Prevalence of Antiviral Drug Resistance in Untreated Patients with Cytomegalovirus Retinitis

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The purpose of this study was to determine the prevalence of UL97 resistance mutations in cytomegalovirus (CMV) DNA amplified from the eyes of patients with AIDS and newly diagnosed CMV retinitis. Relevant segments of the CMV UL97 gene were amplified from vitreous humor, after which restriction digest screening was performed for resistance mutations at codons 460, 520, 591, 592, 594, 595, and 603. Mutations were confirmed by DNA sequencing. Vitreous from 21 eyes with AIDS-related non-CMV viral retinitis served as negative controls. CMV DNA was successfully amplified from 195 of 204 eyes. A resistance mutation was found in only a single eye, a T→G mutation at base 1774, predicting a cysteine to glycine mutation at codon 592. The prevalence of UL97 resistance mutations in the eyes of patients with newly diagnosed CMV retinitis is very low.

Cytomegalovirus (CMV) retinitis is the most common cause of visual loss in patients with AIDS [1]. Current treatment strategies rely on the use of short-term induction therapy with anti-CMV drugs followed by long-term maintenance therapy. As experience with therapy for CMV retinitis has accumulated, it has become evident that long-term maintenance therapy is associated with increased virological resistance and diminished clinical responsiveness to systemically administered anti-CMV drugs [2]. It has been suggested that subtherapeutic drug delivery to the vitreous humor, as achieved by oral or intravenous administration, may be one cause of this problem [3].

In order to determine the rate at which drug-resistant strains of CMV appear in the eye, it is first necessary to know the prevalence of drug-resistant ocular CMV before therapy. Jabs et al. [4] took an indirect approach to this by studying CMV in the blood and urine of 49 patients with new-onset, untreated retinitis. They found a 2% prevalence of resistance to ganciclovir and a 4% prevalence of resistance to foscarnet in their study population. However, different strains of CMV may be present at different sites in the body, and these strains may have different genotypic resistance patterns [5–7]. Assessing the prevalence of drug-resistant virus in the eyes of patients with CMV retinitis would therefore be a more direct and, perhaps, more accurate approach to this problem. Unfortunately, CMV has proven difficult to propagate in culture from ocular tissues.

Recent studies on the molecular mechanisms of CMV antiviral resistance have demonstrated that single-point mutations in the CMV UL97 gene may confer resistance to ganciclovir and that mutations in the CMV polymerase gene may confer resistance to ganciclovir, foscarnet, or cidofovir [8]. We and others have demonstrated the presence of CMV UL97 mutations that may confer resistance to antivirals in the DNA of CMV isolated from the vitreous of the eyes of patients with clinically resistant CMV retinitis [7, 9]. The aim of the present study was to assess the prevalence of genotypic resistance in CMV isolated from the vitreous of patients with newly diagnosed, untreated CMV retinitis.

Patients and Methods

We studied undiluted vitreous specimens from 204 eyes of 185 patients with AIDS and newly diagnosed CMV retinitis. None of the patients had previously received systemic anti-CMV therapy.
and 72°C for the second round of PCR were 94°C for 45 s, annealing at 50°C for 45 s, and extension at 72°C for 45 s. The thermal cycling parameters for the first 5 cycles consisted of denaturation at 94°C for 45 s, annealing at 40°C for 45 s, and extension at 72°C for 45 s. This was followed by 10 cycles of denaturation at 94°C for 45 s, annealing at 50°C for 45 s, and extension at 72°C for 45 s. The final amplified products were subjected to overnight restriction enzyme digestion (table 1), were electrophoresed through polyacrylamide, and were stained with ethidium bromide. Mutations that were identified by restriction digestion were confirmed by repeat amplification and double-strand DNA sequencing.

Double-strand DNA sequencing was performed with a fluorescent dye terminator sequencing kit (AMpliTaqFS; Perkin-Elmer ABI, Foster City, CA) and an automated DNA sequencer (ABI Prism Model 377; Applied Biosystems, Foster City, CA). Amplified PCR products were purified before sequencing with use of the QIAEX II kit (Qiagen, Chatsworth, CA).

Results

We successfully amplified all 3 target regions of UL97 CMV DNA in vitreous from 195 (96%) of 204 eyes. In 186 of 195 eyes, we were able to amplify sufficient DNA copies to enable over, we used a dedicated biosafety hood, gamma ray–sterilized nonaerosolizing tips, and ultraviolet-treated tubes. To monitor for contamination and amplicon carryover, duplicate negative controls were included with each thermocycling run.

