

Detection of herpes simplex virus DNA in donor cornea culture medium by polymerase chain reaction

David J Morris, Graham M Cleator, Paul E Klapper, Robert J Cooper, Emmanuel O E Biney, Carol Dennett, Boris Marcyniuk, Andrew B Tullo

Abstract

Aims/background—Herpes simplex virus (HSV) may establish latent infection in the cornea and therefore be transmissible by corneal transplantation. Monitoring of donor cornea culture medium was evaluated for HSV infection.

Methods—HSV was sought using virus isolation in cell culture, and its DNA was amplified to detectable levels using the polymerase chain reaction (PCR).

Results—Virus isolation in cell culture was negative on neat, cell pellet, and cell free supernatant prepared from the spent culture media of 80 corneas. Three cell pellets (3.8%) were positive for HSV DNA. The PCR positive culture negative results might have reflected latent rather than active HSV infection of the cornea. Post transplant follow up of the three recipients of corneas with HSV PCR positive organ culture media revealed no evidence of HSV induced eye disease or primary graft failure.

Conclusion—Screening of corneal culture medium for HSV by virus culture or for HSV DNA by PCR could not be recommended.

(*Br J Ophthalmol* 1996;80:654-657)

Recent studies indicate that gross corneal endothelial cell necrosis during organ culture before transplantation and primary graft failure may be related to active herpes simplex virus (HSV) infection of the donor cornea.¹

There are no laboratory screening methods currently available to evaluate either donated corneas or corneal donors for active HSV infection other than the testing of spent organ culture medium. Cadaveric donors have the high prevalence of low level HSV antibodies typical of the general population,¹ but HSV shedding from the eye or pharynx is very rare.^{1,2} Although HSV DNA is present in cadaver corneal tissues^{3,4} monitoring of donor corneas for HSV DNA using the polymerase chain reaction (PCR) is precluded by the destructive nature of tissue processing for this assay. False negative and false positive results may arise if only the residual scleral rim is tested.^{1,4} None the less, it is important to establish whether healthy corneas develop active HSV infection in organ culture as a prelude to further investigation of the role of HSV

in precipitating graft failure. We report a prospective study by virus isolation and the PCR of HSV infection in the organ culture media of 80 corneas.

Materials and methods

NORMAL DONOR CORNEAS

Eighty consecutive corneas from 48 cadaveric donors, none of whom had a prior history of herpetic eye disease, were prospectively selected from those issued by the David Lucas Manchester Eye Bank between December 1992 and March 1993. The procurement, processing, and organ culture of the corneas were performed according to standard aseptic protocols in a class II laminar flow cabinet by staff wearing sterile gloves, masks, and gowns.⁵ Eyes trimmed of all adherent conjunctiva and fat were immersed successively in 5% (w/v) polyvinylpyrrolidone-iodine (aqueous Betadine, Napp Laboratories, Watford) for 4 minutes and 0.3% (w/v) sodium thiosulphate for 30 seconds before three washes in normal saline. The corneas together with a 2-3 mm rim of sclera were excised and suspended, via a suture, in 60 ml of culture medium (Eagle's minimal essential medium with Earle's salts containing 2% (v/v) fetal calf serum (FCS), 20 mM HEPES buffer, 24 mM sodium bicarbonate, 2 mM L-glutamine, 100 IU/ml penicillin G, 0.1 mg/ml streptomycin sulphate, and 0.24 µg/ml amphotericin B). The tissues were incubated at 34°C for a minimum of 10 days, and issued for transplantation as an excised 8 mm diameter corneal button when (1) the bacterial and fungal cultures on medium collected 8 days after initial processing and 1 day before issue both proved negative, and (2) trypan blue staining 2 days before issue revealed a minimal endothelial cell count of 2000 cells/mm².

