Ganciclovir Susceptibilities and Analysis of UL97 Region in Cytomegalovirus (CMV) Isolates from Bone Marrow Recipients with CMV Disease after Antiviral Prophylaxis

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Ganciclovir susceptibilities and UL97 sequences were analyzed in 20 cytomegalovirus (CMV) isolates recovered from 15 bone marrow transplant recipients with active CMV infection after prophylaxis with acyclovir (group I; 12 isolates) or after acyclovir prophylaxis followed by ganciclovir therapy (group II; 8 isolates). All group I isolates were susceptible to ganciclovir. Five group II isolates were susceptible to ganciclovir, and 3 isolates (all from the same person) were resistant to ganciclovir (IC\textsubscript{50} > 12 \textmu M). Ganciclovir resistance UL97 mutations were found in 4 group II isolates, including a ganciclovir-susceptible isolate obtained from 1 patient after 41 days of therapy with ganciclovir and 3 ganciclovir-resistant isolates obtained from another patient after 73, 116, and 132 days of treatment with ganciclovir. Ganciclovir-resistant CMV isolates may emerge rapidly in bone marrow transplant recipients who are treated with ganciclovir after receiving prophylaxis with acyclovir.

Cytomegalovirus (CMV) is a major pathogen for bone marrow transplant recipients, in whom it causes severe complications including hepatitis, gastrointestinal disease, and interstitial pneumonitis [1]. Advances have been made in the prevention of CMV disease after bone marrow transplantation, including prophylactic administration of antivirals such as acyclovir and ganciclovir [2, 3]. These two compounds are nucleoside analogues that must be phosphorylated to their nucleotide form (triphosphate) to inhibit the viral DNA polymerase. The initial phosphorylation of acyclovir and ganciclovir to their monophosphates is mediated by a protein kinase encoded by the UL97 gene of CMV [4, 5].

CMV prophylaxis with antivirals in the context of bone marrow transplantation is administered for prolonged periods of time. A potential risk associated with prolonged use of antivirals is the emergence of resistant viruses. Specifically, CMV isolates resistant to ganciclovir have been recovered from immunocompromised patients treated with this compound [6, 7].

In these clinical isolates, resistance to ganciclovir has been related to UL97 mutations at one or several loci [8, 9]. Because acyclovir shares the same mechanism of activation and action as ganciclovir against CMV, it is theoretically possible for UL97 mutations causing resistance to ganciclovir to occur under selective pressure during exposure to acyclovir. To test this hypothesis, we analyzed ganciclovir susceptibilities and UL97 sequences of CMV isolates recovered from bone marrow transplant recipients who developed active CMV infection after receiving prophylaxis with acyclovir.

Materials and Methods

CMV isolates. Viruses studied were selected among those available at the University of Minnesota Clinical Virology Laboratory from bone marrow transplant recipients who developed active CMV infection after receiving prophylaxis with acyclovir (10 mg/kg intravenously [iv] every 8 h starting from up to 10 days before transplantation to 30 days afterward, followed by 800 mg orally 5 times per day for the first 100 days after transplantation). Patients treated with ganciclovir received 5 mg/kg iv every 12 h for 14 days, followed by 5 mg/kg 5 days a week for 4 weeks. Acyclovir and ganciclovir dosages were adjusted for renal function. The first available blood or, if blood isolates were not available, bronchoalveolar lavage or tissue isolates were used for ganciclovir susceptibility studies.

Ganciclovir susceptibilities. Ganciclovir susceptibilities were determined by a DNA-DNA hybridization method (Diagnostic Hybrids, Athens, OH) [10]. Ganciclovir was provided by Julian Verheyden (Syntex, Palo Alto, CA). The CMV laboratory strain AD169 was included in each assay as a susceptible control. CMV isolates with ganciclovir IC\textsubscript{50} > 6 \textmu M were considered resistant.

Sequencing of CMV UL97. Sequences representing codons 418–702 of UL97 were obtained by polymerase chain reaction (PCR), using DNA purified from fibroblasts infected with CMV isolates and primers designed on the basis of published data on
A strain AD169. Primer sequences were 5′-CATCGAGTTCACAGACGAC-3′ (Z2671, forward) and 5′-CCTCAGCAACCGTCAGTTC-3′ (Z2992, reverse). Amplified products were sequenced by a commercial kit (Prism Ready Reaction Dyeediode Terminator Cycle Sequencing kit; Applied Biosystems, Foster City, CA) and primers Z2671 and Z2992. An additional pair of internal sequencing primers, 5′-GCACGGCTCGCCGAATGTTA-3′ (Z6813, forward) and 5′-AGATGAGCAGCCTGCTGACGAC-3′ (Z1600, reverse), was used to generate complete UL97 sequences. UL97 sequences were aligned with existing sequence data to detect previously described drug resistance mutations.

