HSV-1 Migration in Latently Infected and Naive Rabbits after Penetrating Keratoplasty

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Purpose. To investigate the migration of herpes simplex virus type 1 (HSV-1) between latently infected and naive corneal tissues and trigeminal ganglion (TG) in rabbits after penetrating keratoplasty (PKP) and transcorneal epinephrine iontophoresis.

Methods. Two mutants, genetically constructed from HSV-1 strain 17*syn*$^{+}$, were used to inoculate rabbit corneas: 17ΔPst, a latency associated transcript (LAT) negative, low-reactivating virus and 17Pr, a high-reactivating, LAT-positive rescuant of 17ΔPst. Latently infected rabbits were given corneal allografts from naive rabbits, and naive rabbits received grafts from latently infected rabbits. Ninety days after PKP, groups of the transplanted rabbits were induced to reactivate by transcorneal epinephrine iontophoresis, but others were not induced. Viral shedding was monitored by tear film cultures. Rabbits were killed 5 days after iontophoresis. Transplanted grafts, recipient corneal rims, and corresponding TG were obtained. Nucleic acids were extracted and amplified for detection of HSV-1 DNA and viral gene transcription.

Results. In naive rabbits receiving grafts transplanted from rabbits latently infected with 17Pr (LAT^{+}), 3 of 6 corneal rims contained HSV DNA after induction. In contrast, none of the 5 corneal rims from naive rabbits receiving grafts from rabbits latent with 17ΔPst (LAT^{-}) contained viral DNA. Viral DNA and gene transcripts were detected in 2 of 6 TG from naive rabbits that received grafts from 17Pr (LAT^{+}) latently infected rabbits. In recipient corneal rims and TG of latently infected rabbits receiving grafts from naive rabbits, viral DNA concentration was significantly greater with induced reactivation, compared with the results in noninduced rabbits. The amount of viral DNA in naive grafts transplanted into 17Pr (LAT^{+}) latently infected rabbits was significantly higher with induction than without induction ($P = 0.018$). More viral DNA and viral gene transcripts were found in tissues from rabbits latently infected with 17Pr (LAT^{+}) than in rabbits latently infected with 17ΔPst (LAT^{-}).

Conclusions. Corneas from latently infected rabbits contain HSV-1 DNA that can replicate after induced reactivation. Viral migration can occur in both anterograde and retrograde directions between the transplanted graft and the recipient corneal rim and TG. The LAT negative HSV-1 construct 17ΔPst has a significantly reduced ability to replicate and migrate. (*Invest Ophthalmol Vis Sci.* 1999;40:2490–2497)

Herpes simplex virus type 1 (HSV-1) is capable of causing a variety of ocular infections and establishing a lifelong cycle of latency and reactivation.[1–5] HSV-1 reactivates in response to chemical and physical stress,[6] UV irradiation,[7] hypothermia,[8] and hyperthermia.[9] In rabbits, reactivation can be induced by transcorneal epinephrine iontophoresis.[10–17]

Since the isolation of HSV-1 from the sensory ganglion in 1972,[18] investigators have focused on the trigeminal ganglion (TG) as a major site of viral latency. More recently, the development of new biochemical and sensitive molecular biological techniques has stimulated interest in the possibility of detecting extraneuronal sites of HSV-1 latency. In this regard, the cornea has received considerable attention. However, no definitive studies on the molecular state of the virus-host relationship and the dynamics of HSV-1 migration during corneal latency have emerged, and, to date, there has been no single experiment that can prove HSV-1 corneal latency.[19–31]

During the latent phase of HSV-1 infection, viral gene expression is highly restricted. Only the latency associated transcripts (LAT) are produced in abundance during latency, and LAT have become the characteristic marker of latent HSV-1 infection.[1–3,19–23] Also, LAT have been shown to have a significant association with HSV-1 reactivation; numerous studies

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have shown that LAT-deleted viruses have a reduced ability to undergo reactivation in the rabbit.[32–37] Does the LAT-deleted virus also spread from a site of latency to other tissues after reactivation with less efficiency in comparison with LAT^+ virus? To the best of our knowledge, there have been no studies that correlate the ability of an HSV-1 strain to express LAT with the potential of the virus to migrate from a site of latency to other tissues after reactivation.

We designed experiments to study the dynamics of HSV-1 latency using 17ΔPst (LAT^-) and 17Pr (LAT^+), both of which have 17*syn*$^+$ as the parent strain.[19,32,38] The purpose of this study was to test whether the LAT^+ and LAT^- strains of HSV-1 could establish latent infection in rabbit corneas and, if so, whether there is a difference between the two strains in their ability to be reactivated and transferred to naive tissues through corneal grafts. We transplanted corneas from HSV-1 latently infected rabbits to naive uninfected rabbits and from naive uninfected rabbits to latently infected rabbits to investigate the potential for HSV-1 migration with respect to LAT expression status. HSV DNA concentration and productive viral gene transcription were measured in the grafts, recipient corneal rims, and corresponding TG. Our results showed that corneas from latently infected rabbits contain HSV-1 DNA, which is able to replicate and migrate after induced reactivation, and that compared with the LAT^+ construct, the LAT^- mutant has a significantly reduced ability to undergo these processes.

Methods

Cells and Virus

Two HSV-1 genetic constructs were used. 17ΔPst is a LAT^- mutant constructed from the high-reactivation phenotype parent 17*syn*$^+$ by deletion of a 202-bp portion corresponding to bases 118664 through 118866. This LAT^- mutant has previously been shown to exhibit a significantly reduced reactivation frequency.[19,32,38] 17Pr, which is LAT^+, is the rescuant of 17ΔPst (LAT^-), and has the same high-frequency reactivation phenotype as the parent strain 17*syn*$^+$.[19,32,38]

Viruses were propagated on primary rabbit kidney (PRK) cell monolayers in minimal essential medium (MEM; GIBCO Life Technologies, Gaithersburg, MD) with 5% fetal bovine serum and titered by plaque assay on African green monkey kidney cell monolayers.

