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Inhibition of PCR by Aqueous and Vitreous Fluids

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The detection of viral nucleic acids in intraocular fluids and tissues by PCR has become increasingly important in clinical ophthalmology. While much attention has been directed toward minimizing false-positive reactions resulting from specimen contamination or amplicon carryover, relatively little attention has been given to the causes of false-negative PCRs. This report describes a PCR inhibitor in normal aqueous and vitreous fluids that can produce false-negative PCR results. As little as 0.5 μl of vitreous fluid and 20 μl of aqueous fluid can completely inhibit DNA amplification in a 100-μl PCR mixture. This inhibition was not primer specific, nor was it due to chelation of Mg^{2+} ions or DNase activity in the ocular fluid. The inhibitor was completely resistant to boiling for 15 min. However, the inhibitory effects were completely removed by a single chloroform-isoamyl alcohol (24:1) extraction. The extent of PCR inhibition depended upon the type of thermostable DNA polymerase used in the reaction. *Taq* DNA polymerase was very sensitive to the inhibitor, while thermostable DNA polymerases from *Thermus thermophilus* HB-8 (*Tth*) and *Thermus flavus* (*Tfl*) were completely resistant. Thus, the inhibitory effects of intraocular fluids on PCRs can be removed by diluting the specimen, by chloroform extraction, or by using *Tth* or *Tfl* DNA polymerases.

Herpes simplex virus (HSV) and varicella-zoster virus (VZV) are the leading viral causes of uveitis and chorioretinal disease in the United States (6, 9). Primary and reactivated herpesvirus infections have been implicated in a variety of ocular diseases ranging from benign, self-limiting conjunctivitis to sight-threatening infections of the cornea, retina, and choroid. However, a definitive diagnosis of these intraocular diseases is difficult to establish because serological tests for herpesviruses often produce ambiguous results (13). Virus isolation procedures are often unrewarding because of small sample volumes and low virus concentrations (21). In contrast with traditional laboratory methods, PCR appears to be uniquely suited for the detection of intraocular herpesvirus infections because of its exquisite sensitivity and small sample requirements.

Unfortunately, a number of biological fluids contain substances that inhibit PCR (9, 11, 15). Limpens and Kijlstra (10) recently reported that vitreous fluids contain a potent inhibitor of PCR that could not be removed by boiling. This observation was not in agreement with the procedure published by Fox et al. (5), who added 30 μl of boiled aqueous or vitreous fluids to a 70-μl PCR mixture. Our PCR experiences with aqueous and vitreous fluids were similar to those of Limpens and Kijlstra in that boiling did not inactivate the inhibitory substances found in these specimens. Because false-negative PCRs can have significant consequences in the treatment of patients with intraocular infections, we chose to further characterize this inhibitor and to identify methods for removing it before PCR amplification.

(The preliminary findings of this study were presented at the Annual Meeting of the Association for Research in Vision and Ophthalmology, Sarasota, Fla., 1 to 6 May 1994.)

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MATERIALS AND METHODS

Aqueous and vitreous fluids. Aqueous and vitreous fluid specimens that were normally discarded during scleral buckling and vitrectomy surgeries for rhegmatogenous retinal detachments and membrane peeling were collected in sterile syringes and stored at −70°C. All specimens were collected with the informed consent of the patient and the approval of the William Beaumont Hospital Human Investigation Committee. Patients with existing, or a history of, ocular inflammatory disease or diabetes were excluded from the study. Specimens with significant blood contamination were also discarded. Aseptic conditions were maintained during collection, storage, and handling of all specimens.