Screening assay for ganciclovir resistance–related UL97 mutations. Restriction digest screening for ganciclovir resistance UL97 mutations at codons 460 (Met to Val), 520 (Hist to Glu), 594 (Ala to Val), and 595 (Leu to Ser or Phe) was done as previously described [8, 10]. The presence of mutations was determined by distinctive restriction patterns visualized by gel electrophoresis.

Results

Patients and virus isolates. Twenty CMV isolates from 15 bone marrow recipients were included in the study. All patients had active CMV infections. Isolates were recovered from patients after prophylaxis with acyclovir (group I; 12 isolates) or after acyclovir prophylaxis followed by ganciclovir therapy (group II; 8 isolates). CMV isolates from group I were obtained after an average of 58 days (range, 23–118) of acyclovir prophylaxis. CMV isolates from group II were obtained after an average of 46 days (range, 34–76) of acyclovir prophylaxis and of 69 days (range, 9–132) of ganciclovir therapy. Clinical data and results of virologic studies are summarized in table 1.

Ganciclovir susceptibilities. All 12 group I isolates were susceptible to ganciclovir, with a mean $IC_{50}$ of 1.6 $\mu M$ (range, 0.5–4). Five of 8 group II isolates were susceptible to ganciclovir, with a mean $IC_{50}$ of 3.5 $\mu M$ (range, 1.4–5.1). The 3 resistant isolates in this group (all obtained from the same subject) had $IC_{50}$ of 12.2 to >25 $\mu M$. The mean ganciclovir $IC_{50}$ of the susceptible control AD169 was 2.1 $\mu M$ (range, 1.2–4.7).

Analysis of CMV UL97. Five group I isolates contained UL97 mutations, including 2 isolates with a Gln-to-Lys mutation at codon 449 (K449 mutation), 2 isolates with an Asn-to-Ser mutation at codon 510 (S510 mutation), and 1 isolate with a His-to-Tyr mutation at codon 469 (Y469 mutation). Four of the 5 group II isolates that were susceptible to ganciclovir contained UL97 mutations, including a Y469 mutation (1 isolate), an S510 mutation (2 isolates), and a V594 mutation (1 isolate). The 3 remaining group II isolates (15B, 15C, 15D) were resistant to ganciclovir. Isolate 15B contained an Ala-to-Val mutation at codon 594 (V594 mutation); isolate 15C had a His-to-Gln mutation at codon 520 (Q520 mutation); and isolate 15D had both mutations.

Restriction digest analysis of UL97. Results of restriction digest analysis for the most common ganciclovir resistance UL97 mutations were concordant with results of sequencing analysis in all isolates (table 1). Of note is that the S510 mutation had a restriction pattern similar to that of the Q520 mutation (figure 1).

Discussion

Our study suggests that CMV isolates containing UL97 mutations associated with resistance to ganciclovir may emerge in bone marrow transplant recipients who are treated with ganciclovir after receiving prophylaxis with acyclovir. The UL97 mutations we found among these isolates included V594 and Q520. These mutations have been characteristically associated with resistance to ganciclovir in previous studies [8–10].

There is limited information on the incidence of ganciclovir-resistant CMV infections in the context of bone marrow transplantation. In one report, ganciclovir-resistant CMV isolates (IC$_{50}$ of >98 $\mu M$ in a cytopathic effect reduction assay) were recovered from the blood and bronchoalveolar lavage fluid of a patient treated with ganciclovir for >2 months [11]. In a survey by the European Group for Blood and Bone Marrow Transplantation, 23 patients with ganciclovir-resistant CMV infections were reported from 19 of the 68 participating centers [12]. However, resistance to ganciclovir was documented in only 2 cases. In another study [13], a ganciclovir-resistant CMV isolate (IC$_{50}$ of 14.5 $\mu M$ in a DNA hybridization assay) was recovered from a lung autopsy sample in a bone marrow transplant recipient with a second episode of CMV pneumonitis who had received acyclovir prophylaxis and two courses of ganciclovir therapy for a total of 55 days.

An interesting finding in our study was the rapid emergence of viruses containing ganciclovir resistance UL97 mutations in patients who received acyclovir prior to ganciclovir as prophylaxis. Specifically, isolates 12A and 15B were recovered after 46 days (range, 34±76) of acyclovir prophylaxis and of 69 days (range, 9–132) of ganciclovir therapy. Clinical data and results of virologic studies are summarized in table 1.

