CMV retinitis in HIV-positive patients in the pre-HAART era
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CHAPTER IV

CYTOMEGALOVIRUS (CMV) STRAIN DIFFERENCES BETWEEN THE EYE AND THE BLOOD IN AIDS PATIENTS WITH CMV RETINITIS.
Objective: To investigate possible differences in cytomegalovirus (CMV) strain distribution between the eye and blood in AIDS patients with CMV retinitis.

Methods: CMV DNA sequences from aqueous humour and peripheral blood leukocytes (PBL), obtained from 13 AIDS patients with CMV retinitis, were compared. DNA was isolated and the CMV IE-1 sequence (part of the immediate early-1 gene) and the a-sequence (located in the a-region) were amplified by polymerase chain reaction (PCR). The PCR products of the a-sequence were analysed by Southern blotting for amplified fragment-length polymorphisms. The level of divergence between the a-sequences of aqueous humour- and PBL-derived CMV was studied in two patients by cloning these sequences followed by sequence analysis.

Results: CMV DNA could be detected in all aqueous humour samples and in 10 out of 13 paired blood samples. In the 10 patients, with CMV DNA detectable in both aqueous humour and PBL, seven cases showed differences between the amplified products of both compartments. Sequence analysis in two patients revealed that the aqueous humour and PBL of the same patient can harbour both identical, similar and highly divergent CMV a-sequences.

Conclusion: These results indicate that despite haematogenous spread of CMV, the eye, being a relatively shielded organ, may contain CMV strains different from those found in the blood.
INTRODUCTION

Cytomegalovirus (CMV) is the most common opportunistic viral infection in HIV-positive patients and retinitis is the most frequent clinical manifestation of CMV disease. The annual incidence of CMV retinitis is approximately 24% in patients with a CD4+ lymphocyte count below 50 x 10^9/L [1-3]. The diagnosis of CMV retinitis is based on a typical clinical ophthalmologic picture consisting of a “cheese-like” necrotizing retinitis with or without haemorrhage [4]. Additional confirmation of CMV as the causative pathogen can be made by detection of CMV DNA by polymerase chain reaction (PCR) [5-7]. Demonstration of intraocular CMV, by cell culture or shell vial assay out of vitreous samples taken from patients ante mortem, is very rarely successful [5,8,9]. To our knowledge, positive results of virus isolation or shell vial assay out of aqueous humour samples has not yet been reported.

In homosexual men, with and without concurrent HIV infection, restriction fragment-length polymorphism (RFLP) analysis of CMV isolated from blood, urine and semen has shown that infection with multiple CMV strains is relatively common. More than one strain has been demonstrated in individual patients both simultaneously and serially at different sites, such as lungs, prostate, Kaposi’s sarcoma lesions, testis, urine and semen [10-14]. Multiple strains were even found simultaneously at the same site [14].

To distinguish between different CMV strains, several PCR-based polymorphisms have been described. For example, RFLP of amplified coding sequences of CMV glycoproteins revealed at least four different genotypes for glycoprotein B and two for glycoprotein H [15-17]. The most variable part of the CMV genome described so far is the α-region, which is a non-coding region located in the L-S junction [between the long (L) and short (S) isomers] of CMV and is thought to contain signals for cleavage and packaging essential for viral replication [18]. RFLP and amplified fragment-length polymorphism (AFLP) analysis of PCR amplified α-sequences has been widely used to characterize differences between strains in epidemiological studies [19-26].

No data are available concerning analysis of CMV strains recovered from the eyes of patients with CMV retinitis. In this study we therefore compared CMV strains in paired aqueous humour and blood samples obtained from AIDS patients with CMV retinitis. In view of its hypervariability, the CMV α-sequence was used for this study to characterize possible strain differences and relatedness. Because very small differences, such as a single base-pair change, may lead to strain differentiation in RFLP analysis, we used AFLP, which can indicate more drastic variation, such as deletions and insertions.
The results indicated that CMV strain composition in the eye and blood was different based on AFLP in three out of the 10 patients in whom CMV DNA could be detected in both compartments. In four additional patients, the absence of PCR products suggested strain differences between the compartments.

