Evidence for herpes simplex viral latency in the human cornea

S B Kaye, C Lynas, A Patterson, J M Risk, K McCarthy, C A Hart

Abstract
Patients undergoing penetrating keratoplasty for prior herpes simplex keratitis (group A) and corneal disease unrelated to herpes simplex (group B) were investigated to assess whether the cornea is a site for herpes simplex viral latency. All patients were seropositive for herpes simplex viral antibody. Virus was isolated from the tear film postoperatively in one patient and on cocultivation from the cornea of another patient. Herpes simplex viral DNA, however, was detected in the corneas of all patients from group A and half of those from group B by means of the polymerase chain reaction and primers to three well separated regions of the viral genome. Three donor corneas had no evidence of herpes simplex viral DNA. Using RNA polymerase chain reaction, we found evidence of a latency associated transcript and also that of a glycoprotein C coding transcript in two corneas, indicating viral replication. Nine corneas had evidence of a latency associated transcript but no glycoprotein C transcript, which suggests that herpes simplex virus may be maintained in a latent state in the corneas of patients with prior herpes simplex keratitis and in some patients with corneal disease unrelated to the herpes simplex virus.

Herpes simplex virus (HSV) is the commonest infective cause of blindness in many developed countries, where it has a reported incidence of between 5-9 and 20-7 episodes per 100 000 person years.1,2 This appears to be increasing.3 Following primary infection with HSV, the virus becomes latent in sensory nerve ganglia. Recurrent herpetic corneal ulceration is thought to result from reactivation of the virus within the trigeminal ganglion and neuronal spread into the cornea.4 Most blinding ophthalmic disease is a result of repeated recurrent infections.

Recent studies in humans, however, using an operational definition of latency – namely, the absence of virus in a cell free suspension of fluid derived from the corneal tissue, but the isolation of virus following prolonged organ culture of the excised cornea – have pointed to the cornea as a possible site for harbouring HSV in a latent form.5,6 These studies used an explant culture system without a feeder layer, in which the culture medium containing the corneal disc from patients with herpex simplex keratitis was regularly sampled and cultured for HSV. Isolations were made between days 4 and 11 after explantation with a frequency of isolation of between 12 and 29-4% of corneas sampled.4,7 In these reports, however, there was no information on the presence of virus in the tear film prior to or following penetrating keratoplasty. This is of importance if one is to exclude the possibility of corneal seeding from a ganglion derived reactivation via the tear film at or before the time of surgery. Furthermore pre-, per-, and postoperative ocular shedding of HSV has important implications for the management of patients undergoing penetrating keratoplasty. Therefore the characterisation of ocular shedding of HSV in the tear film of patients undergoing penetrating keratoplasty was undertaken in the present study. The release of this labile virus within a corneal disc in vitro may be transient, and it may be present in the explant culture medium in a very low concentration.7 There is therefore a distinct possibility that virus may not be detected.8 The intercellular method of spread of HSV and its suggested origin within the corneal stroma,9 impart a time delay between activation of latent virus and/or amplification of a chronic infection within the stroma and entry into the surrounding medium. Furthermore, by analogy with animal studies, the number of cells capable of supporting viral replication may be insufficient, and thus virus may not reach the medium surrounding the cornea.10 In an attempt to overcome these problems we used an organ cocultivation technique, thereby improving intercellular contact and increasing the surface area of exposure of the corneal tissue. To improve further the possibility of detecting the presence of virus, half of the available tissue from each cornea was cocultivated in the presence of 5-azacytidine, which has been shown to increase viral reactivation in other models of herpes virus latency.11,12

The difficulty encountered by others in demonstrating the presence of HSV in the cornea by conventional culture11,12 suggests that if it is present it must be at very low levels. Despite the refinement of the technique here the possibility remained that the virus might still go undetected by cocultivation. The polymerase chain reaction (PCR) can detect as little as a single copy of a gene in 10^3 cells, and this sensitivity suggested that it might be particularly valuable for the detection of virus in the cornea. We have therefore used primers for the HSV-1 thymidine kinase (TK), glycoprotein C (GC), and major
capsid protein (MCP) genes to screen DNA extracted from corneas for the presence of viral DNA.

