Early Emergence of Ganciclovir-Resistant Human Cytomegalovirus Strains in Children with Primary Combined Immunodeficiency

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Children with primary combined immunodeficiency (CID) and human cytomegalovirus (HCMV) infection often deteriorate despite antiviral therapy. In this study, the emergence of ganciclovir-resistant strains was examined in 6 children with CID and HCMV infection, using sequence analysis of the HCMV UL97 gene and virus susceptibility assays. Mutations in the proposed ATP binding site associated with ganciclovir resistance were found in 4 of the 6 children. In 1 patient with severe CID, an unusual multiplicity of mutations was found in the UL97 substrate binding domain between aa 590–606. All mutations were detected within 10 days to 3 weeks from initiation of therapy. The emergence of resistant strains in children with CID appears earlier than in other groups of HCMV-infected patients. These findings may have relevance to the cellular pathways involved in viral DNA repair and mutagenesis, and they indicate the need for early and frequent genotypic monitoring and prompt therapeutic modification in this patient population.

Materials and Methods

Patients and clinical specimens. Plasma specimens and HCMV isolates recovered from 6 children with primary CID disorders (CID) [1]. These children are increasingly treated by allogeneic bone marrow transplantation (BMT) but remain susceptible to severe HCMV infection and disease even after successful hematopoietic reconstitution.

Ganciclovir is widely used for the treatment and prophylaxis of HCMV disease in immunocompromised persons [2]. However, prolonged ganciclovir therapy can lead to the development of ganciclovir-resistant HCMV strains. Ganciclovir-resistant strains have been recovered mainly from adult AIDS patients who have received the drug for >3 months, and occasionally, it has been recovered from transplant patients [3–6].

Despite the frequent failure of ganciclovir therapy in children with CID and HCMV infection, there is no information about in vitro ganciclovir resistance and its clinical significance in this patient population.

Ganciclovir resistance results from mutations in the HCMV UL97 phosphotransferase gene, which mediates the first step of ganciclovir activation [4, 5, 7, 8]. The clustering of resistance-conferring mutations to defined UL97 sites has allowed for the genotypic analysis of drug resistance rather than the use of conventional culture-based assays, which were not clinically useful [4, 5].

In this study, we used direct genotypic analysis to determine the presence of UL97 gene mutations associated with ganciclovir resistance in HCMV strains from children with CID. We report the early emergence of ganciclovir-resistant strains in CID patients who have received ganciclovir following BMT.
Table 1. UL97 amino acid substitutions in HCMV strains from children with primary combined immunodeficiency (CID).

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age</th>
<th>Underlying disease</th>
<th>Duration of ganciclovir therapy (days)</th>
<th>Amino acid substitution</th>
<th>Specimen analyzed</th>
<th>Ganciclovir ED₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5 years</td>
<td>CID</td>
<td>10</td>
<td>Met-460 → Leu</td>
<td>Plasma</td>
<td>2.5</td>
</tr>
<tr>
<td>2</td>
<td>2 years</td>
<td>HLA class II expression deficiency</td>
<td>21</td>
<td>Met-460 → Val</td>
<td>Plasma, urine isolate</td>
<td>11.3</td>
</tr>
<tr>
<td>3</td>
<td>18 months</td>
<td>HLA class II expression deficiency</td>
<td>14</td>
<td>Met-460 → Val</td>
<td>BAL isolate</td>
<td>NA</td>
</tr>
<tr>
<td>4</td>
<td>5 months</td>
<td>B⁻ SCID</td>
<td>3 (24)</td>
<td>Met-460 → Val, mix; Ala 590 to Ala 606 → multiple, mix</td>
<td>Plasma, leukocyte isolate</td>
<td>16.8</td>
</tr>
<tr>
<td>5</td>
<td>5 months</td>
<td>SCID</td>
<td>5</td>
<td>None</td>
<td>BAL isolate</td>
<td>3.5</td>
</tr>
<tr>
<td>6</td>
<td>20 months</td>
<td>CID</td>
<td>7</td>
<td>None</td>
<td>Urine isolate</td>
<td>5.0</td>
</tr>
</tbody>
</table>

NOTE. All patients except no. 5 received acyclovir therapy after bone marrow transplantation. NA, isolate not available for assay; SCID, severe CID; mix, mixture with wild-type sequences.

