Clinical Failure of CMV Retinitis With Intravitreal Cidofovir Is Associated With Antiviral Resistance

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Objectives: To determine the incidence of clinical resistance to intraocular cidofovir injection for treatment of acquired immunodeficiency syndrome (AIDS)-related cytomegalovirus (CMV) retinitis, and to identify virologic features associated with cidofovir treatment failure.

Patients and Methods: Clinical resistance to intravitreal cidofovir was examined in 64 patients with CMV retinitis who received at least 1 injection of 20 µg of cidofovir. Histopathologic examination, culture, and polymerase chain reaction were used to detect CMV in ocular specimens. Antiviral resistance was assessed by plaque reduction assay and DNA sequencing.

Results: Clinical resistance to intravitreal cidofovir injections was identified in 3 patients (5%) and was associated with prior oral ganciclovir or intravenous cidofovir use. Ganciclovir- and cidofovir-resistant CMV isolates were cultured from 2 patients and harbored resistance-associated mutations in the UL97 and polymerase genes. Resistance mutations were also detected by direct analysis of vitreous. In 1 patient, different resistance mutations were identified in ocular vs extraocular CMV strains.

Conclusions: Clinical failure of intravitreal cidofovir ocurs infrequently, but may be associated with cidofovir-resistant CMV selected by prior ganciclovir or cidofovir treatment. Ocular CMV disease can result from a localized infection with a resistant CMV strain, and antiviral resistance may develop at a local site of infection independently from resistance that develops systemically.


CYTOMEGALOVIRUS (CMV) retinitis is the leading cause of retinal disease and blindness in patients with acquired immunodeficiency syndrome (AIDS), affecting approximately 30% to 34% of patients with absolute CD4+ T-lymphocyte counts of less than 100/µL. Treatment of CMV retinitis consists of intravenous (IV) or oral administration of ganciclovir or intravenous foscarnet sodium or cidofovir. Initially, ganciclovir or foscarnet are effective in controlling retinitis; however, retinitis will relapse in nearly all patients receiving these medications systemically, the time between relapses becoming progressively shorter. The long-term use of ganciclovir and foscarnet has been associated with the development of antiviral-resistant strains, which harbor viral DNA polymerase gene (UL54) or kinase gene (UL97) mutations. Our group initiated the use of intravitreal cidofovir for the treatment of CMV retinitis and has shown that intravitreal injections of 20 µg of cidofovir are effective in the control of newly diagnosed and previously treated CMV retinitis. In this article, we examine the incidence of clinical resistance to intravitreal injections of 20 µg of cidofovir and find antiviral-resistant CMV strains that contain both DNA polymerase and UL97 gene mutations to be associated with the clinical failure of cidofovir.

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CASE 1

A 35-year-old white man who was positive for the human immunodeficiency virus (HIV) was diagnosed with bilateral CMV retinitis in February 1994 and began treatment with IV cidofovir. Before being referred to us, the patient had received more than 20 months of systemic therapy including 4 months of cidofovir, which was discontinued owing to nephrotoxicity; 12.5 months of oral ganciclovir, to which he developed clinical resistance;
PATIENTS AND METHODS

PATIENTS

The study protocol was approved by the US Food and Drug Administration and the Institutional Review Board of the University of California, San Diego. The study population has been previously described in detail, as have the methods of intraocular injections and evaluation of retinitis.\(^{27}\) The study population consisted of all 77 patients with AIDS who were treated with intravitreal injections of 20 µg of cidofovir for CMV retinitis at the AIDS Ocular Research Unit between April 1993 and March 1996; a total of 298 injections with 20 µg of cidofovir were given in 116 eyes. Ophthalmologic data were collected prospectively and included standardized Early Treatment of Diabetic Retinopathy Study tests of visual acuity, slitlamp biomicroscopy, intraocular pressure measurement with applanation tonometry, dilated-pupil indirect ophthalmoscopy with detailed fundus drawing, and wide-angle fundus photography.