During lytic infection HSV displays a co-ordinated cascade pattern of gene expression characterised by the production of immediate-early, early, and late viral mRNA transcripts. Latent infection, however, is characterised by extremely restricted viral transcription. To date the only viral gene transcripts that have been detected in a latent infection are the latency associated transcripts (LATs). To establish whether virus detected in the corneas was indeed latent or merely a low grade persistent infection it was necessary to investigate the pattern of viral gene expression. This was done by RNA PCR.

**Materials and methods**

The study was conducted from June 1989 through June 1990. Informed consent was obtained from and for all patients.

**PATIENTS**

Patients undergoing penetrating keratoplasty for herpetic and non-herpetic related corneal disease as well as a group of corneal donors were studied.

**Group A**

Group A comprised 10 patients (five female and five male) who ranged in age from 26 to 72 years. Each patient had a clinically documented history of herpes simplex keratitis, and this had been confirmed by culture in all except patients A2 and A4 (in Table 1). All had received topical acyclovir (ACV) for previous episodes of dendritic ulceration. All had had at least one previous episode of disciform keratitis, which had been treated with a combination of topical prednisolone and ACV. Patient A5 had had three previous corneal grafts, each failing because of recurrent herpes simplex keratitis.

All patients had been free from recurrence and without therapy for six to 18 months prior to surgery except for patient A6, who developed a descemetocoele requiring acyclovir and urgent penetrating keratoplasty. All had a history of recurrent herpes labialis (HSV-L) (three to six episodes per year), and patients A1 and A3 had had nasal herpes (HSV-N) ipsilateral to the affected eye.

**Group B**

Group B comprised 10 patients (five females and five males) who ranged in age from 27 to 76 years. All had been HSV related corneal opacification and had no history of clinically documented HSK. Patients B3, B5, and B8 had had previous cataract surgery and had developed a bullous keratopathy approximately one to three years before surgery. Patients B1 and B2 had evidence of previous bilateral interstitial keratitis characteristic of syphilis. Patients B1 and B3 also had a history of herpes zoster ophthalmicus ipsilateral to the affected eye. Patients B4, B6, B7, B9, and B10 had keratoconus. All patients apart from B8 and B10 had a history of recurrent HSV-L (1–8 episodes per year).

Postoperatively patients were placed on betamethasone 0.1% eyedrops, which in group A were replaced at six weeks by prednisolone 0.05% eyedrops.

**Group C**

Group C comprised three corneas from donor patients none of whom had a history of corneal disease. They were not suitable for transplantation because of systemic disease.

**TEAR FILM SPECIMENS**

Patients had specimens collected from both eyes three times a week for two to three weeks prior to surgery, three times a week for four weeks after surgery, and then twice weekly for eight weeks. The last preoperative specimen was collected 30 minutes prior to surgery.

The precausal tear film was collected by rotation of a swab along the lower fornix to the medial canthus, where it was held to absorb tears for 10 seconds. Care was taken to avoid swabbing the corneal epithelium. Swabs were immediately placed in a sterile transport medium pack (Virocult, UK) and kept on ice prior to inoculation.

All patients had a detailed ocular examination on each visit. Episodes of HSV-L, HSV-N were recorded and the lesions swabbed.

**VIRUS ISOLATION FROM TEAR FILM SPECIMENS**

Each specimen was assayed for virus in duplicate on confluent Vero cells as previously described. Cultures showing cytopathic effects were maintained until complete cell death and the virus harvested for typing by immunofluorescence with monoclonal antibody (Syva MicroTrak, USA).

**ORGAN COCULTIVATION**

On removal from the patient one half of the corneal disc was snap frozen in liquid nitrogen, the other transported to the laboratory (10–15 minutes) in 5 ml of growth medium with 20% fetal calf serum (FCS). After microdissection into about 0.5–1 mm pieces, the tissue was resuspended in the growth medium and divided in half. Each half was diluted with growth medium (20% FCS) to a volume of 15 ml, but one half also contained a final concentration of 50 μM 5-azacytidine. Aliquots (1 ml) of this suspension were placed in tubes of confluent Vero cells. After three days the medium in tubes containing azacytidine was replaced with growth medium (20% FCS). All cultures were fed weekly with GM (20% FCS) and examined daily after day three for viral CPE.

The rims of the 20 donor corneas were also screened for virus by the same procedure. These were not subjected to PCR.

