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SCREENING OF HUMAN CORNEAS FOR HERPES SIMPLEX VIRUS BY TISSUE CULTURE AND POLYMERASE CHAIN REACTION

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SUMMARY: Superficial eye infections by herpes simplex virus (HSV) constitute a major cause of corneal disease, necessitating the need for corneal transplantation in many patients. Eighty-three corneas from 46 post-mortem donors received from the David Lucas Eye Bank in Manchester were analyzed by Vero cell culture and the polymerase chain reaction (PCR) technique to detect HSV. There was no evidence of a characteristic cytopathic effect in any of the cultures. A 350-bp PCR product corresponding to the HSV thymidine kinase (TK) was detected by Southern blotting in only 2.4% (2/83) of samples. In contrast, approximately 70% of samples yielded a 758-bp PCR product. Although this low prevalence of HSV in corneas may be encouraging, it is high for the actual transplantation program if the viral DNAs maintain their abilities to replicate.

INTRODUCTION

Superficial eye infections caused by viruses result in a broad spectrum of eye diseases in both developed and developing countries throughout the world (1). Almost all of these diseases affect the cornea and may result in ulcers, corneal opacities, scarring, vascularization, perforation, and in some cases, visual impairment.

The most frequently encountered of these viruses are herpes simplex virus (HSV) (2), adenovirus (3,4) and varicella-zoster virus (VZV) (5). HSV is the major cause of corneal inflammation (keratitis), of which type 1 and type 2 account for

90% and 10%, respectively, of all herpetic keratitis cases (6). HSV types 1 and 2 are primarily associated with infections "above the belt" and "below the belt" respectively (7), but can be found in either location.

HSV is characterized by long-term persistence due to establishment of latency in sensory neurons (8) and possibly in nonneuronal tissue (e.g., cornea) (9). Both primary or recurrent infections are common. Primary infections usually occur in infancy or young people, but rarely in adulthood. Recurrent infections occur later in adulthood and are due to reactivation of the latent HSV (10). The exact mechanism of reactivation is, however, unknown.

Recently, an extremely powerful, highly sensitive and specific *in vitro* enzymatic DNA amplification procedure, the polymerase chain reaction (PCR), has been applied to a host of medico-legal situations (11,12), and biomedical research endeavors (13-16), including viral detection in ocular inflammatory diseases (22). This study was undertaken to detect HSV type 1 DNA sequences in corneal tissue destined for corneal transplantation and to evaluate the usefulness of the PCR technique for the routine screening and diagnosis of HSV infections.

MATERIALS AND METHODS

The United Kingdom transplant service is the main body that co-ordinates the activities of the David Lucas Manchester Eye Bank for storage of human corneas awaiting recipient transplantation in the Northern United Kingdom. These corneal discs are stored in organ culture medium and maintained in an incubator at 34 C for up to 30 days. None of the donors was immunocompromised nor had any history of systemic disease such as diabetes or renal failure. Three hundred and thirty two discs were evaluated histologically. Any disc showing endothelial cell loss or herpetic viral particles in the basal cells and keratocytes was rejected. Samples were also screened for bacteria via culture and serologically for antigens of hepatitis B virus (HBV) and Human Immunodeficiency virus (HIV) by an ELISA Test (Abott Laboratories, North Chicago, IL) as both viruses have been demonstrated in the cornea and tears (17,18). Subsequent to the processing of the corneas by the eye bank, samples of 83 corneal organ storage media (not corneal tissue) from 46 cornea donors were received for analysis in the virology laboratory of the School of Diagnostic and Investigational Sciences, University of Manchester Medical School - the basis of this study.

For virus isolation 50- μ l aliquots of samples were inoculated into each of four (4) wells of a 24-well plate monolayer of Vero cells (African green monkey cells) with minimal essential growth medium (MEM) supplemented with 5% fetal

calf serum (FCS), 100 mM glutamine, 100 µg/ml crystamicin. All the preparations were centrifuged onto the Vero cells at $3,500 \times g$ in a 6-liter centrifuge, incubated in a gassed (5% CO₂) incubator at 37 C for one hour and held for 10-21 days, and examined for characteristic HSV CPE. If no CPE was observed during this period, the cultures were considered negative. Media were changed at three-day intervals. Positive herpes simplex virus cultures (control) were identified as described elsewhere (19).