Clinical resistance to intravitreal cidofovir treatment was defined as a lack of healing or stabilization of CMV retinitis despite 3 consecutive injections or the documentation of retinitis progression within 28 days of an intravitreal cidofovir injection. In total, CMV retinitis in 5 eyes of 3 patients demonstrated clinical resistance to intravitreal cidofovir injections. Systemic anti-CMV therapies received by each of these 3 patients prior to intravitreal cidofovir treatment are presented as timelines in Figure 1.

LABORATORY METHODS

Pathologic Examination

A portion of the retinal biopsy specimen collected during pars plana vitrectomy of the left eye of patient 1 was fixed in 4% paraformaldehyde and examined by routine histologic staining and by immunostaining specific for CMV, varicella-zoster virus (VZV), herpes simplex virus (HSV) types I and II, and toxoplasmosis.\(^{28-30}\) Retinal tissue was fixed in 2.5% glutaraldehyde overnight at 4°C, postfixed in aqueous osmium tetroxide, dehydrated in serial alcohol solutions, and embedded in araldite resin using a rapid embedding procedure. Ultrathin sections were stained with uranyl acetate–lead citrate and bismuth subnitrate prior to electron microscopic examination. A vitreous specimen was stained with Giemsa and acid-fast for fungi and mycobacteria.

Drug Susceptibility

Cytomegalovirus isolates cultured from blood, urine, and retinal biopsy tissue were established and maintained in human foreskin fibroblast cells.\(^{17}\) Antiviral drugs were provided by the following sources: ganciclovir, Hoffmann-La Roche Inc, Nutley, NJ; foscarnet, Astra Pharmaceutical Products Inc, Westborough, Mass; cidofovir, Gilead Sciences, Foster City, Calif; and ganciclovir cyclic phosphate, Merck Research Laboratories, West Point, Pa. Drug susceptibilities of both laboratory strains and clinical isolates were determined by plaque reduction assay.\(^{17}\) Drug-resistant isolates are defined as having a median infective dose (ID\(_{50}\)) value of at least 8 µmol/L, at least 324 µmol/L, and at least 2.1 µmol/L for ganciclovir, foscarnet sodium, and cidofovir, respectively.

PCR and DNA Sequence Analysis

DNA was purified by phenol/chloroform extraction and ethanol precipitation from fully cytopathic CMV-infected human foreskin fibroblast cells and from retinal biopsy tissue. Vitreous specimens were stored at –70°C and heated at 94°C for 15 minutes prior to PCR. Approximately 1 µg of infected-cell DNA or 0.1 to 10 µL of vitreous were used in PCR reactions. All PCR reactions were carried out in 100-µL reaction volumes containing 10-mmol/L Tris hydrochloride, pH 8.3; 50-mmol/L potassium chloride; 2.5-mmol/L magnesium chloride; 200-µmol/L deoxynucleotide triphosphates; 2 U of Taq polymerase (Boehringer Mannheim Corp, Ind); and 50 pmol of each primer. The UL97 gene sequences 1203 to 1975 were amplified using primers of 24 nucleotides in length. A nested PCR approach was used to amplify CMV UL97 sequences from vitreous specimens; using 1 µL of product from the above reaction, UL97 fragments 1203 to 1655 and 1378 to 1975 were amplified. Polymerase sequences were amplified as overlapping fragments 244 to 1535, 1433 to 2216, 1807 to 3044, and 2493 to 3744, using primers described previously.\(^{17}\) The UL97 and UL54 (polymerase) gene sequences are numbered from the initiating ATG of each open-reading frame.\(^{31,32}\)

3 months of foscarnet, discontinued owing to toxic effects; and 1.5 months of IV ganciclovir treatment, to which he also demonstrated clinical resistance (Figure 1).

Prior to intravitreal cidofovir injection, visual acuity was 20/25 OU and intraocular pressure was 8 mm Hg OD and 9 mm Hg OS. At 5-week intervals, the patient received 3 intravitreal injections of 20 µg of cidofovir in each eye. Retinitis continued to progress despite this treatment; more than 750 µm within 28 days of the first injection and within 14 days of the third injection (Figure 2). In the left eye, retinitis approached within 1500 µm of the fovea. To rule out the possibility of another infectious cause, a pars plana vitrectomy and endoretinal biopsy were performed on January 24, 1996. Blood and urine specimens were also collected at this time for culture and polymerase chain reaction (PCR) analysis. Despite intravitreal foscarnet and ganciclovir injections, retinitis continued to progress in both eyes, progressing into the macula of the left eye. The patient died of complications of AIDS in May 1996.

