Journal of Medical Virology 52:320–325 (1997)

Low Rate Shedding of HSV-1 DNA, But Not of Infectious Virus From Human Donor Corneae Into Culture Media

Justus G. Garweg* and Matthias Boehnke
Department of Ophthalmology, University of Bern, Bern, Switzerland

Fluid samples derived from 451 organ cultured corneae were tested for the presence of HSV-1 DNA after electroseparation and amplification for fragments of the glycoprotein D- and thymidine kinase-encoding genes. Of the culture media, 134 were processed immediately after withdrawal (Group 1); 100 were stored at ambient temperature for 6 to 60 weeks (Group 2); 90 were stored at –8°C for 4 to 9 weeks (Group 3); and 127 were stored at –20°C for 2 to 30 weeks (Group 4). The degradation of human DNA (marker gene, betaglobin) under these different storage conditions and of human and HSV-1 DNA as a sequential function of time at ambient temperature was gauged by the loss of a detectable signal for the respective component. Endothelial cell density within each of the corneal discs was determined before and after organ culture.

In 7/451 culture fluid samples, HSV-1 DNA corresponding to either the glycoprotein D- or thymidine kinase-encoding genes was detected. In culture fluid samples derived from Groups 2 at ambient temperature, for 6 to 60 weeks) and 3 (at –8°C, for 4 to 9 weeks), complete degradation precluded the detection of human DNA, and hence probably also of HSV-1 DNA; only at –20°C did DNA remain stable for protracted periods of time. Even so, HSV-1 DNA was detected in only 2% of those media in which no degradation was to be expected; additionally, there existed no correlation between its presence in culture fluid samples and the loss of endothelial cells or cytopathic changes.

DNA can be extracted successfully and concentrated twenty-fold from high-volume samples by electroseparation. When shed into culture fluid, it is remarkably prone to a time and temperature dependent degradation, which may lead to false negative results. It is concluded that there is no infectious virus to be expected in the specimens; the occurrence of HSV-1 DNA in donor corneae would not appear to be an important factor influencing their biological quality during the period of organ culture. ***J. Med. Virol. 52:320–325, 1997.*** © 1997 Wiley-Liss, Inc.

KEY WORDS: corneal transplantation; tissue contamination; disease transfer; disease reactivation; virus reactivation; electroseparation

INTRODUCTION

Corneoscleral discs derived from seemingly healthy donors have been shown to contain HSV-1 DNA in 9 to 25% of cases, depending upon the topographic origin of samples taken for analysis (the smaller quota appertaining to centrally derived and the larger to limbally derived tissue). The detection of viral DNA does not allow differentiation between the presence of nonreplicative viral DNA and infectious virus. Therefore, individuals may harbour this pathogen without actually manifesting disease symptoms [Crouse et al., 1990; Cantin et al., 1991; Kaye et al., 1991; Cleator et al., 1994], and their corneae could thus unknowingly serve as a potential vehicle of infection from donor to recipient. Further evidence for such possibility comes from animal studies [Cook and Brown, 1986; Cantin et al., 1992]. Although surgical trauma, such as penetrating keratoplasty, may induce viral reactivation in cases of latent corneal infection [Nicholls et al., 1996], the possibility of donor-to-recipient transmission of HSV-1 DNA via transplanted corneae, and its potential pathogenic consequences, have not been investigated conclusively [Morris et al., 1996].

In 1992, we observed spontaneous endothelial necrosis in a 10-day organ-cultured cornea derived from a 65-year-old donor who had died of acute cardiovascular insufficiency. Immediately after excision, the endothe-

Contract grant sponsor: Swiss National Science Foundation; Contract grant number: 31-33755.92.

*Correspondence to: Justus G. Garweg, M.D., Department of Ophthalmology, University of Bern, Inselspital, CH-3010 Bern, Switzerland.

Accepted 27 February 1997

lium had appeared normal with respect both to morphology and cell density when viewed in the inverted phase-contrast microscope. The subtotal necrosis revealed upon routine examination prior to transplantation could not be attributed to bacterial or fungal infection of the culture, but both the corneoscleral button and the bathing medium were positive for HSV-1 DNA. As a follow-up to this interesting observation, we deliberately infected corneoscleral discs destined for organ culture with the HSV-1 virus in order to test its effect on endothelial viability. As anticipated, total necrosis indeed occurred [Boehnke et al., 1992]. A similar correlation has been reported by other investigators [Tullo et al., 1990; Cleator et al., 1994].

