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An improved technique for the diagnosis of viral retinitis from samples of aqueous humor and vitreous

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Abstract. We applied the technique of DNA amplification with the polymerase chain reaction to nine aqueous humor and five vitreous samples from HIV-1-infected patients with clinically diagnosed cytomegalovirus retinitis. For the amplification, recently published primers specific for herpes simplex virus (HSV), varicella zoster virus (VZV) and cytomegalovirus (CMV-1) were used. Additionally, a newly developed primer pair specific for the main immediately early gene of CMV (CMV-2) was selected and compared with the published one. All primers were tested on noninfected and HSV-, VZV- and CMV-infected human fibroblast cell culture supernatant, thereby excluding cross-reactivity of the chosen primers. In none of 13 aqueous humor and six vitreous samples of healthy controls was any viral DNA amplified. Using the CMV-1 primers, we detected CMV DNA in five of nine aqueous humor and three of five vitreous samples amplifying a DNA fragment 435 base pairs in length. With the CMV-2 primers, we detected a CMV DNA fragment with a length of 110 base pairs in eight of nine aqueous humor and in four of five vitreous samples. Additionally, CMV DNA was found in three of nine urine and two of nine saliva specimens. Both CMV and HSV DNA were amplified in one aqueous sample. Varicella DNA was not detected in any of the specimens. Thus, the polymerase chain reaction is more sensitive than other comparable diagnostic tests and may provide an alternative to conventional virus isolation and in situ hybridization techniques for the laboratory diagnosis of viral ocular disease.

Introduction

The proportion of HIV-infected patients reaching advanced stages of AIDS is increasing [1]. Thus, viral retinitis has been found in up to 25% of AIDS patients [2–4], while various herpesviruses, i.e. herpes simplex virus (HSV) types 1 and 2, varicella zoster virus (VZV) and cytomegalovirus (CMV), have been shown to cause retinal disease [1, 5–10].

Regardless of the virus species involved, retinal disease can show similar clinical profiles. Therefore, an etiologic diagnosis based on ophthalmoscopic observations alone may not be possible. However, an exact etiologic diagnosis is essential for therapeutic and prognostic purposes [11–13].

Antibody titers in systemic viral infections follow a typical time course in immunocompetent individuals, but may not do so in isolated ocular disease [10, 14, 15]. Viral retinitis reflects a severe depression of the immune response [15]. Consequently, negative serological tests for antiviral antibodies do not exclude infection. On the other hand, increased serum antibody levels may be due to nonspecific B-cell stimulation [16]. As an alternative, the aqueous antibody level may be measured. However, the uveal antibody production shows a wide interindividual range [14, 17, 18]. The presence of antibodies in aqueous and vitreous humors additionally depends on the integrity of the blood-uveal barrier [14, 17]. Finally, local antibody levels represent only an indirect measure of the disease. They are dependent on the two factors of barrier destruction and specific local immune reaction to the disease. The varying relation of these factors does not allow a reliable interpretation of the findings [14].

Using the polymerase chain reaction (PCR) technique, we tested aqueous and vitreous humor samples for the presence of viral DNA in order to identify the etiologic agents.

Patients and methods

Patients

Aqueous humor was collected from nine patients (nos. 1–9) and vitreous humor from five patients suffering from AIDS diagnosed as having fresh CMV retinitis (nos. 10–14). Since mild conjunctivitis is a common symptom of these patients [3, 4], we collected

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corneal and conjunctival epithelia from the first nine subjects. Additionally, serum, urine and saliva samples were collected.

Aqueous samples from 13 patients undergoing cataract surgery (nos. 15–27) served as controls.

Methods

DNA was extracted from samples as described by Shibita et al. [19], i.e. the samples were heated to 95° C in a 10 mM Tris buffer (pH 8.0) for 15 min. The supernatant was used for the PCR.

The primers used in our experiments were selected according to published sequences of the expected DNA [20] and synthesized using the beta-cyanoethyl method on an Applied Biosystems 381A DNA Synthesizer. The purification was carried out by high-performance liquid chromatography using a reverse-phase column (Aquapore 300, Applied Biosystems, Weiterstadt, Germany) [21–24].