CASE 2

A 46-year-old, HIV-positive, white man was diagnosed with CMV gastritis in September 1994 and received induction IV ganciclovir followed by oral ganciclovir (3000 mg/d) maintenance therapy. After 5 months, CMV retinitis developed in the right eye, and the patient received reinduction with IV ganciclovir followed by oral ganciclovir. Subsequent reactivations in the right eye were treated during the next 2 months with 330 mg of intra-
ocular fomiviren sodium (ISIS 2922), intraocular foscarnet, and an intraocular ganciclovir device, each in conjunction with oral ganciclovir. Following clinical failure of both oral ganciclovir and the ganciclovir implant, a course of systemic foscarnet induction and oral ganciclovir maintenance therapy was initiated. In October 1995, CMV retinitis developed in the left eye and IV cidofovir treatment (5 mg/kg every other week) was initiated. Both eyes responded to this treatment. After 3.5 months, retinitis reactivated in the left eye. Intravenous cidofovir treatment was discontinued, and this eye was treated with intravitreal foscarnet sodium injections (2400 µg). The CMV retinitis in the right eye remained quiescent.

In February 1996, the patient was referred to us. Visual acuity and intraocular pressure were 20/30 and 11 mm Hg OD and 20/16 and 13 mm Hg OS, respectively. Retinitis continued to progress in the left eye despite an intravitreal injection of 15 µg of cidofovir and concurrent oral ganciclovir therapy. Six weeks later, a second injection was administered; iritis developed 10 days after the injection and was treated with topical corticosteroid drops and cycloplegics. Reactivation of CMV retinitis in the right eye was treated with an injection of 20 µg of cidofovir; however, CMV retinitis progressed by more than 750 µm within 28 days. Neither the left nor the right eye responded to 2 additional injections each, 15 µg and 20 µg, respectively. On June 5, 1996, a second ganciclovir intraocular device was implanted in the right eye. An undiluted vitreous specimen was collected during surgery; blood and urine specimens were also collected for analysis of antiviral resistance. After surgery, foscarnet injections were administered to both eyes; however, retinitis continued to progress.

**CASE 3**

In May 1992, a 31-year-old, HIV-positive, white man developed CMV retinitis in his right eye. During the first year of therapy, IV ganciclovir therapy was given at a continually increasing dose (5 to 15 mg/kg per day) to control progressive smoldering retinitis. After 10 months of therapy, CMV retinitis developed in the left eye. Foscarnet was added to the treatment regimen, but was discontinued after 1 month owing to nephrotoxicity. Despite systemic treatment with high-dose IV ganciclovir (15 mg/kg per day), ganciclovir intraocular implants, and additional injections of 400 µg of ganciclovir, retinitis remained active. After being referred to us, the patient began receiving intravitreal cidofovir injections. At this time his visual acuities were 20/100 OD and 20/25 OS, and intraocular pressure measurements were 9 mm Hg OD and 10 mm Hg OS, respectively. Cytomegalovirus retinitis in both eyes remained active after each of 4 intravitreal cidofovir injections of 20 µg each. Progression was documented by day 42, day 31, and day 21 after the first, second, and third injections, respectively. Subsequent injection with 40 µg of cidofovir in both eyes failed to elicit any response. The patient died of complications of AIDS in August 1994. Specimens were not obtained from this patient for virologic analysis.

**RESULTS**

A total of 77 patients received injections of 20 µg of cidofovir for treatment of CMV retinitis during the study period. Of these 77 patients, 13 had less than 1 month of follow-up and were excluded from further analysis. The study group described in this article is composed of the remaining 64 patients. Two (3%) of the patients were women. One patient was African American (2%), 8 were Hispanic (12%), and the remaining 55 patients were white (86%). The median age of these patients was 39 years.
(mean, 39 years; range, 29 to 58 years) and median follow-up was 128 days (mean, 176 days; range, 31-674 days).