Establishment of Latency

The care and maintenance of all rabbits used in these experiments conformed to the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research. New Zealand White rabbits (1.5–2.5 kg) were inoculated with 25 μl of a viral suspension (1–2 $\times$ 10^6 plaque-forming units [pfu]) of either 17ΔPst (LAT^-) or 17Pr (LAT^+) in both eyes without scarification. The occurrence of corneal lesions confirming acute infection was verified by slit-lamp examination 3 to 8 days after inoculation. Rabbits were also examined 20 days after infection to verify that the corneal lesions had healed. Rabbits in which the corneal lesions had resolved were assumed to be latently infected.

Experimental Design

Ten weeks after inoculation, ocular swabbing was done on all rabbits before penetrating keratoplasty (PKP) to detect spontaneous viral shedding. Rabbits without viral shedding were used for transplantation. Four groups of rabbits underwent penetrating keratoplasty. One group consisted of rabbits latently infected with 17ΔPst (LAT^-) that received grafts from naive rabbits. The second group consisted of rabbits latently infected with 17Pr (LAT^+) that also received grafts from naive rabbits. The third group consisted of naive rabbits that received grafts from rabbits latently infected with 17ΔPst (LAT^-), and the fourth group consisted of naive rabbits that received grafts from rabbits latently infected with 17Pr (LAT^+). In all cases one eye received a graft and the other eye remained untreated.

Approximately 90 days after PKP, all rabbits were swabbed again once daily for 5 consecutive days to detect spontaneous viral shedding. Rabbits that did not shed virus were used for the induced reactivation experiments. Rabbits from groups 2 (rabbits latently infected with 17Pr; naive grafts) and 4 (naive rabbits; grafts from rabbits latently infected with 17Pr) were assigned randomly to an induction or noninduction group. All the rabbits from groups 1 (rabbits latently infected with 17ΔPst; naive grafts) and 3 (naive rabbits; grafts from rabbits latently infected with 17ΔPst) were assigned to induction. The induction groups received transcorneal epinephrine iontophoresis (0.8 mAmp, 8 minutes) once daily for 3 consecutive days. Beginning on the day after the first iontophoresis, tear film samples were obtained daily for 5 days for the detection of infectious virus. Similarly, tear film samples were also obtained from the noninduction groups.

At the conclusion of the tear film–sampling period, all rabbits were killed and the corneas were dissected into two parts, grafts and recipient corneal rims. Corresponding TG were also obtained. Nucleic acids were extracted and amplified by polymerase chain reaction (PCR) for detection of HSV DNA and RNA.

Penetrating Keratoplasty

Penetrating keratoplasty was performed as described by Rootman et al.[39] An 8.0-mm–diameter site was created in the central cornea of the recipient and an 8.5-mm graft was sutured into place. The 0.5-mm larger graft was used to ensure proper wound closure and restoration of the anterior chamber. For 1 week after the transplantation, rabbit eyes were treated twice daily with prednisolone acetate (1%) and tobramycin (0.3%).

Tear Film Cultures

Tear film was collected on a sterile Dacron swab (Puritan; Hardwood Products, Guilford, ME). Each swab was immediately placed into a tissue culture tube containing a confluent PRK cell monolayer in 2 ml of MEM/2% fetal bovine serum and incubated at 37°C and 5% CO_2. The swab was removed 24 hours later and the tube monitored daily for 14 days for the appearance of cytopathic effects indicative of infectious virus.

Transcorneal Epinephrine Iontophoresis

Iontophoresis was performed once daily for 3 consecutive days as previously described.[10–17] A large eyecup (central diameter 12-mm) was used so that the cylinder containing 0.01% epinephrine was in contact with both the graft and recipient corneal rim.

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Table 1. Primer Pairs Used to Detect DNA Replication and Gene Transcription

Primer Pairs*	Sequence Location† (bp)	Size of PCR Product (bp)
Ribonucleotide reductase (RR, U_L39)		
5′-ATGCCAGACCTGTTTTTCAA-3′	88517–88536	243
5′-GTCTTTGAACATGACGAAGG-3′	88759–88740	
Major capsid protein (VP5, U_L19)		
5′-TGAACCCCAGCCCCAGAAACC-3′	35564–35544	149
5′-CGAGTAAACCATGTTAAGGACC-3′	35416–35437	
Rabbit actin		
5′-AAGATCTGGCACCACACCTT-3′	—	110
5′-CGAACATGATCTGGGTCATC-3′	—	

* Primers are listed as the upstream (mRNA sense) primer first followed by the downstream (mRNA antisense) primer.

† Locations of the primer sequence for viral genes are based on the sequence of the 17syn$^+$ strain of HSV-1.

Sample Collection and Tissue Processing

Three days after the last iontophoresis, the rabbits were killed and the grafts, recipient corneal rims, and corresponding TG were removed aseptically. The corneal graft was defined as the transplant excluding the suturing area in latently infected rabbits and including the suturing area in naive rabbits; the corneal rim was the remaining peripheral part. To avoid tear film contamination of corneal samples, the corneal surface was thoroughly washed with sterile balanced saline solution, 5% sodium hypochlorite, 70% ethanol, and diethylpyrocarbonate-treated water before dissection.

The tissues were frozen in liquid nitrogen and stored at −70°C. For analysis, tissues were thawed in Trizol reagent (GIBCO Life Technologies) and homogenized with a PRO 200 homogenizer equipped with a metal probe (PRO Scientific, Monroe, CT). DNA and RNA were extracted with Trizol reagent according to the manufacturer's instructions.