**PATIENTS AND METHODS**

**Patient selection**

Between 1993 and 1997, 58 aqueous humour and paired blood samples of AIDS patients with retinitis were analysed for diagnostic reasons. In several cases, sufficient sample material was still available for further analysis. Patients were included in this study when there was sufficient sample material stored to be analysed and the immediate early (IE)-1 sequence could be detected in the aqueous humour sample. In addition, patients had to have a definite diagnosis of CMV retinitis. A definite clinical diagnosis of CMV retinitis was based on the typical clinical ophthalmologic findings and a favourable response to conventional (re) induction and maintenance therapy with ganciclovir. Thirteen AIDS patients with CMV retinitis diagnosed between 1993 and 1997 were included in this study.

The mean age of the patients was 40 years (range, 27-49 years). All patients were homosexual men. AIDS diagnosis preceded the occurrence of CMV retinitis with a mean of 2.5 years (range, 1-6 years), apart from one patient, whose AIDS-defining diagnosis was CMV retinitis. The AIDS-defining diagnosis was *Pneumocystis carinii* pneumonia in eight cases, Kaposi’s sarcoma in two, central nervous system toxoplasmosis in one, *Candida* oesophagitis in one, and CMV retinitis in one. Mean CD4+ lymphocyte count at the time of diagnosis of CMV retinitis was 35 x 10⁶/l (range, 10-70 x 10⁶/l). Five patients were still living at the end of the study for a mean period of 5.8 months after the diagnosis of CMV retinitis (range, 2-9 months). Mean survival time after the diagnosis of CMV retinitis in the seven patients who died during the study was 9.8 months (range, 1-17 months). With exception of patients 3 and 8, all patients were diagnosed at the Department of Ophthalmology of the Academic Medical Center (University of Amsterdam). In patients 1-10, samples were collected at the first time retinitis was diagnosed, before therapy was started. In patients 11, 12, and 13 there was a 1-week delay in sample collection and patients had already been treated during that week with induction therapy consisting of ganciclovir 5 mg/kg twice daily before samples were obtained.
Cytomegalovirus strain differences.

DNA was isolated from peripheral blood leukocytes (PBL) and aqueous humour samples, as described by Boom et al. [27]. DNA was dissolved in 50–1 water. For PCR analysis, 5–1 of this solution was amplified in a volume of 50–1 containing 10 mmol/l Tris-HCL (pH 8.3), 50 mM KCl, and 1.5 mmol/l MgCl\(_2\) (for CMV IE-1 sequence) or 6 mmol/l MgCl\(_2\) (for CMV a-sequence), 40 pmol dNTP, 2.5 U Taq DNA polymerase (Ampli Taq, Perkin Elmer, Foster City, California, USA) and 10 pmol of the appropriate sense and antisense primers. The PCR reaction was performed in a thermocycler (Trioblock, Biometra, Göttingen, Germany): 5 min at 95°C and for 40 cycles (CMV IE-1 sequence) or 50 cycles at 56°C (CMV a-sequence), 30 sec at 95°C, 30 sec at 56°C (CMV IE-1 sequence) or 55°C (CMV a-sequence), and 1 min at 72°C.

The a-sequence was amplified by using 5’-TTCCCCGGGGGAATCAVACAG-3’ as the first primer and 5’-TTTTTAGCGGGGGTGAAA-3’ as the second primer [26]. The CMV IE-1 sequence was amplified using the primers 5’-GTCAGCTGAGTCTGGGAGAC-3’ and 5’-GATTCTATGCCGCACCATGTCCAC-3’ [28].

Southern blot analysis

Part of the PCR reaction volume was size-fractionated on a 3% agarose gel, denatured in 0.4 mol/l NaOH for 20 min, and blotted to Hybond N (Amersham, International, Little Chalfont, Buckinghamshire, UK) overnight. Blots were hybridized with a random primed labelled IE1-specific probe (the cloned AD 169 IE-1 PCR product), and an a-sequence-specific probe (the cloned AD 169 a-sequence). Hybridization was performed as described elsewhere [29]. After hybridization, filters were washed in 2 x SSC (0.9 M NaCl/0.09 M sodium citrate dehydrate) 1 % sodium dodecyl sulphate at 65°C for 30 min. Filters were exposed to radiographic film at -70°C. After each hybridization, short exposures were made in order to analyse doublets of strong hybridizing signals, and long exposures were made in order to clearly show the presence or the absence of relatively weak signals.

Cloning, sequence analysis, and alignment of the PCR products.