Sixty-one (95%) of 64 patients responded to treatment with cidofovir injections.22-25 In these patients, CMV retinitis remained healed or stabilized with consecutive cidofovir injections. However, 3 (5%) of 64 patients demonstrated clinical resistance. These patients failed to respond at any time to cidofovir injections, suggesting that resistance to cidofovir may have developed during prior treatment for CMV. Each of the 3 patients had received more than 16 months of prior systemic therapy for CMV (Figure 1). Two of the 3 patients with clinical resistance to intravitreal cidofovir had received both oral ganciclovir and IV cidofovir. The third had been treated predominantly with IV ganciclovir.

To determine if there was an association between failure of intravitreal cidofovir and prior use of a specific drug treatment, prior systemic anti-CMV therapeutics received by study participants were examined. Patient histories were available for 63 patients; 22 patients had not received prior anti-CMV therapeutics and all responded to intravitreal cidofovir injections. Forty-one patients had received prior therapy, and were receiving intravitreal cidofovir injections for the treatment of recurrent retinitis. Analysis of the predictor variables (prior use of IV cidofovir therapy, oral ganciclovir, IV ganciclovir, either oral or IV ganciclovir, or IV foscarnet) determined that clinical resistance to intravitreal cidofovir was associated with prior IV cidofovir or prior oral ganciclovir use (P=.02 for each). Clinical resistance to cidofovir was not associated with prior IV ganciclovir or IV foscarnet use (Table 1).

CONFIRMATION OF CMV INVOLVEMENT IN RETINAL DISEASE

Two of the 3 patients with clinical resistance were still receiving treatment at the time of the review. To confirm the clinical diagnosis of CMV retinitis and exclude the possibility that other infectious agents were involved in disease progression, ocular and extraocular specimens from these patients were collected. Hematoxylin-eosin–stained sections of a retinal biopsy specimen from patient 1 showed recognizable retinal tissue adjacent to areas of necrotic debris. The tissue was infiltrated with inflammatory cells, including polymorphonuclear cells and monocytes and macrophages with enlarged cytoplasm, and with eosinophilic debris. Some nuclei had hyaline material resembling ground glass, a feature consistent with viral cytopathic effects. Immunostaining for CMV was positive while stains for HSV, VZV, bacteria, and protozoa were negative. By electron microscopy, herpeslike virus particles were found within the nuclei and the cytoplasm of infected cells (Figure 3) and in disintegrating nuclei of necrotic tissue. Consistent with these findings, CMV was isolated from culture of retinal biopsy tissue on fibroblasts, and CMV DNA sequences were amplified from a corresponding vitreous specimen. For patient 2, CMV was isolated from blood and urine cultures and CMV sequences were amplified by PCR from a vitreous specimen.

### IN VITRO RESISTANCE STUDIES

To assess whether clinical resistance of retinitis in patients 1 and 2 was associated with antiviral-resistant CMV strains, in vitro cidofovir, ganciclovir, and foscarnet susceptibilities were determined for CMV isolates from these patients (Table 2). Late CMV isolates cultured from both patients demonstrated resistance to cidofovir and ganciclovir. In contrast, an early CMV isolate from patient 1, which had been cultured after 4 months of IV cidofovir treatment, demonstrated sensitivity to cidofovir and ganciclovir. All isolates retained sensitivity to foscarnet.

### VIRAL ALTERATIONS ASSOCIATED WITH ANTIVIRAL RESISTANCE

To identify the genetic alterations underlying antiviral resistance, polymerase and UL97 gene sequences were determined.35-38 Isolates from both patients contained UL97 alterations relative to laboratory strain Towne (Table 2). The retinal CMV isolate from patient 1 contained a mutation of UL97 nucleotide G1380 to T. This mutation results in substitution of an isoleucine for methionine at UL97 residue 460 (M460I). The same alteration was detected by direct analysis of CMV in the retinal biopsy specimen and also from a concurrent vitreous specimen; however, the wild-type UL97 sequence M460 was also found in the vitreous specimen. Each of 4 CMV isolates cultured from patient 2 contained UL97 alterations (Table 2). Interestingly, CMV cultured from blood and urine specimens contained a tyrosine for cysteine substitution at residue 607 (C607Y) (nucleotide 1820 G to A), but CMV DNA present in a vitreous specimen lacked nucleotides 1771 to 1782, which encode residues 591 to 594, and was unaltered at residue C607. Each of these alterations has been reported previously in association with ganciclovir-resistant clinical CMV isolates.31,36-40 A silent UL97 mutation, C1329 to T, was identified in CMV DNA present in vitreous, blood, and urine, suggesting that the ocular and extraocular resistant CMV populations may have been derived from the same parental strain.