**SERUM ANTIBODY**

Serum was collected at surgery or within six
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weeks postoperatively and assayed for HSV antibody by a complement fixation test.

**VERO CELL SENSITIVITY**

In order to demonstrate that the sensitivity to HSV-1 infection did not decrease with cell age, tubes containing confluent vero cells were prepared 3 days, 1 week, 2 weeks, and 3 weeks prior to infection. The cells had been re-fed weekly (except for the three-day-old cells) with growth medium (20% FCS). Tubes were inoculated in triplicate with HSV-1 (strain HFEM), 50, 100, and 150 plaque-forming units (PFU) and fed with growth medium (20% FCS). Plaques were counted on day 3 after infection and the results analysed by an F test to test for departure from constancy.

**EXTRACTION OF NUCLEIC ACIDS**

The hemicorneal disc was cut into small fragments with a separate sterile set of instruments for each specimen and digested at 37°C in 0-5 ml of Hirt buffer (0-01 M Tris pH 8, 0-01 M edetic acid (EDTA), 2% sodium dodecyl sulphate (SDS) containing 0.8 g/l proteinase K (BCL) before two rounds of extraction with 50:50::

- phenol:chloroform (nucleic acid grade phenol (Gibco/BRL), saturated with sterile anaR (BDH) water).
- Nucleic acids were precipitated and redissolved in 50 to 100 μl sterile anaR (BDH) water. Nucleic acid content was determined by electrophoresis and spectrophotometry. Dilutions were made so that 5 μl of nucleic acid solution contained 100–200 ng of DNA. Control DNA from fetal kidney, from the bone marrow of a neuroblastoma patient (negative), and positive control DNA and RNA from Vero cells acutely infected with HSV-1, were prepared by ultracentrifugation through caesium trifluoroacetate gradients.

**THE PCR PRIMERS**

Samples of nucleic acid from the corneas of patients A1–A7, B1–B5, and C1 and the control DNA, were subjected to PCR by means of primers for the hypoxanthine-guanine phosphoribosyl transferase (HPRT) gene, present in the genome of all human cells, 1 to confirm that the DNA was relatively intact and free from any inhibitors of the PCR. 4 The primers have the sequences

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5'CTTGCTGGTGAAAAGACCC-3'
5'GTCAGGGCATACTTACAA-3'
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and amplify a fragment of 267 base pairs (bp) from DNA. Since the primers span an intron in the gene, the amplification product from RNA is only 97 bp. The primers used to detect the presence of HSV-1 DNA or RNA were (a) those described previously for the HSV-1 thymidine kinase (TK) gene, 5 which amplify a fragment of 110 bp, (b) those for the glycoprotein C (GC) gene

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5'GTTCACCAACAGTCTCTACGCA-3'
5'ATTGGCGTCGGAGAAGCTC-3'
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which amplify a fragment of 115 bp, (c) those for the major capsid protein (MCP) gene

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5'GGATGGTATGGTCGAGATGC-3'
5'AATCTGCGTGGCCACACGGT-3'
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which amplify a fragment of 244 bp, and (d) those described previously for the HSV-1 latency associated transcript (LAT),6 which amplify a fragment of 195 bp.

**THE DNA PCR**

This was initially performed independently in two different centres (Bristol and Liverpool) on corneas A1–A7, B1–B5, and C1, with the TK primers. All subsequent reactions were in the Liverpool centre. The method was based on that described by Saiki et al. 7,8 In the Bristol Centre the 50 μl reaction mixture contained 10 mM Tris pH 8·3, 50 mM potassium chloride, 1·5 mM magnesium chloride, 33 μM each of dATP, dCTP, dGTP, and dTTP, and was 400 nM with respect to each 20 base primer. In all reactions 5 μl of total nucleic acid mixture and 2·5 units of Taq DNA polymerase (Perkin Elmer) were used. In the Liverpool centre a GeneAmp DNA amplification kit (AmpliTaQ, Perkin Elmer) was used. (For the MCP primers the magnesium chloride was 5 mM.) Automatic thermal cycler (Perkin Elmer: Bristol, and Hybaid: Liverpool) were used.