AD169 was included as a sensitive control with each assay. Assays were done simultaneously for sensitive isolates recovered from patients who responded to ganciclovir (ED₅₀ ≤ 6 µM) and for isolates recovered from the study patients.

Amplification and sequencing of the UL97 catalytic subdomains. DNA extracted from heparinized plasma specimens, viral isolates, and primary cultures was subjected to direct polymerase chain reaction (PCR) sequencing, which was done as previously described [4]. Two noncontiguous UL97 fragments were separately amplified for each sample. Primer UL97-7-5 (5'-CTGCTGTCT-GCTGCCAACACGTCA-3') and primer UL97-8-3 (5'-TCCGAC-ATGCAATAACGCGGTAG-3') amplify a 452-bp fragment (fragment 3–4; nt 1207–1659) encompassing the proposed ATP binding site. Primer UL97 9-5 (5'-TGCGCGAATGTTACCACCTGCTTT-3') and primer UL97 10-3 (5'-TCTGCGAGCATTCGTGGTAGAAGC-3') amplify a 431-bp fragment (fragment 5–6; nt 1548–1979) encompassing the putative substrate binding site.

PCR products, which were visualized as single and clear bands, were resolved by gel electrophoresis and purified from low-melting-point agarose gel. The purified PCR products were subjected to direct PCR sequencing, using the fmol DNA sequencing system (Promega, Madison, WI). The same primers designed to amplify the fragments were used to sequence the sense and antisense strands. Sequence changes were confirmed by sequencing both strands of at least 2 independent PCR products for each fragment.

Glycoprotein B (gB) genotype analysis. The purpose of gB genotyping was to assess genetic relatedness of strains from the same patient and from epidemiologically related patients and to define the presence of one or a mixture of genotypes in a specific patient’s isolate. gB analysis was done on all isolates and plasma samples. For gB analysis, PCR product amplified with primers gB 1319 and gB 1604 [10] was either sequenced directly or digested with restriction enzymes HindIII and Rsal, and the pattern that was obtained was classified on the basis of comparisons with control sequences.

Results
Six children with primary CID who were treated with ganciclovir for HCMV disease were examined for the presence of ganciclovir-resistant HCMV strains by analysis of the UL97 gene and by in vitro susceptibility assays. UL97 mutations associated with ganciclovir resistance were found in 4 of the 6 patients (table 1). All 4 patients excreted strains containing substitutions of the methionine 460. Patient 1 had coexistent wild-type virus (isolate recovered from bronchoalveolar lavage [BAL]) and mutant virus (direct genotypic analysis in plasma) populations after 10 days of ganciclovir therapy. Analysis of the gB gene identified the presence of 2 distinct genotypes in plasma and BAL in patient 1.

Patients 2 and 3, who had received BMT from the same donor, both excreted ganciclovir-resistant virus with a 460 methionine-to-valine substitution in urine (patient 2) and BAL (patient 3) after 21 and 14 days of ganciclovir therapy, respectively. The same mutation was also detected in consecutive plasma specimens from patient 2. gB gene analysis confirmed the presence of the same genotype in patients 2 and 3.

A ganciclovir-resistant leukocyte isolate recovered from patient 4 contained mixed wild-type and mutant UL97 sequences in both methionine 460 and the putative substrate binding site (table 1). In particular, multiple mutations were found between aa 590 and 606 upon analysis of DNA extracted from the leukocyte culture isolate (figure 1). The same mutations could be detected by direct genotypic analysis of the corresponding plasma specimen, thus excluding in vitro selection of the mutations during isolate propagation. The mutations indicated in figure 1 are those that resulted in amino acid substitutions. An additional silent mutation was found at position 1794. Mutations were determined by repeated sense and antisense sequence reading of PCR products obtained from separate amplification reactions of fresh DNA extracts. The mutations were read by independent observers, and the findings were further confirmed by automated DNA sequencing (ABI PRISM 377; Perkin-Elmer ABI, Foster City, CA) using a fluorescent dye terminator cycle. Mutations were detected after 3 days of ganciclovir therapy; however, the patient had received ganciclovir...
for 3 weeks, 2 months previously (total 24 days). In contrast to the multiple mixed UL97 sequences, gB analysis revealed the presence of a single genotype in the isolate and plasma of patient 4.