RNA solution was extensively digested with RNase-free DNase (Sigma Chemical, St. Louis, MO) at 20 to 40 U per 100 μl RNA sample. DNA-free RNA was dissolved in 60 μl of water by heating to 65°C for 10 minutes and placed on ice for 1 minute. The sample was then reverse-transcribed by random primer $p(dN)_6$ using first-strand cDNA synthesis system (Boehringer Mannheim, Indianapolis, IN) at 42°C for 1 hour. After cDNA synthesis, the reaction mixture was diluted to 100 μl with distilled deionized water, and 25 μl was used in each PCR.

PCR Analysis

A primer pair for the HSV-1 ribonucleotide reductase (RR, U_L39) was used for detection of HSV-1 DNA. Two different primer pairs, one specific for RR and one for the major capsid protein gene (VP5, U_L19), were chosen to examine viral transcripts at different phases of transcription.[40] Amplification of the rabbit actin gene was used as a control for DNA recovery and quantitation of the viral DNA. The sequences of the primer pairs are given in Table 1.

Each PCR contained 0.1 μg of extracted DNA or cDNA: 0.2 μM of each primer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 2.5 U of *Taq* polymerase (Perkin-Elmer, Foster City, CA), and 1× PCR buffer in a 50 μl final volume. Cycling reactions were carried out in a thermal cycler (MJ Research, Waltham, MA). Each cycle included denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 60 seconds. PCR primer pairs specific for viral and rabbit cellular gene were used either separately or together. When the actin and VP5 primers were used together, annealing was carried out at 5°C below the actin primer melting temperature. The reaction was terminated with a 10-minute extension at 72°C. Samples underwent 45 cycles of amplification. The products were electrophoresed on 2% agarose gels, stained with ethidium bromide, and visualized using a digital imaging system (Eagle Eye II; Stratagene, La Jolla, CA).

DNA extracted from corresponding viral strain–infected rabbit cornea and TG cell suspensions was used as positive control. DNA extracted from naive uninfected rabbit cornea and rabbit TG homogenates was used as negative control. Reverse transcription–PCR (RT-PCR) controls included positive control of Neo pa RNA provided by manufacturer, RNA blank control, and AMV Reverse Transcriptase blank control. Two separate experiments were done, and all PCR samples were analyzed in triplicate.

DNA Quantitation

To measure the concentration of HSV DNA in grafts, recipient corneal rims, and TG, we amplified both viral DNA using the RR primers and cellular DNA using rabbit actin primers. Gels were digitized and analyzed using a digital imaging system with an image-processing software package (Optimas; Optimas, Seattle, WA). The perimeter of each band was manually traced, and the mean luminescence of the pixels within this region was calculated after subtracting the background luminescence. The HSV DNA concentration was expressed as a ratio of the gel band density of the RR product to rabbit actin product.

Statistical Analyses

Statistical comparisons of HSV DNA concentration between the noninduction groups and the induction groups, as well as between the two virus-phenotype induction groups, were done by conducting an initial one-way ANOVA on the DNA concentration from all the grafts, the recipient corneal rims, or

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TABLE 2. Detection of Viral Shedding in Transplanted Rabbit Eyes

Latency Status			
Recipient*	Graft	Induction	Positive Eyes†/Total Eyes (%)
Retrograde migration			
Naive	17Pr (LAT$^+$)	No	0/6 (0)
Naive	17Pr (LAT$^+$)	Yes	1/6 (17%)
Naive	17ΔPst (LAT$^-$)	Yes	1/5 (20%)
Anterograde migration			
17Pr (LAT$^+$)	Naive	No	1/6 (17%)
17Pr (LAT$^+$)	Naive	Yes	3/6 (50%)
17ΔPst (LAT$^-$)	Naive	Yes	1/5 (20%)

* Naive rabbits received corneal grafts from rabbits latently infected with either 17Pr (LAT$^+$) or 17ΔPst (LAT$^-$); rabbits latently infected with either 17Pr (LAT$^+$) or 17ΔPst (LAT$^-$) received corneal grafts from naive rabbits. Transplanted rabbits were assigned to noninduction and transcorneal epinephrine iontophoresis groups.

† Beginning after the first iontophoresis, tear film samples were obtained from all transplanted eyes once daily for 5 consecutive days for the detection of viral shedding. Data are expressed as number of positive eyes for at least one swabbing.

the TG. ANOVA was performed for the grafts from naive rabbits and separately for grafts from latently infected rabbits. If the ANOVA was significant ($P < 0.05$), comparisons of treatment means were conducted using the overall ANOVA estimate of the treatment group mean error to conduct protected pair-wise *t*-tests.[41] Statistical comparison of the number of samples positive for viral transcripts between noninduction groups and induction groups was done by conducting the chi-square test. $P < 0.05$ defines significance. All data analyses were done using procedures from the Statistical Analysis System (SAS Institute, Cary, NC).

RESULTS

Acute Infection and Corneal Graft Survival

Slit-lamp examination showed that acute infections by the LAT$^-$ mutant 17ΔPst, and the LAT$^+$ rescuant 17Pr, were similar and comparable to our previous results from the parent strain 17*syn*$^+$. This finding is consistent with the results of our acute viral titration (post-inoculation day 7) of rabbit corneal homogenates where we found that there is no statistical difference of acute viral replication in the rabbit cornea between the LAT$^-$ and LAT$^+$ viruses (data not shown). After PKP, the majority of the transplanted grafts remained clear. There were no differences in wound healing or graft survival among the PKP groups whether the rabbit was latent or naive. Also there were no differences regardless of virus strains (data not shown). Rabbits with complications such as corneal opacity or severe neovascularization were excluded from this study. There were 17 naive rabbits receiving grafts from latently infected rabbits and 17 latently infected rabbits receiving grafts from naive rabbits in this study.