Analysis of culture medium samples for the presence of viral DNA is naturally undertaken on the presupposition that if present, this material has remained stable under the storage conditions adopted prior to use [Morris et al., 1996]; but this is an assumption which lacks experimental corroboration. The consideration is an important one since, if degraded, viral DNA will elude detection, thereby giving rise to a false negative result. In the current study, samples withdrawn from the media bathing organ cultured corneae destined for transplantation were tested for the presence of HSV-1-DNA, and these data correlated to the loss of endothelial cells during culture, which would have to be markedly higher in corneae containing replicative infectious virus. The stability of both viral and human DNA as a function of storage time and temperature was also investigated.

MATERIALS AND METHODS

Experimental Set-up

Single 10 ml samples withdrawn from the media of 451 organ cultured corneae destined for transplantation were used in this study. One hundred thirty-four samples were processed immediately after dissection (Group 1); 100 were stored at ambient temperature for 6 to 60 weeks (Group 2); 90 were stored at –8°C for 4 to 9 weeks (Group 3); and 127 were stored at –20°C for 2 to 30 weeks (Group 4) prior to analysis.

Each corneal disc was inspected with the slit lamp, and endothelial morphology as well as cell density were evaluated by inverted phase-contrast microscopy both prior to and after culturing. Organ culture was carried out as outlined in the "Guidelines of the European Eye Bank Association" (1996). Briefly, the culture fluid consisted of Minimal Essential Medium containing Eagle's salts, 2% foetal calf serum, 0.1 μg/ml Penicillin, 0.1 μg/ml Streptomycin and 0.03 μg/ml Amphothericin B. After dissection and examination, corneal discs were immersed in culture medium and stored under sterile conditions at 32°C, in a humidified atmosphere containing 5% CO_2, for 4–42 days until 12 hours prior to transplantation. Tissue buttons were then transferred to fresh culture fluid containing additionally 6% Dextran 500000. A 10 ml sample (representing 25% of the total fluid volume) was withdrawn from each of the Dextran-lacking media under sterile (laminar flow) conditions and subjected to electroseparation either immediately or after storage, according to the strategy outlined above.

The total DNA fraction contained within each 10 ml aliquot of culture medium was electroseparated, and that corresponding to glycoprotein D- and thymidine kinase-encoding genes of HSV-1, as well as that for human betaglobin, amplified. These products then underwent separation by agarose gel electrophoresis, Southern blot transfer, and DNA-DNA hybridization. Amplifications were undertaken in duplicate, and viral DNA was considered to be present in a culture medium if that encoding for either glycoprotein D or thymidine kinase was detected in each case.

Electroseparation of the Total DNA Fraction

A commercially available electroseparation system (Biotrap®, Schleicher & Schüll, Dassel, Germany) was used according to the manufacturer's instructions, but scrupulous care was necessary to avoid DNA being carried over from previous runs. This involved a rigorous cleaning (hydrochloric acid) and decontamination (UV-irradiation) procedure.

Electroseparation membranes permitting the entrapment of DNA fragments larger than 3–5 kDa (BT1®, Schleicher & Schüll, Dassel, Germany) and which were negative for human and viral DNA were attached on each side of the electroseparation chamber, 4 such chambers were inserted in parallel. The running buffer (0.5 × TBE) consisted of 45.5 mM Tris (Hydroxymethylaminomethan), 45.5 mM sodium borate, and 1 mM EDTA (pH 7.6). Electroseparation was performed at ambient temperature either for 6 (200V/100mA) or 12 to 16 hours (100V/50mA). Upon completion of a run, membranes at the anodal end of each chamber were transferred to sterile microtubes containing 200 μl of lysis buffer (10 mM Tris-Cl, 50 mM KCl, 2.0 mM $MgCl_2$, 0.5% Tween 20; pH, 8.3), and the whole was incubated at 62°C for 1 hour and at 95°C for 10 minutes, with continuous rotation. Five μl of the supernatant were used immediately for amplification experiments, as well as for random testing of DNA and protein concentrations.