Different primers were designed or modified as described by Demmler et al. (CMV-1) [21] and Fenner et al. (CMV-2) [22] for the CMV PCR (Fig. 1), Cao et al. [23] and Aurelius et al. [24] for the HSV PCR and Kido et al. [25] for the VZV PCR (Table 1). Two of them had been tested before in cases of acute retinal necrosis syndrome [18, 22].

The amplification mixture contained 75 pM of each primer and 200 µM of each dNTP in 10 mM Tris (pH 8.3), supplemented with

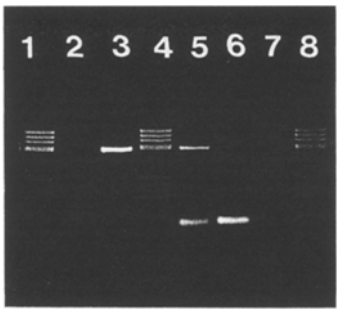

Fig. 1. In a 4% agarose gel two different CMV DNA bands 435 and 110 base pairs in length obtained by 30 amplification cycles are visible. *Lanes 1, 4, 8*, DNA molecular weight marker V, pBR322 DNA (Boehringer Mannheim, Germany); *lane 2*, buffer control; *lane 3*, band of 435 bp in length (CMV-1 primers); *lane 5*, amplification using all four primers in one PCR (two amplification products 110 and 435 bp in length are detected); *lane 6*, band of 110 bp in length (CMV-2 primers); *lane 7*, negative control

50 mM KCl, 2.5 mM $MgCl_2$, 0.02% (wt/vol) gelatin and 2 units of Taq polymerase (Perkin Elmer Cetus, Emeryville, Calif., USA) diluted to a final volume of 50 µl with distilled water. The PCR was performed as follows [26, 27]: DNA samples were heated to 95° C for 5 min for denaturation of the DNA. Then 30 amplifica-

Table 1. Primers

Primers	DNA sequence (from 5′ to 3′)	Product length (bp)	Amplified region
Primers for CMV-DNA			
CMV-1 [22]			
MEE-6	CCAAGCGGCCTCTGATAACCAAGCC	435	1142–1166
MEE-7	CAGCACCATCCTCCTCTTCCTCTGG		1576–1552
Probe	GAGGCTATTGTAGCCTACACTTTGG		1312–1336
CMV-2 [21]			
MIE-5′s	AGTGTGGATGACCTACGGGCCATCG	110	1267–1291
MIE-3′s	GGTGACACCAGAGAATCAGAGGAGC		1376–1352
Probe	GAGGCTATTGTAGCCTACACTTTGG		1312–1336
Primers for VZV-PCR			
VZV-1 [18]			
VZV-11	CAGTTCATCCGCAGACTCCAACGC	126	3260–3283
VZV-12	TACGGACATGAACTTTATCGTACC		3362–3385
Probe	GACTCTCTCACGCAACGCGCGGGCAATACG		3308–3337
VZV-2 [25]			
TS1	ACGATTATTACCGGTACCATGGGAG	959	18422–18446
TS2	CAACACATCTACTGTCTTGACAACA		19383–19359
Probe	AGCTGCAAACTGTTATAGATACAAT		18883–18907
Primers for HSV-PCR			
HSV I [23]			
	CATCACCGACCCGGAGAGGGAC	92	
	GGGCCAGGCGCTTGTTGGTGTA		
Probe	GTCCTCACCGCCGAACTGAGCAGACA		3434–3459
HSV II [24]			
BJHSV1.1	ATCACGGTAGCCCGGCCGTGTGACA	220	19–43
BJHSV1.2	CATACCGGAACGCACCACACAA		239–218
Probe	TACGAGGAGGAGGGGTATAACAAAGTCTGT		96–125

CMV, cytomegalovirus; HSV, herpes simplex virus; PCR polymerase chain reaction; VZV, varicella zoster virus