Detection of Viral Shedding

Eyes with transplanted corneas were swabbed once daily for 5 consecutive days starting after the first iontophoresis. More eyes shed virus in the adrenergically induced groups than the uninduced groups as shown in Table 2. After induction, half of the rabbits latent with 17Pr (LAT$^+$) shed virus, compared with 1 of 5 rabbits latent with 17ΔPst (LAT$^-$); 50% versus 20%, $P = 0.04$, chi-square test.

HSV-1 Retrograde Migration

Naive rabbits were transplanted with corneal grafts from rabbits latently infected with 17ΔPst (LAT$^-$) or 17Pr (LAT$^+$). Rabbits were randomly assigned for noninduction or induced reactivation. Viral DNA concentration was measured in grafts, recipient corneal rims, and corresponding TG. Data are summarized in Table 3, and a representative gel is shown in Figure 1.

All the grafts from latently infected rabbits transplanted into naive rabbits contained HSV-1 DNA; the amount of DNA was 20-fold higher in the eyes subjected to iontophoresis induction, compared with the uninduced eyes. The difference in the amount of viral DNA in the induced versus uninduced 17Pr (LAT$^+$) grafts was significant ($P = 0.038$). Viral migration from the corneal graft into the naive host tissues (as documented by detection of HSV DNA in the recipient corneal rim and TG) was observed with the 17Pr (LAT$^+$) after induction in about half of the animals tested (Table 3). No viral migration occurred with uninduced 17Pr (LAT$^+$) or with induced 17ΔPst (LAT$^-$).

The present results demonstrated that HSV-1 DNA is present in the corneas of latently infected rabbits and that epinephrine iontophoresis significantly increases the amount of HSV DNA in these corneas. The fact that viral DNA of LAT$^+$ virus 17Pr can be detected in the recipient corneal rim and corresponding TG of naive rabbits after induced reactivation indicates that retrograde migration of the virus from the latently infected graft to the naive recipient occurs.

HSV-1 Anterograde Migration

Rabbits latently infected with either 17ΔPst (LAT$^-$) or 17Pr (LAT$^+$) were transplanted with corneal grafts from naive rabbits. Rabbits were randomly assigned to noninduction or induced reactivation. Viral DNA concentration was measured in corneas and TG. The results are summarized in Table 3, and a representative gel is shown in Figure 2.

All the corneal rims and TG of the latently infected rabbits contained HSV DNA; again the concentration was significantly

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Table 3. Quantitation of HSV-1 DNA in Corneas and Trigeminal Ganglia

Latency Status			DNA Concentration (Positive Samples/Total Samples)*		
Recipient†	**Graft**	**Induction**	**Graft**	**Recipient Corneal Rim**	**Corresponding TG**
Retrograde migration					
Naive	17Pr (LAT$^+$)	No	0.02 ± 0.01 (6/6)	0 (0/6)	0 (0/6)
Naive	17Pr (LAT$^+$)	Yes	0.40 ± 0.11 (6/6)	0.10 ± 0.08 (3/6)	0.02 ± 0.02 (2/6)
Naive	17ΔPst (LAT$^-$)	Yes	0.46 ± 0.19 (5/5)	0 (0/5)	0 (0/5)
Anterograde migration					
17Pr (LAT$^+$)	Naive	No	0.002 ± 0.002‡ (1/6)	0.07 ± 0.03 (6/6)	0.12 ± 0.04 (6/6)
17Pr (LAT$^+$)	Naive	Yes	0.11 ± 0.04 (5/6)	1.31 ± 0.24 (6/6)	1.38 ± 0.28 (6/6)
17ΔPst (LAT$^-$)	Naive	Yes	0.004 ± 0.002§ (2/5)	0.53 ± 0.16 (5/5)	0.57 ± 0.19 (5/5)

* Data are based on amplification of DNA samples with both ribonucleotide reductase and rabbit actin primer pairs. DNA concentration is expressed as the ratio of the band intensity of HSV-1 DNA to rabbit actin DNA. Data are expressed as the mean ± SEM in each group. Numbers in parentheses represent the number of positive samples of total sample number.

† Naive rabbits received grafts from rabbits latently infected with either 17Pr (LAT$^+$) or 17ΔPst (LAT$^-$); rabbits latently infected with either 17Pr (LAT$^+$) or 17ΔPst (LAT$^-$) received grafts from naive rabbits. Transplanted rabbits were assigned to noninduction and transcorneal epinephrine iontophoresis induction groups.

‡ One sample in six contained HSV-1 DNA with a concentration of 0.01.

§ Two samples in five had HSV-1 DNA with a concentration of 0.01.

higher in the iontophoresed groups than in those not iontophoresed (17Pr, LAT$^+$, induced versus uninduced, $P = 0.0004$ and $P = 0.0001$ for recipient corneal rim and corresponding TG, respectively; one-way ANOVA and protected pair-wise *t*-test). Also, DNA concentrations were higher in the tissues of animals infected with the 17Pr (LAT$^+$) than in the 17ΔPst (LAT$^-$) strain even after induction (induced 17Pr LAT$^+$ versus induced 17ΔPst LAT$^-$, $P = 0.007$ and $P = 0.013$ for recipient corneal rim and corresponding TG, respectively). The presence of the viral DNA in naive grafts was noted most often (5/6 eyes) in animals latent for the 17Pr (LAT$^+$) after induction but was also seen in animals latent for 17ΔPst (LAT$^-$) with induction (2/5 eyes), and in animals latent for 17Pr (LAT$^+$) without induction (1/6 eyes). The concentration of HSV DNA in the naive corneal grafts was significantly higher for 17Pr (LAT$^+$) after induction than the 17ΔPst (LAT$^-$) with induction ($P = 0.011$) or 17Pr (LAT$^+$) without induction ($P = 0.018$).