PROCESSING OF CORNEAL ORGAN CULTURE MEDIUM

Following removal of the cornea from the culture vessel for transplantation, the residual culture medium was submitted for examination using virus isolation in cell culture for infectious HSV and the PCR for HSV DNA. Neat medium (50 ml) was centrifuged at 1850 g for 5 minutes to separate the cell pellet and the supernatant fluid. Any cell free HSV or its DNA was precipitated from the supernatant fluid by overnight incubation at 4°C with 7 ml 5 M sodium chloride and 8 ml 50% (w/v)

Division of Virology,
Department of
Pathological Sciences,
Medical School,
University of
Manchester

D J Morris
G M Cleator
P E Klapper
R J Cooper
E O E Biney
C Dennett

University
Department of
Ophthalmology, Royal
Eye Hospital,
Manchester
B Marcyniuk
A B Tullo

Correspondence to:
Dr Robert J Cooper,
Division of Virology,
Department of Pathological
Sciences, University of
Manchester, 3rd Floor,
Clinical Sciences Building,
Manchester Royal Infirmary,
Oxford Road, Manchester
M13 9WL.

Accepted for publication
4 April 1996

polyethylene glycol (PEG) 8000 followed by centrifugation at 1850 *g* for 10 minutes.⁶ The cell pellet and the cell free PEG precipitate were resuspended in 800 μ l or 600 μ l respectively of Eagle's minimal essential medium.

VIRUS ISOLATION

Aliquots of each neat medium, resuspended cell pellet and resuspended PEG precipitate were inoculated onto three monolayer cultures of Vero cells in multiwell plastic cell culture trays using centrifugal enhancement of adsorption (3500 *g* for 15 minutes at room temperature) for all preparations except the cell pellets. The Vero cells were propagated in growth medium (Eagle's minimal essential medium supplemented with 10% FCS, 0.1 mM L-glutamine, 100 IU/ml penicillin G, and 100 μ g/ml streptomycin). The inoculated cell cultures were incubated at 37°C in 5% carbon dioxide for 28 days in maintenance medium (Eagle's minimal essential medium supplemented with 1% FCS, 0.1 mM L-glutamine, 100 IU/ml penicillin, and 100 μ g/ml streptomycin) before being discarded as negative. The cell monolayers were examined daily for the appearance of HSV cytopathic effects.

HSV AND HUMAN β -GLOBIN PCRS

The residual cell pellets and cell free PEG precipitates were stored at -20°C between inoculation of Vero cell cultures and PCR testing. The DNA was prepared for PCR amplification by incubation with 0.5% (w/v) sodium dodecyl sulphate (SDS), and 250 μ g/ml proteinase K at 37°C for 2 hours, phenol chloroform extraction, and ethanol precipitation. The pellets were dissolved in 1 \times TE buffer (10 mM TRIS hydrochloric acid, 1 mM ethylenediaminetetra-acetic acid (EDTA) pH 8.0) before DNA amplification.

Primers n7 (5'-CGCGCGGTACCTTATGGCAGCATGA-3') and n8c (5'-CAGGGTAATAACGTGTCCCCGATATGG-3') amplified a 350 base pair fragment of the HSV thymidine kinase gene (detection limit 5 plaque forming units of HSV^{7,8}) whereas primers PC04 (5'-CAACTTCATCCACGTTCCAC-3') and GH20 (5'-GAAGAGCCAAGGACAGGTAC-3') amplified a 268 base pair fragment of the human β -globin gene (detection limit less than 10 copies^{9,10}). Sterile distilled water (SDW) as contamination control or extracted DNA samples (5 μ l) were added to 45 μ l reaction mixture (10 mM TRIS-hydrochloric acid pH 8.3, 50 mM potassium chloride, 2 mM magnesium chloride, 0.02% (w/v) gelatin, 0.2 mM each deoxy-

nucleotide, 0.1 μ M appropriate primers, and 2.5 units Amplitaq DNA polymerase (Perkin-Elmer-Cetus, Emeryville, CA, USA), and evaporation was minimised by an overlay of two drops (50 μ l) of mineral oil. Thermal cycles on a Techne PHC-1 thermocycler comprised 7 minutes at 94°C before 50 cycles of 2 minutes at 94°C, 1.5 minutes at 50°C, and 2 minutes at 70°C. The amplification product was visualised by ethidium bromide staining of DNA bands separated on agarose gel electrophoresis. Rigorous precautions to prevent exogenous contamination of the DNA extracts or PCR reaction mixtures with HSV or its DNA included separate rooms for preparation of reaction mixtures and preparation of and addition of DNA extracts, addition of DNA extracts within a Genesphere (Solotec Ltd, Cambridge), and a third room for product analysis. Plugged pipette tips were used throughout and the operator wore a face mask and gloves.^{1,10,11} The contamination control included in each HSV and β -globin PCR run always gave a negative result.