For patients 5 and 6, who excreted ganciclovir-sensitive wild-type strains, gB analysis identified the presence of 2 distinct genotypes in the BAL and urine in patient 5 and a single genotype in the plasma and leukocyte isolate in patient 6.

Ultimately, all 4 patients who excreted ganciclovir-resistant mutant strains died with clinical and histologic evidence of severe HCMV disease. Of the 2 patients who excreted ganciclovir-sensitive strains, 1 died of HCMV pneumonia, and the other recovered following therapy, with resolution of the cultures and PCR signals.

Discussion

Herein, we describe the appearance of HCMV strains with UL97 gene mutations associated with ganciclovir resistance in 4 children with primary CID. All 4 patients excreted strains with mutations in the methionine 460, which is located in the proposed ATP binding site of the UL97 phosphotransferase. The detected mutations included the well-known Met-to-Val substitution, which has been shown to confer ganciclovir resistance in HCMV laboratory and clinical strains [4, 5, 8, 11], and a Met-to-Leu substitution, which has not been documented previously. In 1 patient, additional mutations were detected in the carboxy-terminal region of the gene between aa 590 and 606. The role of aa 594 (Ala-to-Val substitution), 595 (Leu-to-Phe or Leu-to-Ser substitution), 591–594 deletion, and 603 (Cys-to-Trp substitution) in ganciclovir activation and resistance has been confirmed using marker transfer experiments [4, 5, 7, 12].

Mutations between aa 596 and 607 have also been reported in ganciclovir-resistant clinical isolates. However, the impact of the single substitutions identified at aa 596–599 and 606 remains to be directly established. The coexistence of mutations clustered in proximity in a ganciclovir-resistant strain of a single gB genotype suggests that this larger region could function in drug recognition and binding and in clinical ganciclovir resistance.

Antiviral drug resistance may arise from production of novel mutations during therapy or from selection of mutant virus populations already present before therapy. In AIDS patients, the presence of ganciclovir-resistance mutations correlates with longer duration of therapy, usually exceeding 3 months [3–5, 13]. Of interest, mutations in children with CID were already detected after 10 days to 3 weeks of ganciclovir therapy. This early temporal pattern suggests rapid selection of pretherapy resistant strains. This is further supported by the concurrent excretion of the same mutant variant in the 2 children who acquired HCMV infection from the same donor.

It is important to note that all 4 patients who developed resistant strains had received haploidentical T cell–depleted BMT. Hence, the major contributing factor appears to be the profound immunosuppression associated with the underlying disease (combined with delayed immune reconstitution following bone marrow ablation and T cell depletion) and the associ-
ated graft-versus-host disease in these patients. The high level of immunosuppression may favor the emergence of resistant strains by allowing for increased viral DNA replication and coexcretion of multiple strains. Acyclovir therapy after BMT in the 4 patients could further facilitate the selection of UL97 mutations since acyclovir is also activated by the UL97 gene product.

An unexpected finding in this study was the multiplicity of UL97 mutations in the patient with B− SCID. Of interest, this form of primary SCID is associated with a general cellular defect in DNA repair, leading to marked sensitivity to DNA-damaging agents and increased mutagenesis [14, 15]. It is tempting to speculate that the host’s basic genetic background may affect the viral DNA mutation rate and thus cause a more rapid development of resistant strains during drug selection. Clearly, the concept of increased viral mutagenesis and drug resistance based on impaired cellular repair mechanism requires further investigation. This could contribute to our understanding of the cellular pathways utilized by the virus for DNA repair.

The recognition of early emergence of ganciclovir-resistant strains in children with CID has implications for prophylaxis, monitoring, and treatment of HCMV disease in these patients. While general treatment guidelines should await further prospective studies, our findings suggest the need for early and frequent genotypic monitoring and prompt therapeutic modification in children with CID and HCMV infection.

References