These results demonstrate that the 17Pr (LAT$^+$) migrates from the latent recipient tissues into the naive corneal grafts in greater concentrations and at higher frequencies than the 17ΔPst (LAT$^-$) after induced reactivation. Additionally, the increased concentration of LAT$^+$ viral DNA in the naive grafts after induction corresponds to an increase in the recovery of infectious virus in tear film samples (3/6 eyes) under these conditions, compared with the recovery from uninduced LAT$^+$ (1/6 eyes) and induced LAT$^-$ (1/5 eyes) rabbits (Table 2).

Detection of Viral Gene Transcription

To determine whether the migrated HSV-1 DNA correlated with the presence of productive-phase viral gene transcription, RNA was reverse-transcribed and amplified with both RR and VP5 primer pairs. The results are shown in Table 4, and a representative gel showing the amplification obtained with the VP5 primer pair is shown in Figure 3.

In the two groups not subjected to iontophoresis induction, the total number of transcript-positive samples was low (RR: 1 of 36 samples; VP5: 3 of 36 samples). Only one recipient corneal rim in a 17Pr (LAT$^+$) latently infected rabbit was positive for the RR transcript; and the recipient corneal rim and

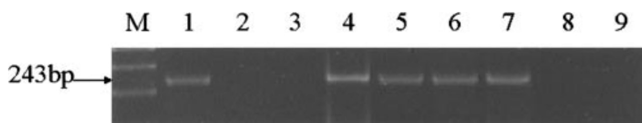

Figure 1. An example of the results of amplification of HSV-1 DNA from grafts, recipient corneal rims, and TG from naive rabbits with grafts from latently infected rabbits. PCR products were visualized by ethidium bromide staining. M, molecular marker. *Lanes 1* through *3* represent graft, recipient corneal rim, and TG, respectively, from a naive rabbit with a graft from a 17Pr (LAT$^+$) latently infected rabbit, without induced reactivation. *Lanes 4* through *6* represent graft, recipient corneal rim, and TG, respectively, from a naive rabbit with a graft from a 17Pr (LAT$^+$) latently infected rabbit, with induced reactivation by transcorneal epinephrine iontophoresis. *Lanes 7* through *9* represent graft, recipient corneal rim, and TG, respectively, from a naive rabbit with a graft from a 17ΔPst (LAT$^-$) latently infected rabbit, with transcorneal epinephrine iontophoresis.

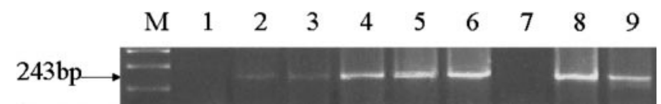

Figure 2. An example of the results of amplification of HSV-1 DNA from grafts, recipient corneal rims, and TG from latently infected rabbits with grafts from naive rabbits. M, molecular marker. *Lanes 1* through *3* represent graft, recipient corneal rim, and TG, respectively, from a 17Pr (LAT$^+$) latently infected rabbit with a graft from a naive rabbit, without induced reactivation. *Lanes 4* through *6* represent graft, recipient corneal rim, and TG, respectively, from a 17Pr (LAT$^+$) latently infected rabbit with a graft from a naive rabbit, with induced reactivation by transcorneal epinephrine iontophoresis. *Lanes 7* through *9* represent graft, recipient corneal rim, and TG, respectively, from a 17ΔPst (LAT$^-$) latently infected rabbit with a graft from a naive rabbit, with transcorneal epinephrine iontophoresis.

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Table 4. Analysis of Viral Gene Transcription in Corneas and Trigeminal Ganglia

Latency Status				Graft*		Recipient Corneal Rim		Corresponding TG	
Recipient†	**Graft**	**Induction**	**n‡**	**RR**	**VP5**	**RR**	**VP5**	**RR**	**VP5**
Retrograde migration									
Naive	17Pr (LAT$^+$)	No	6	0	1	0	0	0	0
Naive	17Pr (LAT$^+$)	Yes	6	0	1	1	1	0	1
Naive	17ΔPst (LAT$^-$)	Yes	5	0	0	0	1	0	0
Anterograde migration									
17Pr (LAT$^+$)	Naive	No	6	0	0	1	1	0	1
17Pr (LAT$^+$)	Naive	Yes	6	1	3	1	2	2	2
17ΔPst (LAT$^-$)	Naive	Yes	5	1	1	0	1	0	1

* RNA was extracted, reverse-transcribed, and amplified by PCR of 45 cycles to detect HSV-1 gene transcription. Primers specific for ribonucleotide reductase (RR) and major capsid protein (VP5) transcripts were used. Data are expressed as the number of positive samples.

† Naive rabbits received grafts from rabbits latently infected with either 17Pr (LAT$^+$) or 17ΔPst (LAT$^-$); rabbits latently infected with either 17Pr (LAT$^+$) or 17ΔPst (LAT$^-$) received grafts from naive rabbits. Transplanted rabbits were assigned to noninduction and transcorneal iontophoresis induction groups.

‡ n = number of rabbits in each group.

corresponding TG of this rabbit and one corneal graft donated by a rabbit latently infected with 17Pr (LAT $^+$) were positive for VP5 transcripts (Table 4). In the iontophoresed groups, more grafts, recipient corneal rims, and TG in 17Pr (LAT$^+$) latently infected rabbits and naive rabbits receiving grafts from 17Pr (LAT$^+$) latently infected rabbits were positive for viral transcripts (RR: 5 of 36 samples; VP5: 10 of 36 samples). A chi-square test showed that the increase for VP5 transcripts after induction was significant ($P = 0.032$), but the increase for RR transcripts was not ($P = 0.24$).

