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Detection of Human Cytomegalovirus Mutations Associated with Ganciclovir Resistance in Cerebrospinal Fluid of AIDS Patients with Central Nervous System Disease

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To examine the involvement of ganciclovir-resistant strains in the development of central nervous system (CNS) disease caused by human cytomegalovirus (HCMV), 14 AIDS patients with CNS disease caused by HCMV were studied for the presence of HCMV strains with UL97 gene mutations associated with ganciclovir resistance by using amplification and direct sequencing of HCMV DNA in cerebrospinal fluid (CSF). The CSF of all seven patients who had not received ganciclovir prior to the development of CNS disease and four patients who had been receiving the drug for 3 to 8 months contained wild-type UL97 sequences. The CSF of three patients who had received ganciclovir for 12 to 30 months contained HCMV strains with nucleotide changes leading to single-amino-acid substitutions within conserved UL97 sites implicated in nucleotide binding (position 460) and substrate recognition (position 591). Patients containing mutant and wild-type strains revealed a similar spectrum of clinical and histopathologic manifestations. These findings indicate that CNS disease in AIDS patients receiving prolonged ganciclovir therapy can be caused by ganciclovir-resistant HCMV strains. Direct genotypic analysis of HCMV DNA within CSF should help to identify ganciclovir-resistant virus and to guide anti-HCMV therapy.

Infection of the central nervous system (CNS) with human cytomegalovirus (HCMV) is common in patients with AIDS and has been documented by autopsy studies in 20 to 30% of persons who have died of AIDS (12). Clinical manifestations cover a broad spectrum of neurologic dysfunctions, and diagnosis during life has been hampered by the lack of definitive tests. Recently, we and others have developed a sensitive and specific PCR-based method for detecting HCMV DNA in the cerebrospinal fluid (CSF) to diagnose HCMV CNS disease (4, 6, 18). The presence of CNS involvement, as indicated by PCR of CSF (CSF-PCR) and by histopathologic studies, has been associated with advanced, late-stage HCMV infection (4, 6, 12, 18). Hence, many of the affected patients have been receiving prolonged antiviral therapy, which could potentially lead to the emergence of drug-resistant virus. HCMV strains resistant to ganciclovir have been recovered from the urine of 8% of the patients receiving the drug for more than 3 months (5). Yet, the capacity of resistant strains to infect the CNS and their pathogenic potential have not been determined. Additionally, because cultures of CSF specimens are frequently negative, despite the presence of CNS disease (6, 18), the detection of phenotypic resistance often is not possible.

Recently, ganciclovir resistance has been shown by sequence analysis to result from mutations within the UL97 gene, a protein kinase homolog which controls ganciclovir phosphorylation in infected cells (1, 2, 8, 9, 13, 15). Resistance-conferring mutations have been mapped to UL97 gene subdomains VI, VIII, and IX, which have been implicated in nucleotide and substrate binding (3, 10, 15–17). In the study described here we used a direct genotypic approach to determine the presence of UL97 mutations in HCMV strains infecting the CNS: the CSF-PCR method was extended to allow rapid and simple sequencing of the viral DNA population in the CSF. We report the detection of mutations associated with phenotypic ganciclovir resistance in strains from patients with AIDS who developed HCMV CNS disease while receiving prolonged ganciclovir therapy for HCMV retinitis.

Patients and clinical specimens. CSF specimens obtained at the time of diagnosis of HCMV CNS disease from each of 14 epidemiologically unrelated patients with AIDS and HCMV CNS disease were studied. The presence of HCMV CNS disease was defined by clinical and laboratory manifestations consistent with CMV-associated CNS disease, with the detection of a positive CSF-PCR signal in the presence of neuropathologic findings compatible with HCMV involvement or a CSF culture positive for HCMV as described previously (18). The clinical manifestations and HCMV-related neuropathologies of the patients have been reported previously (18). Seven patients developed HCMV CNS disease while receiving ganciclovir therapy for HCMV retinitis for 3 to 30 months, while the remaining seven patients had received no prior ganciclovir treatment.