Reactions began with a 3-minute denaturation at 94°C followed by 40 cycles consisting of denaturation for 1 minute at 94°C annealing at 50°C for 1 minute, and primer extension at 72°C for 1 minute. At the Liverpool centre subsequent reactions were modified, so that after a 3-minute denaturation there were 40 cycles consisting of 40 seconds at 94°C, 15 seconds at 55°C, (50°C for LAT and HPRT), and 20 seconds (30 seconds for MCP) at 72°C, followed by 5 cycles: 40 seconds at 94°C, 30 seconds at 55°C and 40 seconds at 72°C. Samples were confirmed as negative only after a further 20 cycles of amplification. In all experiments both positive and negative control DNAs and a no-DNA control were included. Products were analysed on 12% or 8% polyacrylamide or on 0·8% agarose gels.

**RNA PCR**

Prior to RNA PCR, nucleic acid samples were digested with RNase-free DNase (Boehringer Mannheim). The RNA recovered was redisolved in 25 μl anaR water and 5 μl used for each reaction. The mRNA complementary PCR primer was annealed to the template RNA in a 20 μl reaction mix (10 mM Tris-HCL pH 8·3, 50 mM KCl, 5 mM MgCl2, 1 mM each dNTP, 1 μM (0·25 μM LAT) primer, 20 U RNase inhibitor (Boehringer Mannheim) at 45°C for 15 minutes. Reverse transcription by 20 U M-MLV reverse transcriptase (Gibco BRL) followed at 37°C for 60 minutes. After heating to 94°C for 10 minutes, reaction mixtures were prepared for PCR by the addition of 10× PCR buffer (Perkin- elmer), 400 nM antisense primer (100 nm for LAT primer), Taq polymerase and water to a volume of 50 μl. Each sample was subjected to PCR using HPRT primers (which amplify different size fragments from DNA and RNA) to confirm the presence of RNA and to demonstrate the absence of contaminating DNA. Samples were assayed for expression of LAT and GC. The absence of contaminating DNA was also tested.
Table 1 Detection of HSV DNA, RNA, and virus from the cornea and tear film of patients with (group A) and without (groups B and C) prior herpes simplex keratitis

<table>
<thead>
<tr>
<th>Patient</th>
<th>Tear culture*</th>
<th>Corneal Culture*</th>
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<th>RNA PCR</th>
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*Number of isolations. †Complement fixation test; reciprocal of dilution. + = Amplification product present. - = Amplification product absent. NI = not identified as DNA present.

CONFIRMATION OF PCR PRODUCT IDENTITY
The amplified DNA fragments of the expected size were electrophoresed (Biotrap, BT 1000, Schleicher and Schuell, FRG) and their identity confirmed by restriction endonuclease enzyme digestion. With Sma I (TK), Apa I (MCP), Ksp I (GC), Hae III (LAT), and Ava II (HPRT) (Boehringer Mannheim), the following would be generated: 73 bp and 37 bp (TK), 186 and 58 bp (MCP), 59 and 56 bp (GC), 77 bp, 33 bp, and 85 bp (LAT), and 82 and 15 bp (HPRT RNA) respectively. Further confirmation of the 110 bp (TK) amplification product was obtained by hybridisation using a 40 base oligonucleotide of the sequence 5'GAGATGGGGGAGGCTAAC-GAAACAGGGAAGGAGAAATAAA-3'. The TK amplification products and controls were denatured and 5 μl of each sample including positive (HSV-1) and negative (negative PCR reaction) controls spotted on to a nylon membrane (Hybond-N+, Amersham). Blots were ultraviolet fixed and prehybridised at 55°C for 1 h in 5xSSPE (0·9 M sodium chloride, 0·05 M sodium phosphate, and 0·005 M edetic acid), 0·5% (w/v) sodium dodecyl sulphate (0·016% (w/v) denatured salmon sperm, and 0·1% (w/v) each of bovine serum albumin, ficoll (Sigma), and polyvinylpyrrolidone (BDH). Hybridisation was overnight at 55°C with (α-32P)dCTP (Amersham) labelled probe. Blots were washed twice at 20°C in 2xSSPE, 0·1% (w/v) sodium dodecyl sulphate for 2 minutes, followed by two washes in prewarmed 1xSSPE, 0·1% (w/v) sodium dodecyl sulphate at 55°C for 5 minutes and autoradiographed. Blots were stripped with washing in 0·5% (w/v) sodium dodecyl sulphate at 95°C for 10 minutes and rehybridised with an (α-32P)dCTP HLA probe at 68°C. Washing was in 2xSSPE, 0·1% (w/v) sodium dodecyl sulphate for 5 minutes at 20°C followed by two washes in 0·1% SSPE, 0·1% (w/v) sodium dodecyl sulphate at 68°C for 10 minutes.