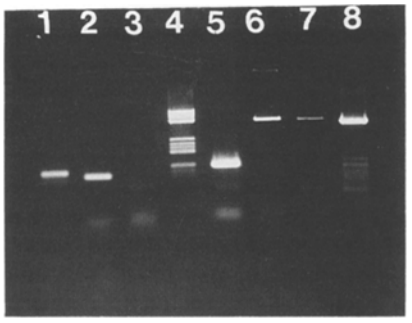

Fig. 2. In a 3% agarose gel (2.5% NuSieve, 0.5% SeaKem, Biozym, Hameln, Germany) the different PCR amplificates are seen. The different fragments were detected by ethidium bromide staining after gel electrophoresis. *Lane 1*, 126-bp fragment = VZV*; *lane 2*, 92-bp fragment = HSV**; *lane 3*, only primers; *lane 4*, DNA molecular weight marker V, pBR322 DNA (Boehringer Mannheim, Germany); *lane 5*, 110-bp fragment = CMV-2***; *lanes 6–8*, 435-bp fragment = CMV-1****. * (PCR protocol and primers of Garweg et al. [18]). ** (PCR protocol modified from Cao et al. [23]). *** (PCR protocol of Fenner et al. [22]). **** (PCR protocol of Demmler et al. [21])

tion cycles were performed. Every cycle consisted of 30 s at 95° C for denaturation, 30 s at 58° C for the annealing of the primers and 90 s at 72° C for the elongation of the amplificates. The same amplification mixture and protocol were used for all amplification experiments, except that for HSV DNA amplification 35 cycles were run (Fig. 2).

To exclude any unspecific DNA amplification, controls using DNA from human embryonal lung fibroblasts were performed in all amplification experiments. Two micrograms of DNA of a line with noninfected cells served as a negative control. The same cell line infected with clinical isolates of human CMV, HSV type 1 and VZV was used for positive controls. In order to exclude a contamination of reagents with DNA from one of the viruses, a buffer control without sample DNA was also run in every amplification experiment. Different rooms were used for handling of samples, for amplification and for preparation of the oligonucleotide primers and PCR buffers.

To exclude cross-reactivity of the primers with DNA of the different viruses or human material, an amplification of each of the virus-infected cell cultures was performed with every primer set. The specific amplified DNA fragments were detected and visualized by liquid hybridization with ^{32}P end-labeled oligonucleotides (probes in Table 1). For the hybridization 10 µl of the amplified DNA sample and 2×10^5 cpm of the labeled oligonucleotides complementary to one of the amplified strands were incubated with 0.15 M NaCl solution at 56° C for 30 min. The samples were loaded onto a 15% polyacrylamide gel (mini electrophoresis chamber, Biorad, Munich, Germany). Electrophoresis was run for 25 min. Bound probe was visualized autoradiographically by exposition to an X-ray film at −70° C for 8–12 h [22, 28]. All results were verified in two control experiments. A sample was regarded as positive if identical bands were seen in three independent amplification experiments.

Results

In our controls we did not detect any target DNA (HSV, VZV, CMV) in uninfected cells or buffer controls, whereas the positive controls (virus-infected cell cultures) showed broad and specific amplification bands (Fig. 2). No cross-reactions between the virus isolates were observed.

The molecular weights of the amplified DNA fragments were compared with a routinely running molecular weight standard [marker V (pBR322 DNA·HAE III), Boehringer Mannheim, Germany; Fig. 2, lane 4]. This agrees with the expected molecular weights.

After DNA amplification as described by Demmler et al. [21] (CMV-1) CMV DNA was detected in five of nine aqueous taps and in three of five vitreous samples. The aqueous humor and vitreous samples of all healthy controls were negative. Sensitivity is therefore 57.1%. There were no false-positive results, indicating specificity of up to 100%.

Using the method published by our group [22] (CMV-2), we detected CMV DNA in eight of nine aqueous humor samples, indicating sensitivity of 88.9% and specificity of 100%. In two of these cases we detected specific DNA additionally in urine and saliva, in one further case only in urine (Fig. 3). In four of five vitreous samples we detected CMV DNA (sensitivity 80%, specificity 100%). Comparing the DNA amplification from both vitreous and aqueous samples with the clinical diagnosis, there is overall sensitivity of 85.4% and specificity of 100%. Comparing the DNA amplification of aqueous humor and vitreous specimens, there is no difference in sensitivity (Fisher's exact test: $P = 0.6044$).

We did not detect VZV DNA in any case of suspected CMV retinitis (0/14) or in any sample of the healthy controls (0/19).