To gain further insight in the relationship between the a-sequences of CMV from the aqueous humour and PBL of the same patient, PCR-amplified a-sequences were cloned and subjected to sequence analysis. For this analysis the a-sequence of patient 2, with a PCR product of identical length in both aqueous humour and PBL and an additional longer PCR product in PBL, and the a-sequence of patient 8, with PCR products of
identical length in aqueous humour and PBL, were used. Part of the PCR reaction (1-1) was used to ligate the amplified $a$-sequence in pGEM-T (Promega, USA). Transformants were screened for the presence of the $a$-sequence by colony hybridization using the $a$-sequence specific probe. Plasmid DNA of positive clones was isolated and digested with $PvuII$ to confirm the presence of an insert. At least 10 clones from each sample were analysed for significant sequence differences using the Sequenase version 2.0 DNA sequencing kit (Amersham). $a$-Sequences were aligned using a multiple sequence alignment program [30].

RESULTS

In 10 out of 13 patients (1-10), CMV DNA could be demonstrated in both the aqueous humour and PBL by the presence of the IE-1 or $a$-sequence-specific PCR products, or both. In the other three patients (11-13), CMV DNA could only be detected in the aqueous humour (Fig. 1).

In seven patients (patients 1-7), CMV PCR products of aqueous humour and PBL were found to be different. In the first three patients (patients 1-3), amplification of the $a$-sequence produced two fragments of different length in one compartment, whereas only one fragment was present in the other compartment. In patients 4 and 5 the IE-1 sequence could be amplified from both aqueous humour and PBL, whereas the $a$-sequence could only be amplified from either PBL (patient 4) or aqueous humour (patient 5). In patients 6 and 7, the IE-1 sequence could not be amplified from PBL, although CMV DNA was clearly present, as demonstrated by the PCR products from the $a$-sequence. In patient 7, the IE-1 sequence could only be amplified from the aqueous humour, whereas the $a$-sequence could only be found in the PBL. In patients 8-10, no differences were found between the CMV PCR products of the eye and blood. In patients 11-13, CMV DNA could only be detected in the eye.

To determine whether the CMV strains found in the aqueous humour and PBL of the same patient were related or highly diverged, the $a$-sequences of two patients were cloned and analysed by sequencing. For this analysis, we used $a$-sequences from patient 2, with two $a$-sequences of different length in the PBL and only one $a$-sequence in the aqueous humour, and the $a$-sequences from patient 8, with $a$-sequences of equal length in both compartments. Ten clones from each compartment were analysed. For comparison the $a$-sequence of the laboratory strain AD169 is shown in addition to the patient-derived sequences (Fig. 2). In the clones derived from the PBL of patient 2, an extended $a$-sequence, explaining the longer $a$-sequence PCR fragment, was found. The 5' part of this sequence (B2.1) was highly homologous to B1, whereas the 3' part appeared to be a partial
Cytomegalovirus strain differences.

Fig. 1 Amplified fragment length polymorphism's of Cytomegalovirus (CMV) α-sequences. Patients were numbered 1-13. E lanes indicate eye-derived CMV sequences and B lanes indicate blood-derived CMV sequences. IE-1 indicates the polymerase chain reaction (PCR) products of the Immediate Early 1 gene sequence and α-seq indicates the PCR products of the α-sequence from each patient. Only one exposure of the Southern blot analysis is shown. The position and the intensity of the various PCR fragments, as deduced from several different exposures, is schematically indicated at the right side of the autoradiograph.

duplicated sequence (B2.2), lacking nucleotides between positions 1 and 51. All α-sequences identified in the aqueous humour-derived clones from patient 2 were identical (E1) and the same sequence was identified in PBL (B3).

The α-sequence-specific PCR products from PBL and aqueous humour of patient 8 were all of similar length as analysed by Southern blot (Fig. 1). All PBL-derived clones were identical (sequence B1). In aqueous humour, the same sequence was found (E2) in addition to the highly similar sequence E1, which showed several small deletions in the 5' end compared with B1 and E2.
patient 2

B1
B2.1
B2.2
B3
E1
AD-169

patient 8

B1
E1
E2
AD-169
DISCUSSION

PCR analysis of the CMV α-sequence and IE-1 sequence of DNA extracted from aqueous humour samples and paired blood samples of AIDS patients with CMV retinitis suggested genomic differences in seven out of 10 patients.