Three grafts transplanted from naive rabbits into rabbits latently infected with 17Pr (LAT$^+$) and one naive graft transplanted into a rabbit latently infected with the 17ΔPst (LAT$^-$) contained detectable VP5 transcripts after induction (Table 4), supporting the concept of anterograde migration provided by the DNA analysis (Table 3). The presence of VP5 transcripts after induction in at least one recipient corneal rim and corresponding TG in a naive rabbit that received a graft from 17Pr (LAT$^+$) latently infected rabbit (Table 4) provides additional evidence for the retrograde viral migration seen in the DNA concentration studies.

In general, tissues from rabbits latently infected with 17Pr (LAT$^+$), including recipient corneal rims and TG, showed greater transcriptional activity than the corresponding tissues from rabbits latently infected with 17ΔPst (LAT$^-$). This difference was also manifested in corneal grafts from latently infected rabbits transplanted into naive rabbits. In addition, there was a correlation between the recovery of infectious virus in the tear film and the detection of viral transcripts.

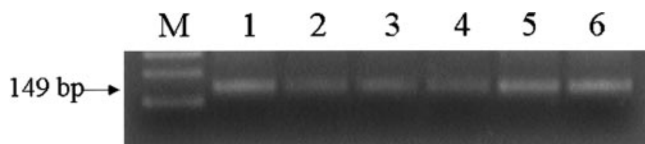

Figure 3. A representative gel showing detection of HSV-1 productive gene transcription. The results of VP5 gene amplification from cDNA generated from RNA are shown. M, molecular marker. *Lanes 1* through *3* represent graft, recipient corneal rim, and TG, respectively, from a naive rabbit with a graft from a 17Pr (LAT$^+$) latently infected rabbit, with transcorneal epinephrine iontophoresis. *Lanes 4* through *6* represent graft, recipient corneal rim, and TG, respectively, from a 17Pr (LAT$^+$) latently infected rabbit with a graft from a naive rabbit, with transcorneal epinephrine iontophoresis.

Discussion

Penetrating keratoplasty of latently infected and naive rabbits made it possible to study the migration of HSV-1 from a site of latency to other tissues. To the best of our knowledge, this is the first study that correlates the ability of an HSV-1 strain to express LAT with the potential of the viral migration after induced reactivation in a rabbit model. We conclude from this study that HSV-1 can migrate in both anterograde and retrograde directions between donor and recipient corneal tissues and corresponding TG in rabbits after induced reactivation.

Viral DNA was recovered in 63.6% (7/11) of the naive grafts transplanted into latently infected rabbits after induced reactivation. Viral migration was also seen in the retrograde direction; HSV-1 DNA was identified in 27.3% (3/11) of the recipient corneal rims and 18.2% (2/11) of the corresponding TG from naive rabbits that received grafts from latently infected rabbits. With respect to LAT gene expression, there was significantly more HSV DNA in naive grafts that were transplanted into rabbits latently infected with the LAT$^+$ virus than in grafts that were transplanted into rabbits latently infected with the LAT$^-$ virus. This difference in migration between LAT$^+$ and LAT$^-$ virus was also noted in naive rabbits that received grafts from latently infected rabbits.

HSV-1 mutants with deletions including the LAT promoter area have significantly reduced ocular reactivation after adrenergic induction.[32–37] 17ΔPst (LAT$^-$) has a 202-bp deletion of the LAT transcription promoter, which significantly reduces LAT transcription. Although this virus establishes latency with the same kinetics as its parent virus, 17*syn*$^+$, spontaneous and induced reactivation is significantly reduced.[19,32,38] Our obser-

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vation suggests that the migration of this LAT$^-$ mutant is also significantly impaired. We believe that the reduced LAT transcription could result in lower yields of the LAT$^-$ phenotype and that this may explain the low recovery of viral DNA and less transcriptional activity in rabbits after induced reactivation; therefore, there was less viral migration.

We postulate that in latently infected rabbits that received grafts from naive rabbits, reactivating HSV-1 traveled from a latent neuronal site (most likely, the TG) into corneal tissue via trigeminal nerves that innervate the cornea. Axoplasmic flow within the sensory and autonomic nerves probably serves as the conduit for the viral migration.[3,5] The virus could migrate into the naive corneal grafts through nerve sprouts or by cell–to–cell infection. In naive rabbits that received grafts from latently infected rabbits, HSV-1 in the grafts could have been reactivated and undergone retrograde migration into the recipient cornea rim and corresponding TG. Again, regenerated corneal nerve endings or cell–to–cell infection could serve as the conduit for this retrograde migration. Nicholls et al.[31] studied recurrent HSV in a corneal transplantation rat model. They found that transplantation trauma–induced HSV-1 reactivation may lack clinical signs of herpetic disease, but that the viral antigen was detectable at the graft-host junction and extended through the stroma to the endothelium, indicating that the graft-host junction is an area in which virus spreads easily. Other possible routes of viral migration could not be ruled out (e.g., the "secondary infection" by the infectious virus reactivated from latent tissues and shed in tear films after induced reactivation).

Our data provide important evidence to support the possibility of HSV-1 corneal latency. The cornea may serve as a reservoir of latent HSV-1 and as a source of viral reactivation. This conclusion is based on our ability to detect viral genomes in recipient corneal rims and corresponding TG in naive rabbits that received grafts from latently infected rabbits and on the recovery of infectious HSV-1 in the tear film of naive rabbits that received grafts from latently infected rabbits. The cornea has been studied for decades for its potential to harbor latent virus and reactivation.[19–31] Openshaw et al.[22] studied the presence and distribution of HSV-1 in eye bank corneas and detected HSV-1 DNA in 10 of 24 corneal samples. Liu et al.[26] PCR-tested 18 corneal buttons obtained from corneal transplantation patients who had quiescent herpes simplex keratitis. Positive HSV-1 DNA was found in 17 samples. Besides DNA detection in patient corneas, Openshaw et al.[22] also used a rabbit animal model by transplantation of corneas containing viral DNA sequences into HSV-1 naive recipients. After 5 months, there was no evidence of HSV-1 shedding in the tear film of the recipient rabbits, but HSV-1 DNA was detected in the corneal grafts at a similar intensity to the PCR signal from the donor rims.