Each electrophoresis run was carried out in the presence of negative and positive controls. The former included buffer or fresh organ culture medium; the latter consisted of culture media negative for viral DNA, but harbouring human genomic DNA, as well as native media or buffers spiked with HSV-1 DNA at a final concentration 10-fold to 100-fold lower than the PCR detection level. Using this dilution, a negative amplification signal would be obtained if either electroseparation or DNA amplification or both were run under suboptimal conditions and thus would permit an independent assessment of both the electroseparation and DNA amplification systems. A second sample withdrawn from each culture medium was subjected randomly to an independent electroseparation run, but this was undertaken as a matter of course if the first was positive for viral DNA.

DNA Amplification and Detection

The amplification procedure has been described fully elsewhere [Garweg and Boehnke, 1996], and only the pertinent details are outlined. Each of the two primers was derived from the glycoprotein D gene of HSV-1 (Aurelius et al., 1991). The amplification mixture consisted of 10 mM Tris (pH 8.3), 50 mM KCl, 2.0 mM $MgCl_2$, 0.02% gelatine (w/v), desoxinucleoside triphosphates at 200 μM, 1 unit of Thermus brockianus DNA polymerase (Finnzymes, Espoo, Finland), 50 pmoles of each primer, and 5 μl of the supernatant derived from lysed, electroseparated samples, the whole made up to a volume of 50 μl. The amplification protocol included denaturation of DNA at 95°C for 5 minutes, then 35 cycles comprised to denaturation at 95°C for 60 seconds, primer annealing at 60°C for 30 seconds, and fragment elongation at 72°C for 90 seconds; this was followed by a final fragment elongation step at 72°C for 7 minutes. All buffers and media employed were run routinely through this protocol (as internal negative controls) in order to exclude the possibility of viral contamination. Controls for specificity included culture supernatants from cells infected with HSV-1, HSV-2, VZV, or CMV; a positive control containing 10 ng HSV-1 DNA was also tested. The amplified DNA fragment consisted of 221 base pairs and was visualized on a 3% NuSieve agarose gel (FMC Bioproducts, Rockland, ME) by staining with 0.01% ethidium bromide. After determining the size of the amplificate according to the mobility of molecular weight markers (pBR322, Hae III Digest; Sigma Chemicals, Louisville, New Haven, CT) run in parallel, DNA was transferred onto nylon membranes (Hybond N^+, Amersham, Arlington Heights, IL) by capillary blotting [Southern, 1975] and fixed with 0.4M NaOH [Reed and Mann, 1985]. For DNA hybridization, a buffer consisting of 50% formamide, 5×SSC, 0.02% SDS, 0.1% N-lauroyl-sarcosine, 0.1% BSA, and 20 μg/ml salmon sperm DNA was used, together with a probe generated by random introduction of Digoxigenin-11-dUTP (Boehringer, Mannheim, Germany) in a nested PCR-setting using internal primers (locations 51–71 and 166–188 [Aurelius et al., 1991]). Bound probe was visualized by light emission from a lumigen (Lumigen PPD®, Boehringer Mannheim, Germany) used as a substrate for alkaline phosphatase bound to anti-Digoxigenin antibody. Amplification of a 110-base pair-fragment of the thymidine kinase gene was likewise performed using the 35-cycle protocol outlined above, but primer annealing was effected at 58°C rather than at 60°C [Lynas et al., 1989].

A 268–base pair fragment of the human betaglobin gene, which was reasonably expected to be present consistently in any sample containing human cells, was amplified from approximately every third culture fluid sample by random selection, in order to assess whether DNA degradation had occurred under the storage conditons adopted; the rate of degradation obtaining at ambient temperature as a function of time was also assessed. The amplification protocol consisted of 45 cycles, and primer annealing was conducted at 60°C for 1 minute [Bauer et al., 1991]. Hybridization of human DNA was deemed to be unnecessary, since samples were expected to contain larger quantities of this material. DNA degradation was considered to have occurred if samples failed to register an amplification signal for human betaglobin gene-DNA. To exclude the possibility of an internal inhibition of amplification, several samples were also spiked with low amounts of HSV-1 DNA prior to this procedure.