Results
VERO CELL RESPONSES
The average numbers of plaques produced on Vero cells monolayers aged 22, 14, 7, and 3 days at inoculation were not signficantly different for each of the three inocula respectively (F test, p>0·1). Thus the sensitivity of Vero cells to infection with HSV did not vary with the age of the cells within the three-week period used in the cocultivation experiments.

PREOPERATIVE PERIOD
In total, 82 and 72 specimens were collected from groups A and B respectively. There was no HSV-1 isolations (Table 1). Patients B1 and B4 had episodes of HSV-L one and two weeks prior to surgery respectively. There were no episodes of HSV-N.

POSTOPERATIVE PERIOD
Two hundred and forty-seven and 220 tear film specimens were collected from groups A and B (Table 1). There were three isolations (all HSV-1). They were made consecutively and were from the affected eye of patient A2. The first isolation was on day 18 after surgery, three days after commencement of topical timolol (0·25%) for raised intraocular pressure. On day 24 an epithelial ulcer developed and topical ACV was started, with resolution of the ulcer over the next five days.

There were no isolations from the donor corneas, C1, C2, and C3. Three patients in group A (3, 4, 7) and three in group B (2, 4, 5) had sutures removed and or replaced. Patients A3 and A1 had one and two episodes of HSV-N (HSV-1) ipsilateral to the affected eye, at four, five, and nine weeks after surgery. There were four episodes of HSV-L (HSV-1).

ORGAN COCULTIVATION
There were two isolations (both HSV-1) on day 4 of tissue incubation. Both were from the corneal disc of patient A5. The isolations were in tubes that did not contain 5-azacytidine. No other isolations were made from either the corneas or the rims of donor corneal tissue.

POLYMERASE CHAIN REACTION
A positive result was taken as a visible band of the predicted molecular weight for the particular set of primers.

DNA PCR
All corneas from those patients in group A (10) as well as five (B1-B5) out of 10 in group B produced a positive result to all three (TK, GC,
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and MCP) primers (Table 1). Corneas C1, C2, C3, and B6-B10 produced a negative result. Both negative and positive results were reproducible in further PCRs (up to three times), and replicate assays in Bristol and Liverpool produced identical results.

RNA PCR
Using the HPRT primers we showed that all but two corneas contained mRNA and no DNA. Corneas from patients A6 and A7 showed evidence of residual DNA by this method, by omission of the reverse transcriptase step and by use of the antisense primer, and were thus excluded from further analysis. All eight of the remaining corneas from group A, as well as three from group B (B1-B3) showed evidence of the presence of a LAT. Among these corneas evidence of GC mRNA was found only in patients A2 and A5 (Table 1).

PRODUCT DIGESTION AND HYBRIDISATION
All amplification products were cleaved as predicted by the relevant restriction enzyme, thus confirming their identities. Similarly, all TK amplification products from positive samples hybridised to the internal oligonucleotide probe, whereas the negative control samples did not. In addition none of the positive samples hybridised to the HLA probe, thus confirming their specificity.

SERUM ANTIBODY
All patients were HSV seropositive apart from C1 (titre less than 1/4) (Table 1). There was no significant difference in antibody levels between groups A and B (t test, p>0.1), nor between patients in group B who had evidence of HSV DNA in their corneas and those who did not (t test, p>0.1).

Discussion
The absence of preoperative shedding of HSV is not unexpected and makes it unlikely that virus was seeded into the cornea at or recently before surgery. The onset of early postoperative shedding on day 18 followed by the development of recurrent herpes simplex keratitis on day 24 (patient A2), has not been reported previously. Early recurrence, that is within 12 months, is usually associated with an event such as rejection or suture removal and is epithelial in nature in greater than 72% of cases. 19 22

Although timolol has been used to induce shedding and recurrent herpes simplex keratitis in rabbits and more recently in a non-human primate, 12 there are at present no studies in humans. Whether timolol induced shedding and recurrence in this patient (A2) is unknown. Acyclovir has been found to reduce herpes simplex keratitis recurrence in rabbit models of penetrating keratoplasty. 27 Although prophylactic antiviral cover after suture removal or replacement has been recommended, 25 26 the absence of shedding and/or recurrence in four patients in group A who had sutures removed or replaced raises questions in this regard. The need for prophylactic antiviral therapy and regular tear film sampling in patients following penetrating keratoplasty for herpes simplex keratitis is debatable and needs further investigation.