With respect to the analysis of viral migration, our results provide additional evidence supporting the concept of HSV-1 corneal latency and its in vivo reactivation. Our experiments showed that HSV-1 in latently infected rabbit corneas could be adrenergically induced to reactivate and undergo migration into other uninfected tissues. Although our results suggest that the cornea is able to serve as a reservoir of latent virus and infectious virions, the TG is not excluded from serving as a major source of latent virus. The presence of a high concentration of viral DNA and productive-cycle gene expression, coupled with a higher rate of anterograde than retrograde viral migration, is consistent with the TG being the main source of viral latency.

Besides true corneal latency, there are two other possibilities that could account for the observed data, the first being that the latent corneal graft transplanted to the naive rabbit may have had coincidental viral shedding at the time of the PKP. This would occur by a small amount of infectious virus shed and present in the nerve endings of the cornea at the time of PKP. This small amount of virus would not be clinically detected by tear film culture, and it would be ultimately cleared by the host immune system; however, there would be sufficient infectious virus to immediately transfer to the rim of the recipient cornea and subsequently to the TG to establish latency after PKP.

A second possibility is that a low-grade chronic infection of the keratocytes, endothelial cells, or both may have been present at the time of transplantation. This would have occurred after the transfer of the PKP in which there was infectious HSV-1 with very slow turnover within the stroma of the graft. This low-grade chronic infection would then be the source of virus, which would then increase and become detectable after epinephrine iontophoresis. The higher titers and viral replication could result in migration to the corneal rim and TG. Such a low-grade chronic infection could be considered another form of "corneal latency," because virus was not detected in our tear film swabs before induced reactivation.

Our results have clinical implications. HSV-1 infection has been suspected to be the cause of both severe endothelial loss during corneal organ culture and some failures of corneal grafting.[27] Under certain conditions, latent HSV-1 can be reactivated and migrate between donor graft and recipient patient corneal tissue. Therefore, the selection of the donor graft with regard to the history of HSV-1 corneal infection is important. Vigilant follow-up after the transplantation is necessary in these instances.

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References

1. Hill JM, Wen R, Halford WP. Pathogenesis and molecular biology of ocular HSV in the rabbit. In: Brown MS, Maclean AR, eds. *Herpes Simplex Virus Protocols.* Totowa, NJ: Humana Press; 1998:291–315.
2. Pereira FA. Herpes simplex: evolving concepts. *J Am Acad Dermatol.* 1996;4:503–521.
3. Liesegang TJ. Biology and molecular aspects of herpes simplex and varicella-zoster virus infections. *Ophthalmology.* 1992;99:781–799.
4. Sawtell NM, Poon DK, Tansky CS, Thompson RL. The latent herpes simplex virus type 1 genome copy number in individual neurons is virus strain specific and correlates with reactivation. *J Virol.* 1998; 72:5343–5350.
5. Pavan–Langston D. Herpes simplex virus ocular infections: current concepts of acute, latent and reactivated disease. *Trans Am Ophthalmol Soc.* 1990;88:727–796.
6. Beyer CF, Arens MQ, Hill JM, Rose BT, Hill GA, Lin DT. Penetrating keratoplasty in rabbits induces latent HSV-1 reactivation when corticosteroids are used. *Curr Eye Res.* 1989;12:1323–1329.
7. Laycock KA, Lee SF, Brady RH, Pepose JS. Characterization of a murine model of recurrent herpes simplex viral keratitis induced

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by ultraviolet B radiation. *Invest Ophthalmol Vis Sci.* 1991;32: 2741–2746.