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An interesting finding in our study was the rapid emergence of viruses containing ganciclovir resistance UL97 mutations in patients who received acyclovir prior to ganciclovir as prophylaxis. Specifically, isolates 12A and 15B were recovered after 41 and 73 days of ganciclovir therapy (table 1). This contrasts with results of a previous study, in which ganciclovir-resistant CMV isolates were recovered from the urine of patients with AIDS after an average of 6.7 months of ganciclovir therapy [7]. Because genetic analysis of the isolates was not done in that study, the possibility that earlier susceptible isolates could contain UL97 mutations cannot be excluded.

Whether prior exposure to acyclovir selects ganciclovir-resistant CMV isolates is not clear. In our initial study of ganciclovir resistance [6], a ganciclovir-resistant isolate (IC$_{50}$ of 14.4 $\mu M$ in a plaque-reduction assay) was recovered prior to ganciclovir therapy from a patient with chronic leukemia who had been treated intermittently with iv acyclovir. This isolate contained a V594 mutation in UL97 [10]. In another study, a CMV isolate with reduced susceptibility to ganciclovir (IC$_{50}$ of 7.1 $\mu M$ in a plaque-reduction assay) was recovered from a solid organ transplant recipient after a 14-day course of treatment with acyclovir [14]. Other studies have suggested that antiviral prophylaxis does not result in the selection of resistant CMV isolates. For instance, none of 6 urine CMV isolates recovered from 6 patients with AIDS treated with acyclovir
Table 1. Summary of clinical, phenotypic, and genotypic data for CMV isolates.

<table>
<thead>
<tr>
<th>Patient/isolate</th>
<th>Site CMV isolated</th>
<th>Days after transplant</th>
<th>Antiviral regimen (days)</th>
<th>CMV syndrome</th>
<th>Ganciclovir IC50 (μM)</th>
<th>Sequence</th>
<th>Restriction digest</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>Blood</td>
<td>66</td>
<td>ACV (66)</td>
<td>Viremia</td>
<td>2.0</td>
<td>K449</td>
<td>Neg</td>
</tr>
<tr>
<td>2A</td>
<td>Blood</td>
<td>56</td>
<td>ACV (70)</td>
<td>Viremia</td>
<td>2.4</td>
<td>S510</td>
<td>ΔS510</td>
</tr>
<tr>
<td>3A</td>
<td>Blood</td>
<td>62</td>
<td>ACV (66)</td>
<td>Viremia</td>
<td>0.7</td>
<td>wt</td>
<td>Neg</td>
</tr>
<tr>
<td>4A</td>
<td>Blood</td>
<td>70</td>
<td>ACV (71)</td>
<td>Viremia</td>
<td>2.7</td>
<td>wt</td>
<td>Neg</td>
</tr>
<tr>
<td>5A</td>
<td>Blood</td>
<td>55</td>
<td>ACV (59)</td>
<td>Viremia</td>
<td>1.6</td>
<td>wt</td>
<td>Neg</td>
</tr>
<tr>
<td>6A</td>
<td>Blood</td>
<td>23</td>
<td>ACV (23)</td>
<td>Viremia</td>
<td>1.6</td>
<td>Y469</td>
<td>Neg</td>
</tr>
<tr>
<td>7A</td>
<td>Blood</td>
<td>41</td>
<td>ACV (41)</td>
<td>Viremia</td>
<td>1.0</td>
<td>wt</td>
<td>Neg</td>
</tr>
<tr>
<td>8A</td>
<td>Blood</td>
<td>66</td>
<td>ACV (70)</td>
<td>Viremia</td>
<td>1.2</td>
<td>wt</td>
<td>Neg</td>
</tr>
<tr>
<td>9A</td>
<td>BAL</td>
<td>137</td>
<td>ACV (118)</td>
<td>Pneumonitis</td>
<td>0.8</td>
<td>wt</td>
<td>Neg</td>
</tr>
<tr>
<td>10A</td>
<td>Lung</td>
<td>41</td>
<td>ACV (41)</td>
<td>Pneumonitis</td>
<td>0.6</td>
<td>K449</td>
<td>Neg</td>
</tr>
<tr>
<td>11A</td>
<td>Blood</td>
<td>46</td>
<td>ACV (47), GCV (42)*</td>
<td>Viremia</td>
<td>1.4</td>
<td>Y469</td>
<td>Neg</td>
</tr>
<tr>
<td>12A</td>
<td>Blood</td>
<td>106</td>
<td>ACV (34), GCV (64)</td>
<td>Pneumonitis</td>
<td>5.1</td>
<td>V594</td>
<td>ΔS594</td>
</tr>
<tr>
<td>13A</td>
<td>Blood</td>
<td>153</td>
<td>ACV (76), GCV (55)</td>
<td>Viremia</td>
<td>2.5</td>
<td>wt</td>
<td>Neg</td>
</tr>
<tr>
<td>14A</td>
<td>Blood</td>
<td>24</td>
<td>ACV (35)</td>
<td>Viremia, pneumonitis</td>
<td>0.5</td>
<td>S510</td>
<td>ΔS510</td>
</tr>
<tr>
<td>14B</td>
<td>BAL</td>
<td>49</td>
<td>ACV (50), GCV (9)</td>
<td></td>
<td>3.7</td>
<td>S510</td>
<td>ΔS510</td>
</tr>
<tr>
<td>14C</td>
<td>Blood</td>
<td>98</td>
<td>ACV (50), GCV (58)</td>
<td></td>
<td>4.7</td>
<td>S510</td>
<td>ΔS510</td>
</tr>
<tr>
<td>15A</td>
<td>Blood</td>
<td>28</td>
<td>ACV (38)</td>
<td>Viremia</td>
<td>4.0</td>
<td>wt</td>
<td>Neg</td>
</tr>
<tr>
<td>15B</td>
<td>Blood</td>
<td>119</td>
<td>ACV (38), GCV (73)</td>
<td></td>
<td>&gt;25</td>
<td>V594</td>
<td>ΔS594</td>
</tr>
<tr>
<td>15C</td>
<td>Blood</td>
<td>162</td>
<td>ACV (38), GCV (116)</td>
<td></td>
<td>12.2</td>
<td>QS20, V594</td>
<td>ΔS20, ΔS594</td>
</tr>
<tr>
<td>15D</td>
<td>Blood</td>
<td>237</td>
<td>ACV (38), GCV (132)</td>
<td></td>
<td>13.4</td>
<td>QS20</td>
<td>ΔS20</td>
</tr>
</tbody>
</table>