PCR. The standard PCR master mixture consisted of 10 mM Tris HCl, pH 9.0 at 25°C, containing 50 mM KCl, 0.1% Triton X-100, 2.5 mM $MgCl_2$, 2.5 U of *Taq* DNA polymerase (Promega Corporation, Madison, Wis.), 1 μM each oligonucleotide primer (Table 1), and 200 μM each deoxynucleotide triphosphate (dNTP). All PCRs were done in 100-μl reaction volumes. Oligonucleotide primers (Table 1) were synthesized by Midland Certified Reagent Company (Midland, Tex.). HSV primers defined a 476-nucleotide segment of the HSV DNA polymerase gene (14), and HSV thermocycling conditions consisted of an initial 5-min soak at 95°C followed by 35 cycles of denaturation at 94°C for 1.5 min, primer annealing at 65°C for 2 min, and primer extension at 72°C for 2 min. An additional 2 s was added to the extension step with each cycle. VZV PCRs were performed with primers that flank a unique 250-nucleotide region of gene 29, which encodes the major VZV DNA-binding protein (19). VZV thermocycling conditions consisted of 30 cycles of denaturation at 94°C for 1 min followed by primer annealing at 40°C for 1 min and primer extension for 1 min at 72°C. The primer extension step was extended an additional 6 s with each cycle. Human β-globin amplifications were performed as described by Saiki et al. (17), with primers that flank a 268-bp region of the gene (Table 1). Specimens were subjected to 35 PCR cycles consisting of 1 min of denaturation at 94°C, 2 min of primer annealing at 55°C, and 1 min of primer extension at 72°C. Thermal cycling was done in a model 480 thermocycler from Perkin-Elmer Cetus (Norwalk, Conn.).

PCRs with the *Taq* DNA polymerase Stoffel fragment (Perkin-Elmer Cetus) were done in 10 mM Tris HCl (pH 8.3 at 25°C) containing 10 mM KCl, 2.5 mM $MgCl_2$, 10 U of AmpliTaq recombinant DNA polymerase Stoffel fragment (Perkin-Elmer Cetus), 1 μM each HSV primer (Table 1), and 200 μM each dNTP. PCRs with the *Tth* DNA polymerase from the thermophilic bacterium *Thermus thermophilus* HB8 were done in 10 mM Tris HCl (pH 9.0 at 25°C) containing 50 mM KCl, 0.1% Triton X-100, 2.5 mM $MgCl_2$, 2 U of *Tth* DNA polymerase (Promega Corporation), 1 μM each HSV primer (Table 1), and 200 μM each dNTP. PCRs were also performed with the recombinant *Tli* DNA polymerase from the thermophilic bacterium *Thermococcus litoralis*. *Tli* PCRs were done in 10 mM Tris HCl (pH 9.0 at 25°C) containing 0.1% Triton X-100, 2.5 mM $MgCl_2$, 1 U of *Tli* DNA polymerase (Promega Corporation), 1 μM each HSV primer (Table 1), and 200 μM each dNTP. PCRs with the *Tfl* DNA polymerase from the thermophilic bacterium *Thermus flavus* were done in 10 mM Tris HCl, pH 9.0 at 25°C, containing 50 mM KCl, 0.1% Triton X-100, 2.5 mM $MgCl_2$, 1 U of *Tfl*

TABLE 1. PCR primers used in inhibitor studies

Target	Product size (bp)	Primer designation	Sequence	Reference
HSV	476	pol-1 pol-2	5′-CAG TAC GGC CCC GAG TTC GTG A-3′ 5′-GTA GAT GGT GCG GGT GAT GTT-3′	14
VZV	240	29-1 29-2	5′-GGT CTT GCC GGA GCT GGT ATT ACC T-3′ 5′-AAT GCC GTG ACC ACC AAG TAT AAT-3′	19
β-Globin DNA	268	PC-04 GH-20	5′-CAA CTT CAT CCA CGT TCA CC-3′ 5′-GAA GAG CCA AGG ACA GGT AC-3′	17

DNA polymerase (Promega Corporation), 1 μM each HSV primer (Table 1), and 200 μM each dNTP.

The amplified DNA sequences were electrophoresed for 90 min in 2% NuSieve 3:1 agarose (FMC Bioproducts, Rockland, Maine) at 6.7 V/cm in 0.89 M Tris HCl containing 0.89 M boric acid, 0.002 M EDTA, and 0.5 μg of ethidium bromide per ml (18). Amplified DNA bands were detected visually with UV transilluminator (Fisher Scientific, Pittsburgh, Pa.).