RESULTS

Quality of Electroseparated DNA

After electroseparation, a 20- to 50-fold increase in DNA concentration was found after spiking culture media with defined amounts of HSV-1-DNA, this discrepancy in the degree of enhancement reflecting the extent to which DNA was reeluted from electrophoresis membranes into the lysis buffer. The protein concentration in culture media ranged between 500 and 1000 μg/ml, whereas that in the lysis fluid after reelution of membrane-bound components was 54 (24 to 62) μg/ml (Micro BCA Protein Assay, Pierce, Rockford, IL). Hence 90% of contaminating proteins were eliminated by electroseparation; the remaining 10% elicited a 2- to 5-fold inhibition of DNA amplification, whereas non-electroseparated culture fluid and Optisol® (a commercial organ culture medium) evoked a 20- to 50-fold inhibition and a complete inhibition, respectively. The proportion of DNA to other contaminating substances was determined by measuring the light absorption at 260 and 280 nm and was found to be 8 (5–21)% (corresponding to an absolute concentration of 0.5–4 μg/ml) and 51 (31–65)% (corresponding to an absolute concentration of 4–26 μg/ml) in culture media and electroseparated eluates, respectively.

Stability of DNA in Culture Fluid

Samples of medium negative for HSV-1 DNA, derived from the fluid bathing 2 partner corneae cultured for 14 days, were used to assess the rate of herpetic DNA degradation by intrinsic DNAse activity. The first medium was spiked with low copy numbers of HSV-1 DNA, while the second remained untreated. After 0, 7, 14, 28, and 60 days of storage at ambient temperature, aliquots were withdrawn for electroseparation and amplification. In the spiked medium, signals corresponding to HSV-1 and human betaglobin DNA were no longer registered after 7 and 60 days, respectively. In the unspiked medium, HSV-1 DNA was, as expected, at no time detectable; that corresponding to human betaglobin was, again, completely degraded after 60 days (Table I, Fig. 1). From the culture media included in this study, human DNA was amplified from samples processed immediately after withdrawal and from those stored for various durations at ambient temperature, −8°C, or −20°C (corresponding to Groups 1, 2, 3, and 4, respectively). The data in Table II reveal that depression of sample temperature to −8°C was insufficient to prevent degradation of human DNA; only at

TABLE I. Degradation of DNA With Time*

Storage time (days)	Culture medium spiked with HSV-1 DNA		Partner medium (unspiked)	
	Human betaglobin gene	HSV-1 glycoprotein D gene	Human betaglobin gene	HSV-1 glycoprotein D gene
0	+	+	+	–
7	+	–	+	–
14	–	–	+	–
28	(+)	–	+	–
60	–	–	–	–

*Storage was at ambient temperature. +, strong signal; (+), weak signal; –, no signal.

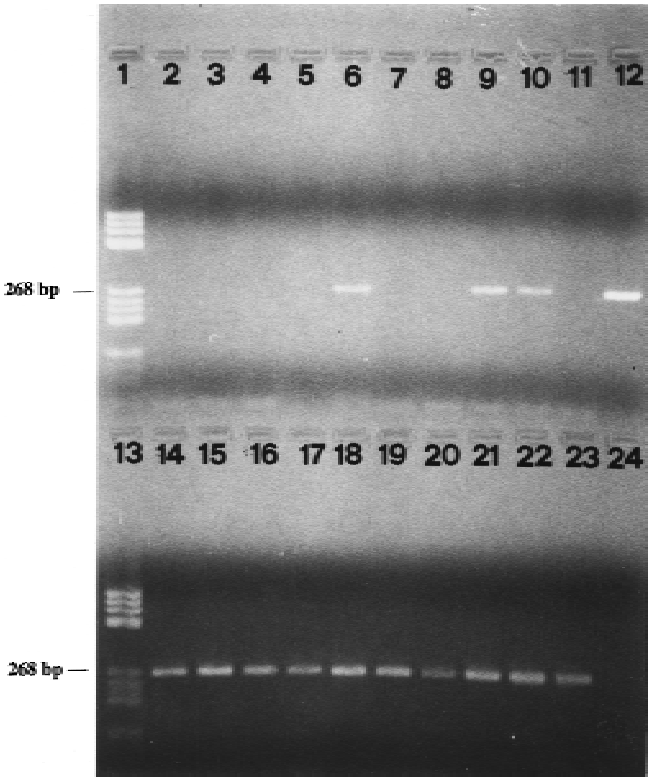

Fig. 1. Agarose gel illustrating the variation in signal intensity for human betaglobin gene DNA amplified from organ culture media stored under different conditions. **Lanes 1, 13:** molecular weight marker (pBR 322.Hae III digest, Boehringer Mannheim, Germany); **lanes 2–11:** media stored at ambient temperature for 6 to 60 weeks (Group 2); **lanes 12, 14–22:** media stored at –20°C for 2 to 30 weeks (Group 4); **lane 23:** positive control (0.1 μg human genomic DNA); **lane 24:** negative (buffer) control.