Screening of amplified CMV UL97 DNA for resistance mutations was accomplished by restriction digest analysis of amplified CMV UL97 DNA after a final short round of PCR amplification by use of intentionally mismatched oligonucleotide primers (table 1). Adapted from a protocol described by Chou et al. [11], the use of these mismatched primers allowed us to introduce specific restriction digest sites into amplified UL97 DNA for the purposes of differentiating wild-type DNA from mutant UL97 DNA and of testing the activity of the restriction enzyme used in the assays. Specifically, 3 μL of UL97 reaction product (from the nested PCR amplification described above) was added to PCR buffer containing dNTPs (50 μM each), formamide (10% final concentration), appropriate primers (table 1), and 0.5 U of Vent (exo-) in a 50-mL volume. Thermocycling parameters for the first 5 cycles consisted of denaturation at 94°C for 45 s, annealing at 40°C for 45 s, and extension at 72°C for 45 s. This was followed by 10 cycles of denaturation at 94°C for 45 s, annealing at 50°C for 45 s, and extension at 72°C for 45 s. The final amplified products were subjected to overnight restriction enzyme digestion (table 1), were electrophoresed through polyacrylamide, and were stained with ethidium bromide. Mutations that were identified by restriction digestion were confirmed by repeat amplification and double-strand DNA sequencing.

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Table 1. Rapid genetic screen for UL97 mutations: sequences, primer sets, and restriction enzymes.

<table>
<thead>
<tr>
<th>Base Codon</th>
<th>Mutations</th>
<th>Primer pairs</th>
<th>Amplicon size, base pair</th>
<th>Restriction enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>1380</td>
<td>ATG→ATT</td>
<td>5′-CGACAGCTACCGACGTCCTTTTGGCATGTT-3′</td>
<td>124</td>
<td>MluIII</td>
</tr>
<tr>
<td>1380</td>
<td>ATG→ATA</td>
<td>3′-AGTCGCACTTGGGCGTGGTG-5′</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1378</td>
<td>ATG→GTC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1560</td>
<td>CAC→CAG</td>
<td>5′-CTATCCGGATTACACAGCTCGTG-3′</td>
<td>127</td>
<td>Abl</td>
</tr>
<tr>
<td>1560</td>
<td></td>
<td>5′-3′</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1772</td>
<td>GCC→GTG</td>
<td>5′-CGGGTCGAGAAGATGCGCAT-3′ [11]</td>
<td>118</td>
<td>FseI and HaeIII</td>
</tr>
<tr>
<td>1781</td>
<td>GGC→GTC</td>
<td>5′-TTCTCGCCGAGTCTGCA-3′ [11]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1783</td>
<td>TCT–TCG</td>
<td>5′-AGTCCTCTGCCCATGCCGCGC-5′</td>
<td>112</td>
<td>MspI</td>
</tr>
<tr>
<td>1808</td>
<td>GCT→GTT</td>
<td>5′-GGACGGTAAAGTCACGCACC-3′ [12]</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NOTE: Intentional base pair mismatches are underlined.
Figure 1. Representative examples of cytomegalovirus (CMV) UL97 resistance mutations, as detected by rapid genetic screening (A–C) and autosequencing (D). A, Polyacrylamide gel electrophoresis of amplified and digested UL97 gene fragments, for the detection of resistance mutations at codons 460, 520, and 603. Lane 1, Molecular weight standards (M); lane 2, undigested 124-base pair (bp) amplicon encompassing codon 460 (U460); lanes 3 and 4, NlaIII digest patterns of wild-type (Wt) and V460 genotypes; lane 5, undigested 127-bp amplicon encompassing codon 520 (U520); lanes 6 and 7, AluI digest patterns of Wt and Q520 genotypes; lane 8, undigested 112-bp amplicon encompassing codon 603 (U603); lanes 9 and 10, MspI digest patterns of Wt and W603 genotypes. B, Polyacrylamide gel electrophoresis of amplified and digested UL97 gene fragments for the detection of resistance mutations at codons 591 and 592. Lane 1, Undigested 118-bp amplicon encompassing codons 591–595 (U591–595); lanes 2 and 3, FseI and HaeIII digest patterns of Wt genotype; lanes 4 and 5, FseI and HaeIII digest patterns of V591 genotype; lanes 6 and 7, FseI and HaeIII digest patterns of G592 genotype; lane 8, M. C, Polyacrylamide gel electrophoresis of amplified and digested UL97 gene fragments for the detection of resistance mutations at codons 594 and 595. Lane 1, Undigested 118-bp amplicon encompassing codons 591–595 (U591–595); lanes 2 and 3, HheI digest patterns of Wt and V594 genotypes; lanes 4 and 5, TaqI digest patterns of Wt and S595 genotypes; lanes 6 and 7, MseI digest patterns of Wt and F595 genotypes; lane 8, M. D. Partial sequence of CMV UL97 DNA from the vitreous humor of patient SLK. Note the T→G substitution in the first base of codon 592 resulting in a TGC→GGC alteration (underlined), thus predicting a cysteine to glycine mutation at this codon. Also note a silent C→A substitution in the third base of codon 591 (*).