Results

VIRUS ISOLATION

None of the organ culture media obtained from 80 corneas yielded HSV in Vero cell culture following separate inoculation of neat medium, cell pellet, and PEG precipitate.

HSV AND HUMAN β -GLOBINS PCRS

Three (3.8%) of the 80 cell pellets from corneas issued for grafting were positive for HSV DNA by PCR. To confirm the presence of HSV DNA in the culture media from which the three HSV DNA positive cell pellets were prepared, PEG precipitates of cell free supernatants from the same three and two other randomly selected culture media were examined using the HSV DNA amplification assay. Positive results were obtained with PEG precipitates of cell free organ culture medium from the three corneas previously positive when cell pellets were tested, but not with the other two samples.

Human DNA amplifiable by PCR was present in most (75 (94%)) of the DNA extracts prepared from the 80 corneal cell pellets subjected to HSV DNA amplification. The absence of human cellular DNA from all five PEG precipitates of cell free medium tested by HSV PCR was confirmed by negative results in the β -globin DNA amplification assay.

TRANSPLANT OUTCOME

All three corneas where the cell pellets and cell free PEG precipitates prepared from spent organ culture medium subsequently shown to contain HSV DNA were issued for transplantation. None had shown unacceptable endothelial cell loss when subjected to trypan blue staining and microscopic examination after organ culture. Table 1 illustrates the clinical outcome of the three recipients of these grafts. None developed primary graft failure or herpes simplex eye disease.

Table 1 Transplant outcome in recipients of donor corneas positive for HSV DNA in spent culture medium

Recipient No	Sex	Age (years)	Diagnosis	Outcome
1	M	54	Keratoconus	Graft clear at 14 months
2	F	82	Pseudophakic bullous keratopathy	Graft failure at 7 months due to vitreous touch
3	F	78	Ocular surface disorder	Graft clear at 18 months

Discussion

The existence of latent HSV infection within the human cornea is currently a matter of debate.^{11 12} Studies utilising organ culture and PCR respectively identified infectious HSV or HSV DNA in corneal tissue.^{1 3 4 13-18} The procedures used were not, however, able to distinguish persistent from latent infection. Japanese workers considered the existence of latent HSV infection of the cornea to be proved when their experimental definition for virus latency was satisfied.¹⁹ None the less, their experiments did not differentiate latent corneal infection from low level virus persistence.¹² Latent infection of the ophthalmic division of the trigeminal ganglion could result in periodic reactivations of the virus from this site. Frequent reactivations might lead to the continuous presence of the virus in the cornea.^{11 12}

We failed to isolate infectious HSV and only rarely amplified HSV DNA from corneal organ culture media despite the use of sensitive culture and PCR techniques and effective procedures for the concentration of both cell associated and cell free virus. The high β -globin PCR positivity rate with the material pelleted from the culture media by low speed centrifugation suggested that most corneas in culture shed cells. Moreover, all five PEG precipitates of supernatant fluid were negative for human DNA by PCR, implying that the cells in corneal organ culture medium were successfully concentrated by low speed centrifugation. Also, three of these precipitates were HSV PCR positive, an observation compatible with efficient concentration of any cell free virus by PEG. Precipitation using PEG proved as effective as ultracentrifugation for concentration of dilute suspensions of human cytomegalovirus from urine specimens, and had the advantage of concentration of viral DNA.⁶

There were several possible explanations for the few HSV PCR positive virus culture negative results on cell pellets of organ culture media. Firstly, the media, DNA extracts, or PCR mixtures might have been contaminated with exogenous virus or DNA when the corneas were in reality free of HSV and its DNA. The three positive and two negative HSV PCR results obtained with both cell pellets and cell free PEG precipitates derived from the same culture media, and the negative results with a contamination control in every PCR run made contamination of culture media, DNA extracts, or PCR mixtures after the receipt of the culture media in the virology laboratory unlikely. The risk of contamination of the corneal cultures during processing at the eye bank was minimised by the techniques used.¹ Other explanations for the viral DNA positive infectious virus negative results included false negative virus culture experiments, the presence of inactivated virus, or latent virus infection manifest as shedding of viral DNA but not infectious virus. The first of these was implausible given the high sensitivity of Vero cells for primary isolation of HSV and the almost certain absence of interfering virus neutralising antibodies in the culture media

which contained only fetal calf and not human serum. However, the true explanation remained elusive given the controversy surrounding the existence of latent HSV in the cornea.