–20°C did this species remain stable for a protracted period of time (Table II).

Detection of HSV-1 DNA in Culture Fluid

HSV-1 DNA from the gene encoding for glycoprotein D was detected in one of the culture media samples processed immediately after withdrawal (Group 1) and in one of those maintained at –20°C (Group 4), but in none of those stored at ambient temperature (Group 2) or at –8°C (Group 3). Two samples in each of Groups 1 (immediate proceeding), 2 (storage at ambient temperature), and 4 (storage at –20°C), but none in Group 3, registered the presence of thymidine kinase-encoding herpetic DNA (Table III); in one instance (Group 4), both markers for HSV-1 DNA were detected in the same sample.

TABLE II. Detection of Human DNA Under Different Storage Conditions

Group	Storage condition	Human betaglobin gene
1	Immediate processing	33/34
2	Ambient temperature for 6 to 60 weeks	2/29
3	–8°C for 4 to 9 weeks	0/32
4	–20°C for 2 to 30 weeks	38/38
Total		73/133

Endothelial Cell Counts

Estimates of endothelial cell density in corneae derived from HSV-1 DNA-positive and negative media did not differ from one another, either before or after organ culture; the cell losses incurred during the period of incubation were also comparable (χ^2-test: $P = 0.44$; Table IV).

DISCUSSION

In the current study, electroseparation of necessarily large volumes of media derived from organ cultured corneae permitted a twenty-fold enhancement in DNA concentration upon reelution into lysis buffer and a concomitant elimination of contaminating proteins to the extent of 90%.

A somewhat disturbing finding was that DNA is remarkably prone to degradation in stored samples of culture media (probably by intrinsic DNAase activity), since this is both time (Table I) and temperature (Table II) dependent in the case of that encoding for the human betaglobin gene. Early loss of the amplification signal for the HSV-1 glycoprotein D gene in culture media spiked with low copy numbers of HSV-1 DNA (Table I) and the higher incidence of detection of the thymidine kinase gene fragment (which is shorter and probably more stable than the glycoprotein D one; Table III), supports this interpretation. A storage of culture media under conditions which are not well defined, may, as shown in this study, lead to false nega-

TABLE III. Detection of HSV-1 DNA in Organ Culture Media

Group	Storage condition	HSV-1 glycoprotein D gene	HSV-1 thymidine kinase gene
1	Immediate processing	1[a]/134	2/134
2	Ambient temperature for 6 to 60 weeks	0/100	2/100
3	−8°C for 4 to 9 weeks	0/90	0/90
4	−20°C for 2 to 30 weeks	1/127	2[a]/127
Total		2/451	6/451

[a]In one sample, HSV-1 DNA corresponding to both glycoprotein D- and thymidine kinase-gene fragments were detected.

TABLE IV. Endothelial Cell Counts of Corneal Discs

	Before culture cells/mm^2	After culture cells/mm^2	Cell loss/mm^2
Cultures harbouring HSV-1 DNA	2304 ± 283	2078 ± 276	399 ± 641
HSV-1 DNA-negative cultures	2800 ± 623	2650 ± 492	150 ± 168
			$P = 0.44$

tive results, which we were not aware when designing the study. After we had found a complete degradation under a storage at ambient temperature, and, in parallel, an almost complete one at 4°C, we decided to compare a storage at −20°C (at which temperature most enzyme activity has quietened down) and an immediate work up of materials, which we now believe to be the best investigational protocol. However, the data showed that if an immeidate work-up is not possible, storage at −20° may be appropriate to inhibit DNA degradation for a limited period of time. The stability of DNA in culture media has important implications for all investigations in which the concentration of target DNA is low or in which maintenance of its integrity influences the outcome of results. Regarding this point, the study of Morris et al. [1996] lacks information about storage conditions and the time from end of culture to extraction of DNA. These probably were not documented because the relevance of these factors has not been recognized. Consequently, the suitability of their results to estimate the risk of viral contamination of corneal donor tissue cannot be evaluated with the information provided.