Inhibitor assay. The PCR inhibitor assay consisted of the HSV PCR master mixture described above and 5,000 50% tissue culture infectious doses ($TCID_{50}$s) of an HSV type 2 (HSV-2) clinical isolate. After the potentially inhibitory substances were added to the reaction mixture, water was added to produce a 100-μl final volume. Thermal cycling was done as described above. Amplified DNA sequences were electrophoresed as previously described, and DNA bands were detected visually with a UV transilluminator. The absence of the 476-bp HSV nucleic acid band indicated the presence of a PCR inhibitor.

RESULTS

Effect of increasing concentrations of vitreous and aqueous fluids. Our initial attempts to amplify HSV sequences from aqueous and vitreous fluids by the method described by Fox et al. (5) were unsuccessful and closely paralleled the experiences described by Limpens and Kijlstra (10). Aqueous and vitreous fluid specimens spiked with 500 to 50,000 $TCID_{50}$s of HSV-2 and boiled for 15 min could not be amplified by PCR. Positive control specimens without eye fluids produced the expected 476-bp amplification products after identical treatments (data not shown). Follow-up experiments were done to determine if the PCR inhibition was dependent upon the concentration of aqueous or vitreous fluid in the PCR mixture. In these experiments, 0.5, 1.0, 2.0, 5, 10, and 20 μl of normal vitreous or aqueous fluid were added to the HSV inhibitor assay as described above. The results (Fig. 1) indicate that as little as 0.5 μl of vitreous and 20 μl of aqueous fluid completely inhibited PCR product formation. These results were not patient specific, as the same results (±1 dilution) were obtained with 10 normal aqueous specimens (10 different patients) and 12 normal vitreous specimens (12 different patients).

Specificity of PCR product inhibition. Because our initial experiments were performed with a single virus and primer set, we could not rule out the possibility that the inhibition could be primer or virus specific. To address this issue, PCR inhibition assays were performed with human β-globin and VZV target DNA. For the β-globin assay, human leukocytes were purified by Ficoll-Hypaque banding (22), extracted with phenol and phenol-chloroform, and precipitated with ethanol. The extracted leukocyte DNA (6,800 leukocyte equivalents) was amplified as described above. In the VZV PCR, a clinical VZV isolate was passaged in CV-1 cells and the cultures were extracted with phenol-chloroform. The VZV PCR mixture contained 134 focus-forming units of VZV. PCRs were performed in duplicate; 5 μl of untreated vitreous fluid was added to one reaction mixture and 5 μl of water was added to the second reaction mixture. The PCR conditions are described above. All of the positive control reactions (no vitreous fluid) amplified DNA normally and produced the expected PCR products (Fig. 2). However, the HSV, β-globin, and VZV reactions were completely inhibited by 5 μl of vitreous fluid. These results suggest that the PCR inhibition was not primer or virus specific but rather, the inhibitor acted independently of the DNA sequence being amplified.

Effect of extraction methods. To determine the effect of selected sample preparation protocols, a single vitreous specimen was spiked with 5,000 $TCID_{50}$s of HSV-2 and divided into two aliquots. One aliquot was boiled for 15 min while the other specimen was extracted once with chloroform-isoamyl alcohol (24:1) (18). Ten microliters of the resulting nucleic acid preparations was added to PCR master mixtures, and temperature cycling was done as previously described. The boiled (15 min) HSV positive control, which contained no vitreous fluid, produced the expected 476-bp PCR product. However, the PCR mixture containing the boiled vitreous fluid was completely inhibited, and no PCR product was observed. The PCR mixture containing chloroform-extracted vitreous fluid amplified DNA normally.