AFLP demonstrated the presence of an additional strain in one of the compartments in patients 1-3. In patients 4-7, loss of detectable PCR product, either α-sequence or IE-1 sequence, occurred in one of the compartments. Repeating the PCR and Southern blot analysis of these four patients led to the same results (data not shown). These results indicated nucleotide variations in the CMV sequences complementary to the PCR primers that were sufficient to inhibit PCR amplification. In previous reports, others have also assumed mutations at the primer sites to be a probable cause of negative PCR results [22,23,26].

PCR analysis of the α-sequence located within the L-S junction is considered a reliable method by which to compare CMV strains for epidemiological studies [22-26]. This method makes it possible to use original clinical specimens directly, and enabled us to compare CMV strains from the eye and blood for the first time.

The CMV strains detected in the aqueous humour are most likely identical to the strains causing retinitis. False positive results are unlikely to account for the detection of CMV DNA in aqueous humour in HIV-positive patients with CMV retinitis. This is supported by two previous studies using PCR-based analysis of aqueous humour samples in the differential diagnosis of retinitis in AIDS patients [6,7]. In addition, in patients without ocular infection (n=38), and HIV-uninfected patients with intraocular infections such as acute retinal necrosis (n=16) or toxoplasmic retinitis (n=22), CMV DNA could not be detected by PCR analysis of ocular fluids in our laboratory (data not shown).

Strain differences between blood and the eye, as observed in this study, can be explained in several ways. After infection with CMV and during continuing replication, different local evolution possibly leads to strain differences between the eye and blood. Alternatively, after a first infection with CMV, infection with a new CMV strain can occur, which, although present in blood, does not reach the eye. If genomic differences develop in the course of time between the CMV strain(s) in the eye and blood, they can persist for a long period of time because the eye is relatively shielded from the blood by the presence of the blood-retinal, and blood-aqueous barrier [31].

Both mechanisms, different local evolution or reinfection with a new CMV strain, were supported by the sequence analysis of the α-sequence of patient 2. The 5' part of the partially duplicated α-sequence of clone B2127
was almost identical to the sequence of clone B1, suggesting that the strain with the B2 sequence had evolved from B1 by incomplete duplication. In vivo, strain differentiation has been described after inoculation of volunteers with the human CMV Towne strain and comparing the original strain with the strains that could be cultured afterwards [32]. The presence of highly divergent a-sequences in the same compartment, suggesting infection with different strains, was also supported by the sequence data from patient 2. The a-sequences B1/B2 and the a-sequences B3/E1 only showed high homology in the PAC-2 region, while many small deletions and insertions were present at both the 5' and 3' side of this region. This suggests that the CMV strain with the B1/B2 sequence has not (yet) reached the eye and represented a reinfection of the blood. Reinfection with a second CMV strain has been previously described in homosexual HIV-positive patients [12,13].

The sequence analysis of the a-sequences confirmed the differences between the strains, as found by AFLP analysis. The most informative restriction enzyme sites in the a-sequence for differentiation of CMV strains are MnII and BssHII [26]. Additional RFLP analysis of the cloned a-sequences using these enzymes would not have added much to the already detected strain differences found by AFLP (Fig. 2).

The absence of detectable CMV DNA in the blood of patients 11-13 was most probably due to the fact that they had already been treated with ganciclovir at the time of sampling. The rapid disappearance of detectable CMV DNA in the blood of patients treated for CMV retinitis has been reported previously [33].

Differences in the a-region found between CMV strains do not necessarily reflect differences in the remainder of the genome. The coding regions of the genome in particular are more conserved. Nevertheless, the different strains as observed in this study can have different biological properties such as tropism, virulence and drug resistance. Some CMV strains could be more ophthalmotropic, and could still be present in the eye, although absent from the blood. Additionally, determination of drug resistance of blood-derived CMV strains is therefore not necessarily representative of resistance of the intraocular CMV strain(s). Patients with progressive CMV retinitis have been reported despite standard treatment with ganciclovir, although CMV recovered from the blood of these patients showed normal sensitivity to ganciclovir [34]. Limited ocular penetration of systemically administered ganciclovir is one explanation, but strain differences between blood and eye, as demonstrated in this study, may also account for these observations.
REFERENCES:

Chapter IV