The mechanisms underlying the latency and reactivation of HSV-1 infection in humans are but poorly understood. The existence of herpetic DNA in the absence of manifested symptoms of its activity has been demonstrated in both normal and diseased corneal tissue in human [Rong et al., 1988; Crouse et al., 1990; Cantin et al., 1991; Holbach et al., 1991; Kaye et al., 1991; Ohashi et al., 1991; Rong et al., 1991] and animal studies [Openshaw, 1983; Cantin et al., 1992]. Viral antigen, presented to the immune system by corneal epithelial cells [Fahy et al., 1993], triggers a cytokine-induced overexpression of class I and II human leukocyte antigens in the central corneal epithelium and stroma [Pepose et al., 1985], where it attracts CD4-positive T cells and Langerhans cells [Hendricks et al., 1992]. Instigation of this train of events may contribute to an increased risk of graft rejection [Missotten, 1994], although the expression of class II antigens and the number of Langerhans cells present in corneal discs are known to undergo a gradual regression during the course of organ culture [Boehnke, 1991]. We did not test for the presence of viral antigens in the culture fluid nor for the expression of HLA class II antigens in our transplant tissues. Nevertheless, in our series the post-transplantation history was obtainable corresponding to positive cultures from 5 of 7 corneal grafts. It showed a completely uneventful postoperative course without graft rejection episodes or signs of herpetic activity in any of the four grafted corneal transplants, as did the corresponding partner tissues. The fifth corneoscleral disc was not grafted, but it showed a vital and stable endothelium and normal stromal keratocytes at the final examination after more than 2 months of culture.

We demonstrated the release of HSV-1 DNA into the media of organ cultured corneae in less than 2% of cases, but its presence was not, as suspected on the basis of previous data [Boehnke et al., 1992], correlated to the loss of endothelial cells (Table IV). This gives strong evidence that there was not a replicative infectious virus.

For corneal organ culture, standard cell culture methods are used. If infectious virus were present in the culture, it would produce cytopathic changes to the graft and the epithelial cell layer on the bottom of the culture, both of which have regularly been examined throughout organ culture. Because the sensitivity of such culture system is identical to conventional shell vial virus culture technique (except for the additional use of immunofluorescence technique), there was no rational base to include virus culture into our protocol. However, not even this would, of course, have allowed us to exclude definitely the possibility that infectious herpes virus existed in transplant tissue, since targeting of endothelial cells may represent an advanced stage in corneal infection. But that such was the case seems unlikely: if donor corneae had indeed harboured HSV-1, then organ culture should have precipitated the reactivation of latent infection during the period of incubation adopted by ourselves (4–42 days [O'Brien and Taylor, 1989]); inevitably, such replicative virus would have impaired the tissue and thus have been detected not only by the presence of its DNA. The possibility of a donor-to-recipient transmission of replicative HSV-1 can therefore be excluded with reasonable certainty.

REFERENCES

Aurelius E, Johannson B, Sköldenberg B, Staland A, Forsgren M (1991): Rapid diagnosis of herpes simplex encephalitis by nested polymerase chain reaction assay of cerebrospinal fluid. Lancet 337:189–192.

Bauer HM, Ting Y, Greer CE, Chambers JC, Tashiro CJ, Chimera J, Reingold A, Manos MM (1991): Genital human papillomavirus in female university students as determined by a PCR-based method. JAMA 256:472–477.

Böhnke M (1991): Preservation of Donor Corneae. Current Opinion in Ophthalmology 2:432–442.

Böhnke M, Garweg J, Germann D (1992): Spontaneous endothelial necrosis in human donor cornea. International Conference on Herpetic Eye Disease, New Orleans, LA; Abstract 23:4.

Cantin EM, Chance J, McNeal J, Willey DE, Openshaw H (1991): Detection of herpes simplex virus DNA sequences in corneal transplant recipients by polymerase chain reaction assays. Current Eye Research 10 (Suppl.):15–21.

Cantin E, Chen J, Willey DE, Taylor JL, O'Brien WJ (1992): Persistence of herpes simplex virus DNA in rabbit corneal cells. Investigative Ophthalmology and Visual Science 33:2470–2475.

Cleator GM, Klapper PE, Dennett C, Sullivan AL, Bonshek RE, Marcyniuk B, Tullo AB (1994): Corneal donor infection by herpes simplex virus: Herpes simplex virus DNA in donor corneas. Cornea 13:294–304.

Cook SD, Brown SM (1986): Herpes simplex virus type I persistance and latency in cultured rabbit corneal epithelial cells, keratocytes, and endothelial cells. British Journal of Ophthalmology 70:642–650.