NOTE. BAL = bronchoalveolar lavage fluid; ACV = acyclovir; GCV = ganciclovir; wt = wild type sequence; Δ = mutation present at indicated codon; Neg = no mutation detected.
* This patient had CMV pneumonia (documented by biopsy) and completed 42-day course of GCV therapy before bone marrow transplantation.

In a study by our group, none of 17 CMV isolates from solid organ transplant recipients who developed active CMV infections after receiving prophylaxis with acyclovir were resistant to ganciclovir [16]. However, these results were based on ganciclovir susceptibility testing alone. These isolates are currently being analyzed to determine whether any of the viruses contain ganciclovir resistance UL97 mutations.

In the current study, all CMV isolates from patients who had received only acyclovir prophylaxis were susceptible to ganciclovir, but analysis of UL97 revealed the presence of mutations (K449, Y469, S510) in 5 of the isolates. In a previous report [8], a Gln-to-Arg mutation at UL97 codon 449 (R449 mutation) was found in 11 ganciclovir-susceptible isolates and 1 ganciclovir-resistant isolate, and the Y469 mutation was found in a ganciclovir-susceptible isolate and a ganciclovir-resistant isolate [9]. These data suggest that K449 and Y469 are not related to antiviral resistance and most likely represent UL97 mutations that occur naturally among clinical CMV isolates.

The significance of S510 is less clear. This mutation has been found in one clinical CMV isolate resistant to ganciclovir (IC50 of 10.1 μM in a plaque-reduction assay) that also contained a deletion of UL97 codons 591–594 [9]. In our study, we found the S510 mutation in 4 isolates, all susceptible to ganciclovir. Whether S510 is a natural mutation or it is selected under antiviral pressure requires further investigation.
The S510 mutation has diagnostic importance. As shown in figure 1, the restriction pattern it causes can be mistaken with that of the true ganciclovir resistance mutation Q520. Therefore, laboratories using restriction digest analysis to screen for ganciclovir resistance UL97 mutations should include appropriate wild type and mutant CMV controls for each of the mutations being analyzed.

Three of the 4 isolates in our study that contained ganciclovir resistance UL97 mutations were resistant in the susceptibility assay we used. It is unlikely that this discrepancy in the estimated variance of the DNA hybridization assay was 0.05 μM and that the assay discriminated well between ganciclovir-resistant and susceptible controls [16]. The discrepancy between a resistant genotype (i.e., mutant UL97 sequence) and a susceptible phenotype (ganciclovir IC₅₀ ≤ 6 μM) could indicate that the sensitivity of the susceptibility assay for detection of isolates containing ganciclovir resistance UL97 mutations is lower than that of PCR sequencing methodologies. Because immunocompromised patients can harbor multiple CMV isolates, it is also possible that isolates selected by culture and tested in susceptibility assays were representative of a different virus population than that selected by PCR and characterized in sequencing studies.

In summary, our data indicate that CMV isolates containing ganciclovir resistance UL97 mutations may emerge rapidly in bone marrow transplant recipients treated with ganciclovir. Therefore, active CMV infections that occur in this setting should be considered as potentially caused by ganciclovir-resistant viruses. Whether these resistant isolates are selected for during prior prophylaxis with acyclovir would require additional studies.

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References