Using the primers published by Cao et al. [23], HSV DNA and CMV DNA were found in two aqueous humor specimens (patients 5 and 9) and one vitreous specimen (patient 11). In none of the other samples was HSV

Table 2. Detection of CMV-DNA in the different samples of 14 patients with cytomegalovirus retinitis

Number of patients	Aqueous humor	Vitreous	Corneal epithelium	Conjunctival epithelium	Urine	Saliva
5	+	n.t.	−	−	−	−
2	+	n.t.	−	−	+	+
1	+	n.t.	−	−	+	−
1[a]	−	n.t.	−	−	−	−
4	n.t.	+	n.t.	n.t.	n.t.	n.t.
1[b]	n.t.	−	n.t.	n.t.	n.t.	n.t.

n.t., Not tested

[a] After 3 months of antiviral therapy with ganciclovir

[b] Final state with progression of retinitis during 7 months' antiviral therapy with ganciclovir, total retinal necrosis

Table 3. Results of the PCR experiments from aqueous humor and vitreous

Patient number	Diagnosis	PCR results for		
		CMV	VZV	HSV
1	CMV Retinitis, chorioretinitis	+	−	−
2	CMV Retinitis	−	−	−
3	CMV Retinitis	+	−	−
4	CMV Retinitis, state after toxoplasmosis[a]	+	−	−
5	CMV Retinitis	+	−	+
6	CMV Retinitis	+	−	−
7	CMV Retinitis	+	−	−
8	CMV Retinitis	+	−	−
9	CMV Retinitis	+	−	−
10	CMV Retinitis	−	−	−
11	CMV Retinitis, vitreitis	+	−	−
12	CMV Retinitis	+	−	−
13	CMV Retinitis	+	−	−
14	CMV Retinitis	+	−	−
15–27	Noninfected controls, aqueous	−	−	−
28–33	Noninfected controls, vitreous	−	−	−

[a] Patient 4 had suffered an episode of ocular toxoplasmosis. At that time urine culture was positive for CMV. CMV DNA was detected using PCR in saliva but not in aqueous humor. At the time of CMV retinitis, CMV DNA was detected in aqueous humor, saliva and urine

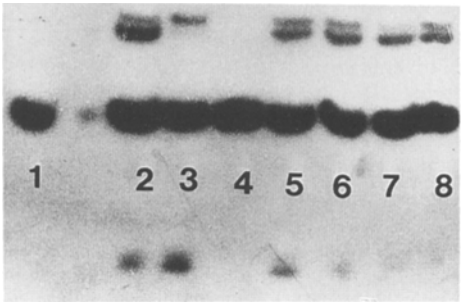

Fig. 3. The specific amplified DNA fragment was detected by liquid hybridization with a ^{32}P end-labeled oligonucleotide. The broad lower bands contain the nonbound radioactivity. *Lane 1*, buffer control; *lane 2*, CMV-positive control; *lane 3*, CMV-positive vitreous specimen; *lane 4*, negative control; *lanes 5, 6*, CMV-positive aqueous humor; *lane 7*, CMV-positive urine; *lane 8*, CMV-positive saliva

DNA detected. Therefore, HSV DNA amplification was controlled using a system modified from Aurelius and coworkers [24] (Table 1). In this experiment HSV-1 DNA was detected in only one sample (patient 5).

We did not detect viral DNA in conjunctival and corneal epithelium in any of the tested samples (0/9). Contamination of aqueous with viral DNA from the epithelium was thus excluded.

Discussion

Usually serologic and virologic tests fail to identify the etiologic agent in viral ocular disease [14, 17]. This diagnostic problem may be overcome by the amplification and visualization of viral DNA from ocular fluids with the PCR method [18, 22, 29–31]. This method detects DNA in quantities smaller than 50% of the amount that can be detected by cell culture [21, 23, 28]. The small sample volume needed for analysis is a second advantage of this method [22, 26, 27]. However, the method can be hampered by secondary DNA microcontamination or unspecific background amplification [32, 33], which was ruled out here by running appropriate controls in every amplification experiment.

We were able to demonstrate CMV DNA in eight of nine aqueous humor and four of five vitreous samples from patients with CMV retinitis by amplification of a DNA fragment, 110 base pairs in length, of the main immediate early gene of CMV. These results establish the PCR as a powerful tool for the laboratory diagnosis of CMV retinitis from aqueous and vitreous samples, equivalent to clinical diagnosis.