Discussion

Despite aggressive antiviral therapy, the course of CMV retinitis in patients with AIDS can be relentless and can result in profound visual loss. Although many factors are likely to contribute to the progression of CMV retinitis in the face of seem-
ingly adequate therapy, the development of antiviral drug resistance clearly plays a role in this process [2, 4, 7, 9]. In a study, 27.5% of patients treated with ganciclovir for 9 months developed at least 1 resistant isolate of CMV in the blood or urine, which correlates with the development of contralateral retinitis [2].

Although the incidence of CMV retinitis in patients with AIDS appears to be in decline since the introduction of highly active antiretroviral therapy, CMV retinitis remains an important clinical problem. Patients with AIDS may fail or stop responding to highly active antiretroviral therapy, and those patients who develop CMV retinitis are living longer with their disease. Furthermore, there appears to be a recent increase in the number of cases of CMV retinitis associated with bone marrow and solid organ transplantation [12, 13]. In the current study, we have taken advantage of a relatively large set of clinical samples obtained before the recent decline in the incidence of AIDS-associated CMV retinitis to begin to study the prevalence of antiviral drug-resistant CMV in the eyes of patients before the onset of antiviral therapy. Our data indicate that the prevalence of CMV UL97 resistance mutations in the eyes of drug-naive patients is low. These findings are consistent with the reported prevalence of drug-resistant virus in the blood, urine, and semen of AIDS patients with no previous anti-CMV therapy [4, 14].

Because we were not able to amplify all of the UL97 CMV DNA target regions from all of the vitreous samples that we tested, it is possible that we missed resistance mutations in this small group of eyes (9 [4.4%] of 204 of eyes). There are several possible reasons for this. First, we have observed that the virus load in the vitreous of eyes with CMV retinitis can be quite variable. Second, 8 of 9 vitreous samples from which we had difficulty amplifying CMV DNA were from remote clinical sites, which makes it likely that there were errors in the handling and shipping of these specimens. Third, the viral DNA in the eyes of some of the patients may have had polymorphisms at targeted primer binding sites, leading to inefficient PCR amplification. Similarly, the intentional mismatching of the final set of primers in our assays inevitably results in inefficient amplification of targeted sequences. This final consideration prompted us to attempt to amplify and to directly sequence CMV DNA from all samples in which we could not detect a visible PCR reaction product by ethidium-bromide staining. With the recent decrease in cost and increase in efficiency of automated DNA sequencing, direct sequencing would now probably be a more effective way to analyze viral DNA in samples of vitreous. However, the current study was begun before we had access to affordable automated sequencing.

In the current study, we assumed that the viral DNA amplified from the vitreous is identical to that of the viral genome responsible for retinitis. However, it is possible that CMV in the vitreous and retina represent 2 different genotypic pools, either as a consequence of inflammatory breakdown of the blood-eye barrier or of surgical contamination of the vitreous from the blood or conjunctiva [15]. To minimize the chances of this, all our assays were developed using vitreous from AIDS patients with low CD4 cell counts and non-CMV retinitis as negative controls.

Clearly, the experimental approach that we took in this study may have missed some UL97 resistance mutations in our study samples. We specifically chose not to assay for the Y607 and Q520 (CAC->CAA) resistance mutations because of technical difficulties encountered in developing a rapid, yet sensitive, screen for these mutations in vitreous. Fortunately, the incidence of these mutations in resistant clinical isolates appears to be very low [11]. For obvious reasons, we also chose not to assay for UL97 mutations that have been identified in resistant clinical isolates but have yet been shown to confer ganciclovir resistance in marker transfer experiments. Because we found a very low prevalence of UL97 resistance mutations in our study population, and because most ganciclovir-resistant isolates of CMV have been associated with the UL97 mutations assayed for in our study [11], it is likely that screening for additional potential resistant genotypes in the UL97 coding region would have little effect on the outcome of our study.

Here we describe the first substantive prevalence data of UL97 resistance mutations in direct ocular specimens from untreated patients with CMV retinitis, which will enable for future objective assessment of the effect of various local and systemic treatments on the development of resistance in the ocular virus. These data may also prove useful in planning logical treatment strategies to minimize the development of antiviral drug resistance and to improve the visual outcome of patients with CMV retinitis.

References


7. Liu W, Kuppermann BD, Martin DF, Wolitz RA, Margolis TP. Mutations