DNA was extracted with 1% (w/v) sodium dodecyl sulfate (SDS) and 200 µg/ml proteinase K. This was followed by phenol:chloroform (1:1) extraction and ethanol precipitation, respectively. Following centrifugation at 3,000 rpm for 5 min, the precipitates were dissolved in 10 µl of $1 \times$ TE (Tris-EDTA) buffer (3 mM Tris-HCl, 0.2 mM Na₂EDTA), pH 8.0.

To amplify HSV DNA sequence, the PCR procedure was performed according to methods described by Saiki and associates (20). In brief, 10 µl of DNA extract was subjected to the PCR reaction on 100 µl of PCR buffer (10 mM Tris-HCl, pH 8.0, 50 mM MgCl₂, 0.01% gelatin) in the presence of 80 µM deoxynucleoside triphosphate (dATP, dCTP, dGTP, dTTP), 20 pmol each of 5' and 3' primer, and 2.5 units of *Thermus aquaticus* (Tag) DNA polymerase (Perkin-Elmer Cetus, Emeryville, CA). A pair of primer sequences, Nest 7 and Nest 8c, that bracketed a 350-bp fragment of the HSV thymidine kinase genome (Research Genetics, Huntsville, AL) was used. The sequences were:

Nest 7: 5' CGCGCCGTACCTTATGGGCAGCATGA and

Nest 8c: 5' CAGGTTAAATAACGTCGTCCCGATA

The β-globin primer pair, Globin 1N and Globin 2, would initiate the amplification of a 268-bp sequence DNA fragment that is part of the sixth codon human β-globin gene.

The sequences were:

Globin 1N: 5' CAACTTCATCCACGTTCCACC and

Globin 2: 5' GAAGAGCCAAGGACAGGTAC

An overlay of 50 µl of mineral oil was added to the top of the reaction mixtures and placed in a thermocycler (Techne PHC-1). The PCR was simultaneously carried out for the β-globin primer pair to serve as an internal control primarily for the integrity of the cellular DNA and to demonstrate that there were no PCR inhibitors in the DNA extracts. Amplification consisted of one cycle of 7-minute denaturation at 94 C, 1.5-min annealing at 50 C and 1.5-min extension at 70 C. The subsequent 50 cycles consisted each of 2-min denaturation at 94 C, 2-min annealing at 50 C and 2-min extension at 70 C.

Ten-microliter aliquots of the amplified DNA products were electrophoresed on 1.5% agarose in $1 \times$ TAE buffer pH 8.0 (20 mM Tris, 5.7% acetic acid, 50 mM Na₂EDTA, pH 8.0) and stained in a solution of ethidium bromide (0.5 µg/ml), visualized and photographed under short-wave ultraviolet transillumination. To confirm that the amplified DNA bands were specific to HSV, Southern blot hybridization was done by using a biotinylated UTP HSV probe, as described elsewhere (21).

Fig. 1. Agarose gel electrophoresis of polymerase chain reaction products. Lane 1: 1 kb DNA ladder, lane 8: contamination control, lane 2A: amplification control for HSV DNA, lane 9B: amplification control for human DNA, lanes 3A through 7A and 10B through 14B are corneal samples.

RESULTS

There was no evidence of characteristic HSV CPE in the Vero cell culture of the 83 specimens studied over the 10-to 21-day incubation period. There was, however, evidence of HSV DNA in two specimens via the PCR technique. The generation of a 350-bp fragment (Fig. 1A, lanes 3 and 7) indicates the presence of HSV thymidine kinase (TK). Hybridization of the PCR products from the two positive samples showed that the 350-bp fragment is sequences complementary to HSV TK DNA. Only one sample was shown with hybridization of an anomalous 758-bp fragment (Fig. 3 left, lane 2). Thus 2.4% of virus culture that was negative for HSV possessed DNA that was complementary to HSV TK DNA in the PCR technique.

Fig. 2. Determination of the sensitivity of polymerase chain reaction (PCR) amplification with serial dilutions of purified HSV DNA stock containing 2.7×10^7 molecules/ μ l (Fig. 2A). Agarose gel electrophoresis of PCR products. Lane 1: 1 kb DNA ladder, lane 2A through 7A serial dilutions of HSV DNA (10^{-1} to 10^{-6}), lane 8: contamination control (Fig. 2B), lanes 9B through 14B serial dilutions of HEL DNA (10^{-1} to 10^{-6}).