Cytomegaloviral DNA was detected in 30% of urine and saliva samples. This is not surprising, as there are only short phases of viremia and viruria in systemic CMV disease [21, 34]. There were no false-positive results, whereas in two retinitis cases (one aqueous and one vitreous specimen) CMV DNA was not detected. CMV DNA was not detected in ocular epithelial surface cells. This may indicate that conjunctivitis of these patients is mainly of unspecific origin, in contrast to published single case reports of CMV conjunctivitis [4]. Our data regarding sensitivity and specificity of CMV DNA detection with the PCR are supported by published data [27, 34, 35]. The results are in agreement with the findings of Olive et al. [34, 35] and Jiwa et al. [36].

In five of nine aqueous samples we detected CMV DNA amplifying a segment described by Demmler et al. [21]. The sensitivity of the method was improved by amplifying a smaller DNA product of only 110 base pairs [22]. A reduction of specificity, which seemed to be a major problem in other series [30, 37], was not observed. The first trial systematically investigating the sensitivity and specificity of PCR by blinded testing of

blood samples in laboratories with extensive experience in PCR testing was published recently. In five laboratories, average sensitivity of 99% and a specificity of 94.7% were found for the amplification of HIV-1 proviral DNA. After analysis of misclassifications the sensitivity of PCR was above 95% and specificity varied between 90% and 100%, mainly due to laboratory errors due to nonstandardized PCR techniques. Therefore the authors emphasize the need for standardization of PCR testing [37].

In contrast to the PCR system described by Fox et al. [30], we chose another DNA fragment and did not find false-positive results, which is an improvement [32, 33, 37]. On the other hand, we were not able to detect target DNA in two samples. This reduced sensitivity may be related to the absence of a verifying system for the clinical diagnosis or may be specific to the amplified DNA segment [37]. The main immediate early region of the CMV genome is a preserved region of the genome. Thus, strain-specific differences should not be the explanation for a negative result.

All samples were tested for the presence of DNA of other viruses of the herpesvirus family. We found no cross-reaction between CMV and HSV in our positive control series (virus-infected cell cultures). Nevertheless, in three ocular fluid samples of patients with the clinical picture of CMV retinitis we found two amplification products of CMV DNA and HSV DNA using the primers published by Cao et al. [23]. Most likely these results have to be interpreted as cross-reactions between HSV and CMV genome [20, 38]. Unspecific background amplification is another possibility [26, 32]. A PCR product carryover [33] was excluded by the two control amplification experiments with identical results and by buffer controls, which were always negative. Double infection with HSV and CMV is also possible. Such was found by Pepose et al. [1] in one in a group of 19 cases of viral retinitis via immunohistochemistry, but seems unlikely to occur in three of 14 cases.

Thus, using the primers of Cao et al. [23], PCR must be expected to be less useful to detect HSV in retinitis patients. Using a pair of primers that amplifies a different region of the glycoprotein D gene [24], HSV DNA was detected in only one of these samples, which seems more likely.

A central problem arises from the lack of established diagnostic control systems for PCR investigations [28, 37, 39]. The standard for comparison used is the clinical diagnosis. However, a clinical diagnosis may not be reliable enough for use in assessing the sensitivity and specificity of a laboratory method [28, 30]. We do not know the causative agent in the cases of negative or double amplification. Furthermore, in systemic CMV disease without ocular involvement, CMV DNA might be detected in aqueous humor with the PCR. These two limitations should be kept in mind when using the terms "sensitivity" and "specificity" here. Several attempts have been made to detect local antibody production for confirmation of the diagnosis [40]. Antibody investigations are well established, sensitive and specific. They are quickly and easily performed and of low cost. To date, however, the time course of the development of a local humoral immune response is not known. Furthermore, as mentioned above, in AIDS patients there may be unspecific B-cell stimulation as well as the total absence of a specific immune response. Therefore these investigations do not necessarily lead reliably to the diagnosis, as has been shown in systemic CMV disease [14, 16, 18].

In conclusion, there is no in vivo method to demonstrate the causative agents of viral retinitis except the endoretinal biopsy [41], which is rarely indicated. In 12 of our 14 cases, the results of DNA amplification corresponded with the clinical diagnosis in patients with CMV retinitis. The polymerase chain reaction is thus an alternative to established diagnostic tests in the laboratory diagnosis of CMV ocular disease.

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