8. Varnell ED, Kaufman HE, Hill JM, Thompson HW. Cold stress-induced recurrences of herpetic keratitis in the squirrel monkey. *Invest Ophthalmol Vis Sci.* 1995;36:1181–1183.
9. Sawtell NM, Thompson RL. Rapid in vivo reactivation of herpes simplex virus in latently infected murine ganglionic neurons after transient hyperthermia. *J Virol.* 1992;66:2150–2156.
10. Hill JM, Shimomura Y, Kwon BS, Gangarosa LP. Iontophoresis of epinephrine isomers to rabbit eyes: induced HSV-1 ocular shedding. *Invest Ophthalmol Vis Sci.* 1985;26:1299–1303.
11. Hill JM, Dudley JB, Shimomura Y, Kaufman HE. Quantitation and kinetics of induced HSV-1 ocular shedding. *Curr Eye Res.* 1986;5: 241–246.
12. Hill JM, Haruta Y, Rootman DS. Adrenergically induced recurrent HSV-1 corneal epithelial lesions. *Curr Eye Res.* 1987;6:1065–1071.
13. Hill JM, Rayfield MA, Haruta Y. Strain specificity of spontaneous and adrenergically induced HSV-1 ocular reactivation in latently infected rabbits. *Curr Eye Res.* 1987;6:91–97.
14. Shimomura Y, Dudley JB, Gangarosa LP, Hill JM. HSV-1 quantitation from rabbit neural tissues after epinephrine-induced reactivation. *Invest Ophthalmol Vis Sci.* 1985;26:121–125.
15. Hill JM, Kwon BS, Shimomura Y, Colborn GL, Yaghmai F, Gangarosa LP. Herpes simplex virus recovery in neural tissues after ocular HSV shedding induced by epinephrine iontophoresis to the rabbit cornea. *Invest Ophthalmol Vis Sci.* 1983;24:243–247.
16. Kwon BS, Gangarosa LP, Green K, Hill JM. Kinetics of ocular herpes simplex virus shedding induced by epinephrine iontophoresis. *Invest Ophthalmol Vis Sci.* 1982;22:818–821.
17. Kwon BS, Gangarosa LP, Burch KD, deBack J, Hill JM. Induction of ocular herpes simplex virus shedding by iontophoresis of epinephrine into rabbit cornea. *Invest Ophthalmol Vis Sci.* 1981;21:442–449.
18. Stevens JG, Newburn AB, Cook ML. Latent herpes simplex virus from trigeminal ganglia of rabbits with recurrent eye infection. *Nature (New Biology).* 1972;235:216–217.
19. Devi–Rao, Aguilar JS, Rice MK, et al. Herpes simplex virus genome replication and transcription during induced reactivation in the rabbit eye. *J Virol.* 1997;71:7039–7047.
20. O'Brien WJ, Tsao LS, Taylor JL. Tissue-specific accumulation of latency-associated transcripts in herpes virus-infected rabbits. *Invest Ophthalmol Vis Sci.* 1998;39:1847–1853.
21. Perng GC, Zwaagstra JC, Ghiasi H, et al. Similarities in regulation of the HSV-1 LAT promoter in corneal and neuronal cells. *Invest Ophthalmol Vis Sci.* 1994;35:2981–2989.
22. Openshaw H, McNeill JI, Lin XH, Niland J, Cantin EM. Herpes simplex virus DNA in normal corneas: persistence without viral shedding from ganglia. *J Med Virol.* 1995;46:75–80.
23. Gordon YJ, Romanowski E, Araullo–Cruz T, McKnight JL. HSV-1 corneal latency. *Invest Ophthalmol Vis Sci.* 1991;32:663–665.
24. Shimomura Y, Mori Y, Inoue Y, Kiritooshi A, Ohashi Y, Manabe R. Herpes simplex virus latency in human cornea. *Jpn J Ophthalmol.* 1993;37:318–324.
25. Cook SD, Brown SM. Herpes simplex virus type 1 latency in rabbit corneal cells in vitro: reactivation and recombination following intratypic superinfection of long term cultures. *J Gen Virol.* 1987; 68:813–824.
26. Liu Z, Li S, Chen J, Liang S. Detection of herpes viral genome in corneal buttons of quiescent herpes simplex keratitis with polymerase chain reaction. *Yen Ko Hsueh Pao.* 1995;11:183–185.
27. Cleator GM, Klapper PE, Dennett C, et al. Corneal donor infection by herpes simplex virus: herpes simplex virus DNA in donor corneas. *Cornea.* 1994;13:294–304.
28. Cantin EM, Chen J, McNeill J, Willey DE, Openshaw H. Detection of herpes simplex virus DNA sequences in corneal transplant recipients by polymerase chain reaction assays. *Curr Eye Res.* 1991;10(suppl):15–21.
29. Biney EE, Orrett FA. Screening of human corneas for herpes simplex virus by tissue culture and polymerase chain reaction. *Jpn J Ophthalmol.* 1997;50:151–160.
30. Morris DJ, Cleator GM, Klapper PE, et al. Detection of herpes simplex virus DNA in donor cornea culture medium by polymerase chain reaction. *Br J Ophthalmol.* 1996;80:654–657.
31. Nicholls SM, Shimeld C, Easty DL, Hill TJ. Recurrent herpes simplex after corneal transplantation in rats. *Invest Ophthalmol Vis Sci.* 1996;37:425–435.
32. Bloom DC, Devi–Rao GB, Hill JM, Stevens JG, Wagner EK. Molecular analysis of herpes simplex virus type 1 during epinephrine induced reactivation of latently infected rabbits in vivo. *J Virol.* 1994;68:1283–1292.
33. Hill JM, Sedarati F, Javier RT, Wagner EK, Stevens JG. Herpes simplex virus latent phase transcription facilitates in vivo reactivation. *Virology.* 1990;174:117–125.
34. Perng GC, Dunkel EC, Geary PA, et al. The latency-associated transcript gene of herpes simplex virus type 1 (HSV-1) is required for efficient in vivo spontaneous reactivation of HSV-1 from latency. *J Virol.* 1994;68:8045–8055.
35. Trousdale MD, Steiner I, Spivack JG, et al. In vivo and in vitro reactivation impairment of a herpes simplex virus type 1 latency-associated transcript variant in a rabbit eye model. *J Virol.* 1991; 65:6989–6993.
36. Hill JM, Maggioncalda JB, Garza HH, Su YH, Fraser NW, Block TM. In vivo epinephrine reactivation of ocular herpes simplex virus type 1 in the rabbit is correlated to the 370-base-pair region located between the promoter and the 5′ end of the 2.0-kilobase latency-associated transcript. *J Virol.* 1996;70:7270–7274.
37. Loutsch JM, Perng GC, Hill JM, et al. Identical 371-base-pair deletion mutations in the LAT genes of herpes simplex virus type 1 McKrae and 17*syn*$^{+}$ result in different in vivo reactivation phenotypes. *J Virol.* 1999;73:767–771.
38. Devi–Rao GB, Bloom DC, Stevens JG, Wagner EK. Herpes simplex virus type 1 DNA replication and gene expression during explant-induced reactivation of latently infected murine sensory ganglia. *J Virol.* 1994;68:1271–1282.
39. Rootman DS, Haruta Y, Hill JM, Kaufman HE. Corneal nerves are necessary for adrenergic reactivation of ocular herpes. *Invest Ophthalmol Vis Sci.* 1988;29:351–356.
40. Wagner EK. Herpesvirus transcription-general aspects. In: Wagner EK, ed. *Herpesvirus Transcription and Its Regulation*. Boca Raton, FL: CRC Press; 1991:1–15.
41. Milliken GA, Johnson DE. *Analysis of Messy Data.* New York: Van Nostrand Reinhold; 1984.

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