and 8 of 8 examined by RT-PCR expressed LATs. It is likely that the difference in incidence of LAT detection is caused in part by the difference in sensitivity between RT-PCR and in situ hybridization. In addition, differences in tissue processing make direct comparison of these studies difficult. In situ hybridization was performed on archived fixed tissues, whereas RT-PCR was conducted using snap-frozen, fresh tissues. The latter is less likely to result in RNA degradation. In the studies of Kaye et al. infectious virus and gC transcripts were both detected in only 1 of 10 cases. In one additional case gC transcripts were detected in the absence of infectious virus, strongly suggesting that virus was latent in the remaining cases.

Reports of animal models of herpetic eye disease also contain conflicting data. Cook et al. using virus strains known to reactivate spontaneously in the rabbit, detected LATs in 2 of 22 rabbit corneas in the absence of transcripts for TK, whereas all TG contained LATs. Thymidine kinase transcripts were not detected in 4 of the 22 corneas tested. In the data reported here, we were unable to detect LATs in the corneas of rabbits latently infected with a strain of virus (RE)
that does not reactivate spontaneously. The same primer sets for LAT and gC were used in both studies, which makes primers or the PCR procedure an unlikely source of differences. In a third study using a rabbit model, investigators examined expression of HSV genes in corneas after epinephrine-induced reactivation. Latency-associated transcripts were detected in 4 of 4 corneas from rabbits killed before reactivation and in only 2 of 21 corneas after reactivation, whereas transcripts for VP5, a late capsid protein, were detected in 13 of 21 corneas after reactivation. In this third study, strain 17syn* was used, a strain known to reactivate spontaneously in approximately 10% to 15% of eyes. The reason for detection of LATs in corneas not stimulated to reactivate and the low frequency of detection in the reactivated corneas is unclear, especially at times between 72 and 168 hours after reactivation, when it would be expected that LATs would be expressed as a late gene in productive-cycle replication. These studies together suggest that there may be a difference between corneal LAT expression during latency depending on virus strain.

Studies in which mouse models were used for latency have also produced conflicting data. Abghari et al.27 used in situ hybridization to detect LATs in 25% of the pools of cells harvested from the corneas of mice latently infected with the McKrae strain of virus, a strain known to reactivate spontaneously in rabbits. Only 3% of these LAT-positive samples also possessed ICP0 transcripts, which suggests that virus had reactivated in only a fraction of these corneas. Tanaka et al.44 were unable to detect LATs or ICP0 transcripts in the eyes of mice infected by intracameral injection of virus. In both of these studies Balb/c mice were used, but the strains of virus used were different.

In all animal model systems the frequency with which LATs accumulate to detectable levels in corneas of latently infected animals with a history of herpetic keratitis is low (10%). In human tissues, results in the few available studies have also produced conflicting data. Abghari et al. 27 used in situ hybridization to detect LATs in 25% of the pools of cells harvested from the corneas of mice latently infected with the McKrae strain of virus, a strain known to reactivate spontaneously in rabbits. Only 3% of these LAT-positive samples also possessed ICP0 transcripts, which suggests that virus had reactivated in only a fraction of these corneas. Tanaka et al.44 were unable to detect LATs or ICP0 transcripts in the eyes of mice infected by intracameral injection of virus. In both of these studies Balb/c mice were used, but the strains of virus used were different.

It is clear that the latent virus in the cornea conforms to the classic definition of latency: the viral genome persists, transcription is extremely limited, and infectious virus can be reactivated from the latently infected cells. Some investigators have extended the definition of latency to include the expression of LATs311.20, however, numerous studies have shown that latency can be established and maintained in neurons in the absence of LAT expression.28 Therefore, it does not seem warranted to include this expression in the definition of latency. Because LAT expression improves the efficiency of reactivation from latency, it is possible that the rate of reactivation seen in the corneal cultures is negatively influenced by the absence of LAT expression.

The important question that still remains is whether this latent virus in the cornea can contribute to herpetic eye disease. The expression of HSV genes, for example, TK, could contribute to antigenic stimulation in the absence of reactivation. With our current methods, it was not possible to determine whether the TK transcripts detected are authentic, were perhaps read-through transcripts from other promoters, or were translated into proteins. The local expression of viral genes within the cornea and immune presentation of their products certainly has the potential to provide a stimulatory event that could lead to the immune response characteristic of herpetic stromal disease.

References