The isolation of HSV from one of the corneas of those patients with prior herpes simplex keratitis (one out of 10) is in accord with some previous reports, 12 which are in turn at variance with others in which no isolations were reported. 11 12

The infrequency of an isolation in this study may reflect the use of acyclovir during active herpes simplex keratitis and the long period of quiescence (six to 18 months) prior to surgery. The absence of an isolation in the 5-azacytidine treated cultures may be due in part to its effect on viral replication. This could be investigated by reducing the concentration of 5-azacytidine to 15 μM, at which concentration its effect on replication is markedly reduced. 10

We have demonstrated that HSV-1 DNA sequences are present more frequently in diseased human corneas than previously thought. Patients with previous herpes simplex keratitis are highly likely to have HSV-1 DNA in their corneas. This is consistent with previous reports which have demonstrated HSV viral antigens in corneal discs taken from patients with previous herpes simplex keratitis. 11 12 26 Furthermore we have shown that patients with no documented history of herpes simplex keratitis, who have non-HSV related corneal disease and who are HSV seropositive, may have HSV DNA within their corneas. We used primers that amplify three segments that are widely separated on the HSV-1 genome. In each case when DNA products were found all three segments were present. Thus it is reasonable to assume that the whole of the HSV-1 genome was present. That HSV-1 DNA was found in herpes simplex keratitis corneas implies that its presence is not always associated with typical clinically recognisable disease. Indirect support for this occurrence may be adduced from the finding that secretory anti-HSV antibody can be found in the tear film of individuals who do not have serum anti-HSV antibody, and raises the possibility that the eye may be a primary portal of entry for HSV, 25 and that spread beyond the eye need not necessarily occur.

The presence of HSV-1 DNA in these corneas could be explained by the presence of replicating virus in a low grade persistent infection, by viral DNA remaining from previous infection, or by latent virus. The first possibility appears unlikely, since infectious virus was isolated by cocultivation from only one of the 15 corneas containing HSV DNA. In an attempt to demonstrate that the DNA was from latent virus it was necessary to investigate the pattern of viral gene transcription, which so far appears to be confined to LATs during latent infection. Because of the limited amount RNA available it was not possible to study the expression of a wide range of HSV genes, and investigations were confined to the LAT and GC genes. Of the genes for which primers were available, GC was selected, since it is a late or δ gene, and so its expression is likely to
be a marker for the production of infectious virus.

Two of the corneas were found to contain GC transcripts, and one of these was the cornea from which virus was isolated by cocultivation. Although no virus was isolated from the other, the viral DNA detected in both was assumed to be present as actively replicating virus. A further two corneas apparently contained GC mRNA, but the signal was probably due to a minute amount of remaining DNA. The samples still gave a positive result for GC, when reverse transcription was omitted or the antisense GC primer used, and RNA PCR with the HPRT primers produced bands of expected size for amplification from both DNA and RNA templates.

In nine of the 11 remaining corneas which did not contain evidence of GC mRNA we could amplify a fragment of LAT. The presence of LAT transcripts in the absence of GC mRNA suggests that viral gene transcription is limited in these corneas, and, within the limits of this study, follows the pattern of a neuronal type of latent infection. Our results must be further evidence for the possibility of corneal latency, though they are not unequivocal proof. Further investigation of the gene expression is necessary, particularly of other genes previously postulated to have a role in the establishment and maintenance of latency, such as infected cell protein 0 (ICPO), TK, and ribonucleotide reductase. Truly latent virus should be capable of reactivation, which we were unable to detect under the conditions described. Different cocultivation conditions may promote reactivation and improve detection of the low levels of virus likely to be involved. Nevertheless, it appears that in some patients HSV may be maintained in a latent state in the cornea, and this is not always related to prior herpes simplex keratitis. The implication of these findings to our understanding of the pathogenesis of herpes simplex keratitis is not yet clear.


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