If the DNA positive culture negative results in this study reflected possible latent corneal infection, positive PCR tests on corneal culture media could have provided a marker for such latent infection. The reported prevalence of apparently latent HSV infection in human corneas varied widely in previous studies. Organ culture experiments suggested persistent or possibly latent infection in up to 67% of human corneas from patients with chronic stromal keratitis.^{13 16} Polymerase chain reaction studies gave positivity rates of eight (73%) or four (36%) of 11 in corneas from patients transplanted for herpes keratitis or pseudophakic corneal oedema,¹⁷ and in one (3%) of 32,¹⁸ five (50%) of 10,³ three (30%) of 10,¹ or 10 (42%) of 24⁴ cadaver corneas. This large range of reported prevalences of arguably latent HSV infection in donor corneas could not be explained by the choice of PCR technique. One of the three studies employing only one set of PCR primers³ gave at least as high a positivity rate (50%) as that using a possibly more sensitive nested protocol (42%).⁴ However, different prevalences of covert previous herpes simplex eye disease in the corneas considered suitable for transplantation in the four centres cannot be excluded. Therefore, we do not know whether the relatively low rate of HSV PCR positivity in corneal organ culture media in the present study suggested the true prevalence of latent HSV infection in the donor corneas selected or in reality underestimated that prevalence. Failure to remove PCR inhibitors during DNA extraction was a possible cause of the low positivity rate for the HSV DNA with the cell pellets derived from corneal organ culture media. Most of the extracts were positive for β -globin DNA. However, the residual activity inhibitory to Taq polymerase in the extracts could have been insufficient to prevent successful amplification of relatively large quantities of human DNA even if detection of low concentrations of HSV DNA was rendered impossible.

Routine monitoring for infectious HSV of organ culture medium from corneas being stored before issue for transplantation seems difficult to justify in the absence of positive results. Active HSV infection may cause very rare episodes of severe corneal endothelial cell destruction during organ culture,¹ but affected corneas should be identified by microscopic examination and discarded. Primary failure has been described in a corneal graft with an acceptable endothelial cell count after storage when gross endothelial cell necrosis was identified in the fellow cornea from the same donor.¹ However, this combination of events is also probably exceptional, and would not of itself justify monitoring of the organ culture media of all donor corneas for infectious HSV. Testing of similar material for HSV DNA using the PCR would merely identify a few latently infected corneas (if such infection exists) and document rare instances of HSV transmission by corneal transplantation (if this event oc-

curs). The low rate of positivity for HSV DNA in spent culture media from donor corneas is consistent with the fact that ocular herpes has been described following penetrating keratoplasty only rarely in patients with no preoperative history of this disease.^{20 21} The incidence of primary graft failure following corneal transplantation²² is similar to the prevalence of HSV PCR positivity (3.8%) in spent corneal organ culture medium in the present study. Though trauma to the cornea during retrieval, handling, storage, or transplantation, and in particular poor eye bank²³ or surgical technique,²² may be the major causes of primary graft failure, a role for HSV cannot be excluded. None the less, detection of HSV DNA in a corneal organ culture medium could not at present be used as an indication for discarding the donor cornea. In this study none of the three recipients of HSV DNA positive corneal grafts has developed clinical consequences attributable to HSV at up to 18 months after transplantation.

We are grateful to Ms Pamela Gregory for assistance with the preparation of this manuscript.