### Table 1. Clinical Resistance to Intravitreal Cidofovir Injection Is Associated With Prior Oral Ganciclovir or Intravenous Cidofovir Treatment

<table>
<thead>
<tr>
<th>Prior Systemic Treatment†</th>
<th>Total No. of Patients</th>
<th>No. With Clinical Resistance to Intravitreal Cidofovir</th>
<th>P†</th>
</tr>
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<tbody>
<tr>
<td>No anti-CMV therapy</td>
<td>22</td>
<td>0</td>
<td>...</td>
</tr>
<tr>
<td>Any anti-CMV therapy</td>
<td>41</td>
<td>3</td>
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<td>Intravenous cidofovir</td>
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<td>2</td>
<td>.02</td>
</tr>
<tr>
<td>Intravenous ganciclovir</td>
<td>41</td>
<td>3</td>
<td>&gt;.50</td>
</tr>
<tr>
<td>Oral ganciclovir</td>
<td>4</td>
<td>2</td>
<td>.02</td>
</tr>
<tr>
<td>Intravenous foscarnet sodium</td>
<td>24</td>
<td>3</td>
<td>.25</td>
</tr>
</tbody>
</table>

†Fisher exact test. Ellipses indicate not applicable.

*CMM indicates cytomegalovirus.
Cytomegalovirus isolated from both patients also contained polymerase alterations relative to the Towne strain (Table 2). The retinal CMV isolate from patient 1 contained substitution of glutamate for lysine at residue 513 (K513E). The polymerases of blood and urine isolates from patient 2 contained 3 substituted residues: serine to leucine at residue 655 (S655L) and glycine to arginine at residue 874 (G874R), which have been identified previously in pretherapy clinical CMV isolates; and a novel alteration, serine for glycine at residue 678 (G678S). A pretherapy CMV isolate was unavailable to determine if the G678S alteration was selected during anti-CMV therapy. However, reduced susceptibility to ganciclovir cyclic phosphate (a monophosphate derivative of ganciclovir that has UL97-independent anti-CMV activity) suggests that ganciclovir resistance of the G678S-containing isolate is conferred, at least in part, by a polymerase alteration (Table 2).

Intravitreous cidofovir therapy given every 6 weeks is highly effective in the therapy of CMV retinitis, even in patients who have failed with or are intolerant of other therapies. The median time to failure exceeds the 120 days to progression seen with IV cidofovir. Although initially it appeared that the ophthalmologic adverse effect profile of IV cidofovir was better than that of intravitreous cidofovir, a recent report suggests that this may not be true and that the incidence of cidofovir-related intraocular inflammation and hypotony may be similar with both forms of administration. The growing use of local therapy, including ganciclovir implants and intravitreal injections of ganciclovir, foscarnet, and cidofovir, prompted us to examine the incidence of and virologic features associated with clinical resistance to local cidofovir therapy.