Amplification and sequencing of the UL97 catalytic subdomains in CSF. Samples were maintained at −70°C until assayed. CSF (10 μl) was freeze-thawed three times, digested with proteinase K (120 μg/ml; Sigma Chemical, St. Louis, Mo.) in 1× PCR buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 2 mM $MgCl_2$) at 60°C for 1 h, heated at 95°C for 10 min, centrifuged at 12,000 × *g* for 5 min, and directly amplified by PCR.

Two noncontiguous UL97 fragments were separately amplified for each sample. The primers defining the fragments were derived from the published AD169 sequence (2). The sequences of the primers and the amplified fragments are as follows. Primer UL97-7-5 (sense; 5′-CTGCTGTCTGCTGCA CAACGTCA-3′) primer UL97-7-3 (antisense; 5′-ACACAGC GCTCGTTGTAATCCGGA-3′) amplify a 284-bp fragment (fragment 3; nucleotides 1207 to 1491) encompassing catalytic subdomains V to VII, with the proposed ATP-binding site

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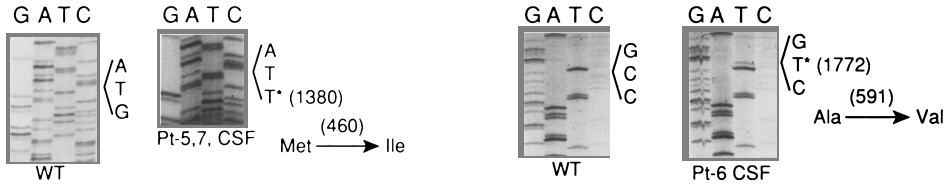

FIG. 1. Nucleotide changes in the UL97 catalytic domain of HCMV DNA sequences in strains from the CSF of three patients which resulted in amino acid substitutions when compared with the AD169 (wild-type [WT]) sequence. Nucleotide changes are indicated. Strains from patients (pt) 5 and 7 and from patient 6 contained changes in the proposed nucleotide and substrate binding sites, respectively. Sequences were obtained by direct PCR sequencing by using ^{32}P-end-labeled primers.

being in catalytic subdomain VI (2, 7). Primer UL97-10-5 (sense; 5′-CACGGAGGCGTTGCTCTTTAAGCA-3′) and primer UL97-10-3 (antisense; 5′-TCTGCGAGCATTCGTGGTAGA AGC-3′) amplify a 238-bp fragment (fragment 6; nucleotides 1741 to 1979) encompassing catalytic subdomains IX to XI containing the putative substrate binding site in catalytic subdomain IX (2, 7).

To amplify each fragment, the template was combined with the appropriate primer pair (50 pmol each), deoxyribonucleoside triphosphates (200 μM each; Pharmacia LKB, Piscataway, N.J.), and *Taq* polymerase (2.5 U; Perkin-Elmer Cetus, Norwalk, Conn.) in a total of 100 μl of 1× PCR buffer, and each fragment was amplified by 35 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 15 s, and extension at 72°C for 1 min in the Gene Amp PCR system 9600 (Perkin-Elmer Cetus) as described previously (18).

HCMV strains from 14 patients with HCMV CNS disease were examined for the presence of strains with UL97 mutations associated with ganciclovir resistance. All seven patients who had not received ganciclovir prior to the development of HCMV-related neurologic dysfunction contained strains with wild-type sequences. Of seven patients who had been receiving ganciclovir for 3 to 30 months, CSF specimens from three patients revealed strains with single nucleotide changes leading to an amino acid substitution within conserved UL97 sites (Fig. 1 and Table 1); the HCMV DNA sequences of strains in the CSF samples from two patients (patients 5 and 7) receiving ganciclovir for 12 and 30 months contained a G-to-T change at position 1380, resulting in a methionine-to-isoleucine substitution at position 460 (11). The methionine at residue 460 is located in a region corresponding to catalytic subdomain VI, which has been implicated in ATP binding (2, 7). The third patient (patient 6), who had received ganciclovir for 12 months, had a CSF specimen which contained a strain with a C-to-T change at position 1772, leading to an alanine-to-valine substitution at position 591 in the proposed substrate binding site (2, 7). CSF specimens from four patients (patients 1 to 4) who had received ganciclovir for 3 to 8 months contained strains with wild-type UL97 sequences.