Nuclease activity check. PCRs can be inhibited if the specimen contains nucleolytic activity. Nucleases disrupt PCRs by

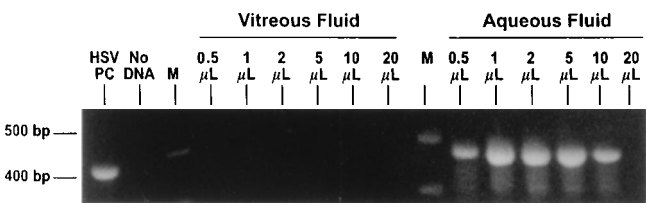

FIG. 1. Effect of increasing concentrations of vitreous and aqueous fluids on PCR product formation. Increasing volumes of normal vitreous or aqueous fluid were added to an HSV PCR master mixture containing 5,000 $TCID_{50}$s of HSV. Intraocular fluid was not added to the HSV-positive control (HSV PC). Lanes M, molecular size markers.

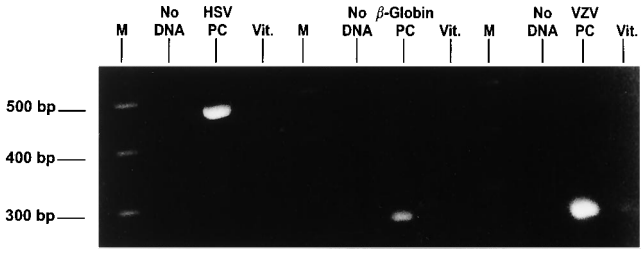

FIG. 2. Specificity of PCR product inhibition. To determine if the inhibitory effects were primer or agent specific, PCR inhibition assays were performed with 5,000 $TCID_{50}$s of HSV-2, 6,800 leukocytes for human β-globin, or 134 focus-forming units of VZV. Nucleic acids were amplified in the presence (Vit.) or absence (PC) of vitreous fluid. Lanes M, molecular size markers.

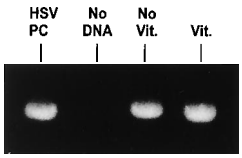

FIG. 3. Nuclease testing. To determine if the PCR inhibitor acted as a nuclease, duplicate HSV PCR master mixtures containing 5,000 $TCID_{50}$s of HSV-2 were prepared without *Taq* polymerase. Five microliters of vitreous fluid was added to one tube (Vit.), and 5 μl of water was added to the second tube (No Vit.). Both tubes were incubated at 37°C for 1 h, extracted with phenol-chloroform-isoamyl alcohol (25:24:1), and the DNA was precipitated with ethanol. The DNA pellet was suspended in 100 μl of fresh PCR master mixture containing *Taq* polymerase, and the nucleic acids were amplified as described previously. The untreated HSV-positive control (HSV PC) contained no vitreous fluid.

degrading target nucleic acids, PCR primers, and/or PCR products. To determine if vitreous fluids contain a thermostable nuclease, duplicate PCR master mixtures (without *Taq* polymerase) containing HSV primers and 5,000 $TCID_{50}$s of HSV-2 target DNA were prepared and 5 μl of untreated vitreous fluid was added to one reaction mixture and 5 μl of distilled water was added to the other. The mixtures were incubated for 1 h at 37°C. The potential inhibitor was removed by phenol-chloroform extraction, and the nucleic acids were precipitated in the presence of glycogen and ethanol. DNA pellets were solubilized in a fresh PCR master mixture containing *Taq* polymerase and subjected to thermal cycling as described above. No PCR inhibition was observed in the control tubes or in the tubes containing vitreous fluid (Fig. 3). These results indicate that the PCR inhibition was not due to degradation of the nucleic acids in the reaction mixture.

Effect of increasing magnesium concentrations. PCRs can also be inhibited by chelating divalent cations such as Mg^{2+}. To determine if the observed inhibition was magnesium dependent, increasing concentrations of $MgCl_2$ (2.5, 5, 7.5, 19, 12.5, and 15 mM) were added to duplicate HSV PCR mixtures. Five microliters of vitreous fluid was added to one set of tubes, and 5 μl of water was added to the other set. PCR amplifications were done as described above. The results (Fig. 4) indicate that increasing magnesium ion concentrations reduced the amount of PCR product, and no PCR product was detectable in the reaction mixture containing 15 mM $MgCl_2$. The inhibitory effect of vitreous fluids did not appear to be magnesium dependent because the presence of 0.5 to 10 times the normal $MgCl_2$ concentrations failed to rescue the PCR activity of specimens containing 5 μl of vitreous fluid.