Figure 3. A. Electron micrograph showing the nucleus of an infected cell from the patient's retinal biopsy specimen. Arrow indicates viral particles located close to the nuclear membrane. Cytoplasm is observed in the upper right corner of the picture (original magnification ×16 360). B. Micrograph of many viral particles in the outer portion of the nucleus (center right). Disintegrating chromatin appears in the left portion of the picture. Arrow indicates viral particles adjacent to the nuclear membrane (original magnification ×16 360). C. Many viral profiles appear in the clumped degenerating chromatin (arrows) (original magnification ×16 360). D. Virus particles (arrow) appear in the cytoplasm of a largely necrotic cell. Profile of an erythrocyte is seen left of center (original magnification ×16 360).
Clinical failure of intravitreal cidofovir treatment was documented for 3 (7%) of 41 patients who had received prior systemic anti-CMV therapy, while 22 patients who received intravitreal cidofovir as a first-line therapy responded well to intravitreal cidofovir injections. Statistical analysis identified an association between clinical resistance of retinitis to intravitreal cidofovir and prior use of IV cidofovir or oral ganciclovir. Moreover, in the 2 patients whose CMV isolates were examined, clinical resistance to intravitreal cidofovir was associated with CMV strains that demonstrated in vitro resistance to both cidofovir and ganciclovir.

The 3 resistant patients failed to respond at any time to cidofovir injections, suggesting that resistance to cidofovir may have developed during prior anti-CMV treatment. Although both patients whose cultures yielded cidofovir-resistant CMV isolates had received 4 months of IV cidofovir treatment, an early CMV strain that was isolated from patient 1 immediately after cidofovir treatment retained cidofovir sensitivity. This suggests that IV cidofovir treatment alone was not responsible for selection of the cidofovir-resistant strain in patient 1. An early-treatment CMV isolate is not available to determine if IV cidofovir contributed to selection of the cidofovir-resistant CMV sample isolated from patient 2.

A key issue in the management of CMV retinitis is whether early treatment regimens can select for CMV strains that are cross-resistant to alternate therapies. Several studies have examined the development of cidofovir resistance during treatment of retinitis with ganciclovir. To date, all polymerase alterations known to confer ganciclovir resistance also confer cidofovir resistance. We have previously demonstrated that high-level ganciclovir-resistant isolates (mean ganciclovir ID_{50} values ≥ 30 μmol/L) cultured from ganciclovir-naïve patients who received IV ganciclovir typically harbor both UL97 and polymerase mutations and demonstrate in vitro cross-resistance to cidofovir.

Moreover, although antiviral susceptibility analysis of CMV cultured from patients receiving oral ganciclovir prophylaxis therapy suggests that the development of antiviral resistance is rare (1%), in 1 case, a CMV strain with high-level ganciclovir resistance and cross-resistance to cidofovir was isolated after 9 months of oral ganciclovir treatment and was associated with later treatment failure.

All 3 patients with clinical resistance to intravitreal cidofovir received courses of ganciclovir, and CMV isolates from patients 1 and 2 demonstrated high-level resistance and harbored polymerase mutations. An association between polymerase residue 513 alterations and in vitro ganciclovir and cidofovir resistance has been reported. The involvement of the polymerase G678S alteration in antiviral resistance has not been described previously. This residue does not lie within a conserved polymerase region; however, alteration of residue S676 has been associated with resistance. Cytomegalovirus isolates were not available to examine the virologic features associated with clinical resistance of patient 3 to intravitreal cidofovir injection; however, the systemic treatment received by this patient was almost exclusively IV ganciclovir. Thus, prior systemic ganciclovir treatment for CMV retinitis appears to have contributed to the development of antiviral-resistant CMV and the clinical failure of intravitreal cidofovir treatment in all 3 patients.

In patient 2, CMV populations in ocular and extraocular specimens appeared to be derived from the same parental strain; however, different UL97 mutations were
The CMV variant identified in the ocular specimen could not be cultured from blood 7 weeks after the population was known to be present in the eye. This suggests that ocular CMV infection may be a highly compartmentalized event. Our findings emphasize that blood and urine isolates may not be representative of the CMV population responsible for retinitis. Additionally, they suggest that the most accurate methods for determining antiviral resistance during retinitis treatment would require the examination of CMV present in ocular specimens.

In summary, we have demonstrated that clinical resistance of CMV retinitis to intravitreal cidofovir treatment can be associated with antiviral-resistant CMV strains that may have been selected by prior anti-CMV therapy. Moreover, we have demonstrated that CMV isolates cultured from blood and urine specimens may not be representative of the CMV populations in the eye. This finding indicates that a more comprehensive understanding of factors influencing CMV pathogenesis, dissemination, and antiviral resistance is required to better understand and monitor CMV retinitis.

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