The sensitivity of the PCR was tested prior to the actual runs with serial dilutions of purified HSV-1 thymidine kinase DNA solution (courtesy of Yousif Khodari, the virology laboratory, University of Manchester). This stock of HSV-1 DNA contained 2.7×10^7 molecules/ μ l and was diluted (10^{-1} to 10^{-6}) for the sensitivity assay. The PCR HSV sensitivity was 13.5×10^3 molecules (Fig. 2A). The sensitivity of β -globin PCR was determined with serial dilutions (10^{-1} to 10^{-6}) of human embryo lung (HEL) DNA containing 7.3×10^5 molecules/ 5μ l (courtesy of Lorraine Mac Elhinney, virology laboratory, University of Manchester). The sensitivity of the β -globin PCR was only 7.3×10^3 molecules (Fig. 2B). Figure 3

Fig. 3. Left: agarose gel electrophoresis of polymerase chain reaction for herpes simplex virus showing the 758 bp anomalous band. Lane 1: 1 kb DNA ladder, lanes 2 through 7 are corneal samples, lane 8: contamination control. Right: autoradiograph showing Southern immunoblot analysis of DNA products amplified for herpes simplex confirms this as herpetic.

shows a 758-bp anomalous band identified via Southern blot hybridization as possibly herpetic.

DISCUSSION

Herpes simplex virus is known to be a major cause of keratitis. The isolation and identification of the virus is rare irrespective of the state and type of ocular tissue (normal or diseased) used for the cell culture (22). The transplant program was designed to use clinical material with minimal interference to routine

corneal transplantation as well as improving the sensitivity of detection of the virus. In this study, of the total 83 medium specimens (media in which corneas were kept) screened by both virus isolation and the PCR technique, none of the samples showed any characteristic HSV CPE via culture. Two (2.4%) of the samples were, however, HSV positive by the PCR. This may indicate that infectious virus was not present and that viral sequences amplified by the PCR were from latent virus. Although the results obtained by the PCR technique may be positive indicators for the success of a transplant program, this rate may be high for the actual transplantation if viral DNAs maintain their abilities to replicate. The two HSV positives may suggest a possible corneal latency because virus was not isolated from the Vero cell culture. This may be due to rapid loss of viral infectivity in the organ culture media before transfer to the cell layers.

These two HSV positives were, however, confirmed by Southern blot hybridization to be specific to the HSV TK. The sensitivity of the particular PCR is crucial in determining the PCR result. In the HSV PCR, the sensitivity was only 13.5×10^3 molecules and thus would require large numbers of viral DNA molecules in the specimens for a positive result. The sensitivity may require improvement by the use of nested primers, or altering the amplification conditions. In the β -globin PCR, 7.3×10^3 molecules of HEL DNA were detectable. Although this sensitivity may be low, many of the clinical samples contained detectable levels of human DNA (β -globin). Perhaps failure to detect human DNA in the other reactions may have been due to insufficient DNA in the samples.

A large number of the samples generated an anomalous band of approximately 758 bp with the HSV primers and not with the β -globin primers. The origin of this band is unknown. The HSV primers should have very minimal or no homology to cellular sequences. No sequence homology to human genes was revealed following exhaustive computer search. The limited amount of sequence information on the human genome presently available prevents this approach from being conclusive.

However, the band could not be produced with HEL DNA, even in very large quantities (up to 2 μ g/assay). The conditions of incubation for the PCR procedures were carefully checked especially since HSV DNA contains high GC content when compared to the other viruses. It is unlikely, therefore, that the band is generated from human DNA. Since this anomalous band was widespread in the samples, and was not present in the positive controls, the possibility of this product being a new herpes virus or a new virus altogether may be worth considering. It is unlikely that the band is of human herpes viruses 6 or 7 (HHV 6, HSV 7) be-

cause their genome sequences do not have homology to the HSV TK gene. It is well known that, if HHV 6 possesses TK, it is not in the expected location on its genome (9). The band may have homology with herpes viruses (VZV and EBV) or non-herpes viruses (Adenovirus) but these were not used in the amplification procedures. Nevertheless, from the Southern blotting result, this 758 bp may be herpetic because the product was hybridized by biotinylated probe specific to the HSV TK.

The frequent presence of this band especially since it hybridized with the HSV TK probe encourages doubts about the specificity of the amplification process and could indicate contamination of the sample. It may also be an aberrant amplification product due to the unusually high number of cycles. We did not attempt to modify this amplification condition.

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