Effect on other thermostable polymerases. Several recent articles have demonstrated that many substances can directly inhibit the activity of the thermostable DNA polymerase (1, 2, 4, 7–9, 11, 15, 20). We tested this possibility by performing

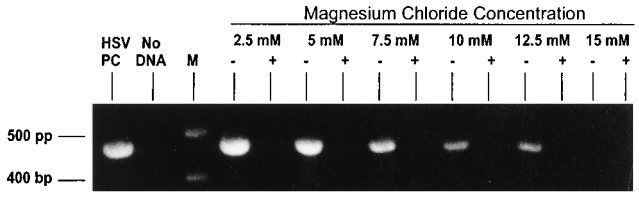

FIG. 4. Effect of increasing magnesium concentrations. Duplicate PCR mixtures were established with an HSV PCR master mixture containing 5,000 $TCID_{50}$s of HSV and increasing magnesium ion concentrations. Five microliters of vitreous fluid was added to one set of tubes (+), and 5 μl of water was added to the remaining tubes (−). The untreated HSV-positive control (HSV PC) contained 2.5 mM $MgCl_2$. M, molecular size markers.

PCR inhibition studies with several thermostable DNA polymerases. A single vitreous specimen was diluted 1:2 to provide sufficient volume for this testing. Increasing volumes of vitreous specimen were added to appropriate PCR master mixtures containing *Taq* DNA polymerase, the Stoffel fragment of *Taq*, *Tli* DNA polymerase, *Tth* DNA polymerase, or *Tfl* DNA polymerase. The results (Fig. 5) indicate that the Stoffel fragment was more sensitive to the inhibitory effects of the vitreous fluid than the intact *Taq* polymerase. Vitreous fluid inhibited the *Tli* polymerase reaction in a dose-dependent manner and 20 μl of vitreous fluid was sufficient to completely inhibit this reaction. In contrast with these observations, the *Tth* and *Tfl* DNA polymerase reactions were completely resistant to the inhibitory effects of 20 μl of vitreous fluid. These results suggest that the resistance of DNA polymerases can vary significantly from species to species. A recent report by Katcher and Schwartz (8) provides further support for this observation. These authors have shown that *Tth* DNA polymerase can function normally in the presence of 5% phenol, whereas traces of phenol completely inactivate *Taq* polymerase.

DISCUSSION

PCR has been used to detect infectious agents in a wide variety of ocular specimens. While much attention has been directed toward minimizing false-positive reactions resulting from specimen contamination or amplicon carryover, relatively little attention has been given to the causes of false-negative PCRs (3). The elimination of false-negative PCRs is extremely important because negative test results imply that the organism in question is not responsible for the patient's illness. False-negative results can influence therapeutic decisions and may cause the clinician to alter or withhold antibiotic or antiviral drugs. The medical consequences of false-negative PCR results are especially serious for ocular diseases because serological and culture results are often unrewarding (21).

False-negative reactions can occur through a number of mechanisms. Specimens that contain EDTA can chelate divalent cations such as Mg^{2+} that are necessary for the PCR. The presence of RNase or DNase can also disrupt PCRs by degrading nucleic acid targets and/or primers. However, direct inhibition of the DNA polymerase is the best-known mechanism of PCR inhibition. Several recent articles have shown that heme (11, 15), heparin (2, 7), phenol (8), polyamines (1), plant polysaccharides (4), urine (9), and calcium alginate (20) inhibit PCRs in this manner.