Crouse CA, Pflugfelder SC, Pereira I, Cleary T, Rabinowitz S, Atherton SS (1990): Detection of herpes virus genomes in normal and diseased corneal epithelium. Current Eye Research 9:569–581.

Easty DL, Shimeld C, Claque CP, Menage M (1987): Herpes simplex isolation in chronic stromal keratitis: Human in laboratory studies. Current Eye Research 6:69–74.

European Eye Bank Association (1996): "Directory of the European Eye Bank Association." Pels E, Tullo AB (eds). 4th edition.

Fahy GT, Hooper DC, Easty DL (1993): Antigen presentation of herpes simplex virus by corneal epithelium–an in vitro and in vivo study. British Journal of Ophthalmology 77:440–444.

Garweg J, Böhnke M (1996): Slow viral replication of HSV-1 is responsible for early recurrence of herpetic keratitis after corneal grafting. Graefe's Archives for Clinical and Experimental Ophthalmology 234 (suppl):133–138.

Hendricks RL, Janowicz M, Tumpey TM (1992): Critical role of corneal Langerhans cells in the CD4- but not CD8-mediated immunopathology in herpes simplex virus-1-infected mouse corneas. Journal of Immunology 148:2522–2529.

Holbach LM, Font RL, Bear W, Tittler SJ (1991): HSV-antigens and HSV-DNA in avascular and vascularized lesions of human herpes simplex keratitis. Current Eye Research 10 (Suppl.):63–68.

Kaye SB, Lynas C, Patterson A, Risk JM, McCarthy K, Hart CA (1991): Evidence for herpes simplex viral latency in the human cornea. British Journal of Ophthalmology 75:195–200.

Lynas C, Cook SD, Laycock KA, Bradfield JW, Maitland NJ (1989): Detection of latent virus mRNA in tissues using the polymerase chain reaction. Journal of Pathology 157:285–289.

Missotten L (1994): Immunology and Herpetic Keratitis. Eye 8:12–21.

Morris DJ, Cleator GM, Klapper PE, Cooper RJ, Biney EO, Dennett C, Marcyniuk B, Tullo AB (1996): Detection of herpes simplex virus DNA in donor cornea culture medium by polymerase chain reaction. British Journal of Ophthalmology 80:654–657.

Nicholls SM, Shimeld C, Easty DL, Hill TJ (1996): Recurrent herpes simplex after corneal transplantation in rats. Investigative Ophthalmology and Visual Science 37:425–435.

O'Brien WJ, Taylor JL (1989): The isolation of herpes simplex virus from rabbit corneas during latency. Investigative Ophthalmology and Visual Science 30:357–364.

Ohashi Y, Yamamoto S, Kohj N, Okamoto S, Kinoshita S, Hayashi K, Manabe R (1991): Demonstration of herpes simplex virus DNA in idiopathic corneal endotheliopathy. American Journal of Ophthalmology 112:419–423.

Openshaw H (1983): Latency of herpes simplex virus in ocular tissue of mice. Infection and Immunity 39:960–962.

Pepose JS, Gardner KM, Nestor MS, Foos RY, Pettit TH (1985): Detection of HLA class I and II antigens in rejected human corneal allografts. Ophthalmology 92:1480–1484.

Reed KC, Mann DA (1985): Rapid transfer of DNA from agarose gels to nylon membranes. Nucleic Acids Research 13:7207–7221.

Rong BL, Kenyon KR, Bean KM, Langston DP, Dunkel EC (1988): Detection of the HSV genome in human corneal buttons. Investigative Ophthalmology and Visual Sciences 29 (suppl):158.

Rong BL, Pavan-Langston D, Weng QP, Martinez R, Cherry JM, Dunkel EC (1991): Detection of herpes simplex thymidine kinase and latency-associated transcript gene sequences in human herpetic corneas by polymerase chain reaction amplification. Investigative Ophthalmology and Visual Sciences 32:1808–1815.

Shimeld C, Tullo AB, Easty DL, Thomsitt J (1982): Isolation of herpes simplex virus from the cornea in chronic stromal keratitis. British Journal of Ophthalmology 66:643–647.

Southern EM (1975): Detection of specific sequences among DNA fragments separated by gel electrophoresis. Journal of Molecular Biology 98:503–517.

Tullo AB, Marcyniuk B, Bonshek R, Dennett C, Cleator GM, Lewis AG, Klapper PE (1990): Herpes virus in a corneal donor. Eye 4: 766–767.