- 1 Cleator GM, Klapper PE, Dennett C, Sullivan AL, Bonshek RE, Marcyniuk B, *et al.* Corneal donor infection by herpes simplex virus: herpes simplex virus DNA in donor corneas. *Cornea* 1994;13:294-304.
- 2 Kaye SB, Madan N, Dowd T, Hart C, McCarthy K, Patterson A. Ocular shedding of herpes simplex virus. *Br J Ophthalmol* 1990;74:114-6.
- 3 Kaye SB, Lynas C, Patterson A, Risk JM, McCarthy K, Hart CA. Evidence for herpes simplex viral latency in the human cornea. *Br J Ophthalmol* 1991;75:195-200.
- 4 Openshaw H, McNeill JI, Lin XH, Niland J, Cantin OM. Herpes simplex virus DNA in normal corneas: persistence without viral shedding from ganglia. *J Med Virol* 1995;46:75-80.
- 5 Ridgway AE, Marcyniuk B. The UK cornea transplant service and the establishment of the David Lucas Manchester Eye Bank. *Dev Ophthalmol* 1991;22:44-9.
- 6 Kimpton CP, Corbitt G, Morris DJ. Comparison of polyethylene glycol precipitation and ultracentrifugation for recovery of cytomegalovirus from urine prior to detection of DNA by dot-blot hybridisation. *J Virol Methods* 1990;28:141-6.
- 7 Klapper PE, Cleator GM, Dennett C, Lewis AG. Diagnosis of herpes encephalitis via Southern blotting of cerebrospinal fluid DNA amplified by the polymerase chain reaction. *J Med Virol* 1990;32:77-82.
- 8 Dennett C, Klapper PE, Cleator GM, Lewis AG. CSF treatment and the diagnosis of herpes encephalitis using the polymerase chain reaction. *J Virol Methods* 1991;34:101-4.
- 9 Bauer HM, Ting Y, Greer CE, Chambers JC, Tashiro CJ, Chimera J, *et al.* Genital human papillomavirus infection in female university students as determined by a PCR-based method. *JAMA* 1991;265:472-7.
- 10 McElhinney LM, Cooper RJ, Morris DJ. Multiplex polymerase chain reaction for human herpesvirus-6, human cytomegalovirus, and human β -globin DNA. *J Virol Methods* 1995;53:223-33.
- 11 Cook SD, Hill JH. Herpes simplex virus: molecular biology and the possibility of corneal latency. *Surv Ophthalmol* 1991;36:140-8.
- 12 Gordon YJ, Romanowski E, Araullo-Guz T, McKnight JLC. HSV-1 corneal latency. *Invest Ophthalmol Vis Sci* 1991;32:663-5.
- 13 Shimeld C, Tullo AB, Easty DL, Thomas J. Isolation of herpes simplex from the cornea in chronic stromal keratitis. *Br J Ophthalmol* 1982;66:643-7.
- 14 Tullo AB, Easty DL, Shimeld C, Stirling PE, Darville JM. Isolation of herpes simplex virus from corneal discs of patients with chronic stromal keratitis. *Trans Ophthalmol Soc UK* 1985;104:159-64.
- 15 Coupes D, Klapper PE, Cleator GM, Bailey AS, Tullo AB. Herpes virus simplex in chronic human stromal keratitis. *Curr Eye Res* 1986;5:735-8.
- 16 Easty DL, Shimeld C, Clauoe CMP, Menage M. Herpes simplex virus isolation in chronic stromal keratitis: human and laboratory studies. *Curr Eye Res* 1987;6:69-74.
- 17 Cantin EM, Chen J, McNeill J, Willey DE, Openshaw H. Detection of herpes simplex virus DNA sequences in corneal transplant patients by polymerase chain reaction assays. *Curr Eye Res* 1991;10(suppl):15-21.
- 18 Crouse C, Pflugfelder SC, Pereira IE, Cleary J, Rabinowitz S, Atherton S. Detection of herpes viral genome in normal and diseased corneal epithelium. *Curr Eye Res* 1990;9:569-81.
- 19 Shimomura Y, Mori Y, Inoue Y, Kiritooshi A, Ohashi Y, Masabe R. Herpes simplex virus latency in human cornea. *Jpn J Ophthalmol* 1993;37:318-24.
- 20 Salisbury JD, Berkowitz RA, Gebhardt BM, Kaufman HE. Herpes virus infection of corneal allografts. *Ophthalmic Surg* 1984;15:4058-60.
- 21 Beyer CF, Byrd TJ, Hill JM, Kaufman ME. Herpes simplex virus and persistent epithelial defects after penetrating keratoplasty. *Am J Ophthalmol* 1990;109:95-6.
- 22 Mead MD, Hyman L, Grimson R, Schein OD. Primary graft failure: a case control investigation of a purported cluster. *Cornea* 1990;13:310-6.
- 23 Buxton JN, Seedor JA, Perry HD, Eagles RC, Pecego JA. Donor failure after corneal transplantation. *Cornea* 1988;7:89-95.