Conventional methods for removing inhibitory substances and extracting nucleic acids from tissues include proteinase K digestion(s) followed by multiple phenol and chloroform-isoamyl alcohol (24:1) extractions (18). The resulting nucleic acids are precipitated in the presence of salts and cold ethanol. The DNA pellet is washed with cold 70% ethanol to remove any contaminants, dried, and dissolved in a suitable buffer system for the ensuing procedures. While this lengthy extraction procedure completely removes the inhibitory effects of aqueous and vitreous fluids (data not shown), many laboratories use abbreviated extraction protocols (5) that may not remove the inhibitory substances. Abbreviated procedures that employ boiling steps have no effect on the inhibitory activity of intraocular fluids. In contrast, a single chloroform-isoamyl alcohol (24:1) extraction will completely remove the inhibitory effects of these specimens. Therefore, nucleic acid extraction methods for intraocular fluid specimens should include at least one chloroform-isoamyl alcohol (24:1) extraction.

Aqueous and vitreous fluid specimens contain a heat-stable PCR inhibitor. While the specific inhibitory substance(s) have

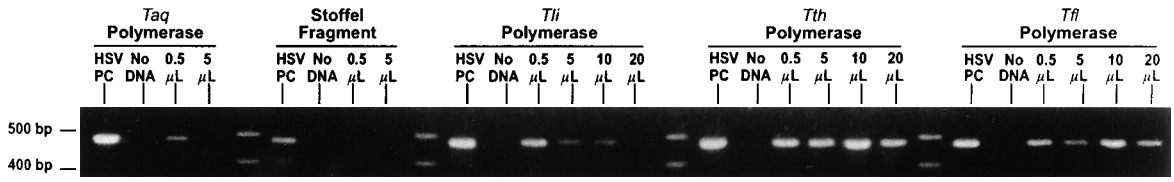

FIG. 5. Effect on other thermostable polymerases. The inhibitory effect of vitreous fluids on other thermostable DNA polymerases was tested by adding an increasing volume of vitreous fluid directly into HSV PCR master mixtures containing *Taq* DNA polymerase, the Stoffel fragment of *Taq*, *Tli* DNA polymerase, *Tth* DNA polymerase, or *Tfl* DNA polymerase. HSV PC, HSV-positive control.

not been identified, we have determined that normal vitreous fluids contain at least 40 times more inhibitory activity than aqueous fluids. We can hypothesize that the inhibitor may be present in higher concentrations in vitreous fluids than in aqueous fluids or that vitreous fluids may contain an entirely different inhibitor. The vitreous inhibitor does not appear to act as a nuclease or a chelator of magnesium ions but rather seems to act directly on the *Taq* polymerase. It was interesting that *Tth* and *Tfl* DNA polymerases were completely resistant to the inhibitory effects of vitreous fluids. Therefore, laboratories performing PCRs on intraocular fluids may prefer to use *Tth* DNA polymerase rather than *Taq*. The use of *Tth* DNA polymerase has a number of advantages over the use of *Taq*. Katcher and Schwartz (8) recently reported that the *Tth* polymerase is extremely hardy and can function in the presence of 5% phenol whereas *Taq* polymerase can be inactivated by a trace of phenol. In addition, the thermostable *Tth* DNA polymerase can act as a reverse transcriptase or a DNA polymerase at elevated temperatures (12, 16). This activity can be especially useful during testing for RNA viruses that have extensive secondary structures at lower temperatures (12).

The inhibitory effects of intraocular fluids are concentration dependent. Therefore, PCR inhibition can be eliminated by diluting aqueous fluids 1:4 and vitreous fluids 1:50 in a suitable buffer. While specimen dilution provides an inexpensive mechanism for eliminating PCR inhibition, dilution procedures can also contribute to false negative PCRs. Our observations and those of Limpens and Kijlstra (10) suggest that the specimens used in the study by Fox et al. (5) may have been diluted during specimen collection.

In summary, aqueous and vitreous fluids contain a heat-resistant inhibitor of *Taq* polymerase. The inhibitory effects of intraocular fluids can be eliminated by dilution of the specimen, by chloroform extraction, or by the use of *Tth* or *Tfl* DNA polymerases. These studies also indicate that abbreviated extraction protocols may contribute to false-negative PCR results.

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