The patients who harbored resistant viral strains, as indicated by genotypic analysis, did not differ from the patients who were infected with wild-type virus strains in clinical manifestations or the extent of neuropathologic findings (Table 1); one patient (patient 6) presented with dementia associated with the presence of microglial nodule encephalitis. Two patients (patients 5 and 7) presented with HCMV-related polyradiculopathy, with one of them demonstrating ventriculitis, encephalitis, and meningitis on histopathologic examination.

In this report, we have described three patients with CNS disease related to mutant HCMV variants. Mutations were identified in two conserved UL97 domains. Strains from two patient CSF specimens had a single nucleotide change, resulting in a methionine to isoleucine substitution at position 460, which is within the putative ATP binding site of the enzyme. The same mutation has recently been reported in a ganciclovir-resistant laboratory-derived strain, and its direct effect on the development of resistance has been demonstrated by marker transfer (11). A substitution of the methionine at residue 460 by valine has also been found in a few ganciclovir-resistant clinical isolates (3, 17). The third patient contained a strain with a mutation in the proposed substrate recognition and binding subdomain, with an alanine-to-valine substitution at position 591. The substituted alanine at residue 591 is one of four amino acids that was found to be deleted from a previously characterized ganciclovir-resistant laboratory strain and is located in proximity to the mutation at residue 595, which has recently been associated with clinical resistance to ganciclovir (15, 17) and is thus likely to confer ganciclovir resistance. Moreover, we have recently identified the same mutation in a strain in the plasma of a patient with HCMV retinitis who exhibited disease progression during therapy (17), further suggesting a role for the alanine substitution at residue 591 in the development of clinical resistance. Interestingly, the two substituted amino acids are conserved in the UL97 homolog en-

TABLE 1. Genotypic analysis of UL97 sequences from strains from CSF of seven patients who developed HCMV CNS disease while receiving ganciclovir therapy

Patient no.	Duration of therapy (mo)[a]	Amino acid substitution	Clinical manifestation	HCMV-related neuropathology
1	3	None	Somnolence, hallucinations	Ventriculitis
2	5	None	Polyradiculopathy	NA[b]
3	7	None	Mental deterioration	Encephalitis
4	8	None	Seizures, coma	Ventriculitis, encephalitis
5	12	Met-460→Ile	Disorientation, polyradiculopathy	Ventriculitis, encephalitis, meningitis
6	12	Ala-591→Val	Dementia	Encephalitis
7	30	Met-460→Ile	Polyradiculopathy	NA

[a] Prior to the development of CNS disease.
[b] NA, not available.

coded by human herpesvirus 6, which is also susceptible to ganciclovir (2). This might indicate the presence of a similar mechanism for drug activation.

In HCMV and human herpesvirus 6, the clustering of resistance mutations to the same residues in epidemiologically unrelated patients might reflect the limited number of functional UL97 sites and may further support a conserved role for the putative nucleotide and substrate binding subdomains in vivo. However, despite the occurrence of mutations in conserved UL97 residues, the pathogenic potential of the mutant variants appears to be unaffected, as indicated by their capacity to infect the CNS and cause progressive disease. Thus, one could speculate that the mutant UL97 product might retain its original, as yet uncharacterized role in the life cycle of the virus even though it is selectively unable to phosphorylate ganciclovir.

The limited number of functional mutations also has relevance for the potential reliable prediction of ganciclovir resistance by genotypic analysis. However, it should be noted that other unidentified mutations may lead to the development of clinical resistance to ganciclovir, as recently suggested by the detection of DNA polymerase mutations in resistant laboratory strains (11, 14).

As the number of patients with AIDS increases and their life expectancies improve, more patients are likely to develop advanced stages of HCMV infection that often require prolonged ganciclovir therapy. We have shown that the involvement of the CNS in these patients can be related to drug-resistant virus. The presence of strains with resistance-conferring mutations appears to correlate with longer periods of therapy. With the availability of CSF-PCR for the detection of HCMV infection of the CNS, direct genotypic analysis of viral DNA within CSF will allow for the detection of resistant strains without the need to culture CSF specimens for the recovery of virus. This approach could help to guide the treatment of persons with AIDS with HCMV-related CNS disease.

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