Emergence of Multiple Human Cytomegalovirus Ganciclovir-Resistant Mutants with Deletions and Substitutions within the UL97 Gene in a Patient with Severe Combined Immunodeficiency

DANA G. WOLF,* ISAAC YANIV, SHAI ASHKENAZI, AND ALIK HONIGMAN

Department of Clinical Microbiology and Infectious Diseases, Hadassah University Hospital, Jerusalem, and Schneider Children Medical Center, Petach Tiqwa, Israel

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Human cytomegalovirus (HCMV) is a major pathogen in immunocompromised individuals, especially in transplant recipients, patients with AIDS, and children with congenital immunodeficiency disorders (1, 10). With the availability of effective antiviral drugs, prolonged prophylactic and therapeutic regimens are increasingly employed. Ganciclovir, a nucleoside homologue, is the most widely used anti-HCMV drug (11). However, prolonged ganciclovir therapy can lead to the development of ganciclovir-resistant strains. HCMV ganciclovir resistance results mainly from impaired phosphorylation of the drug, caused by mutations in the HCMV UL97 phosphotransferase (2, 3, 7, 8, 21).

Until recently, ganciclovir-resistant strains have been recovered mainly from AIDS patients who received the drug for more than 3 months. With the widespread use of ganciclovir prophylaxis among transplant recipients, ganciclovir resistance is increasingly reported in the transplant setting, especially among solid-organ transplant recipients who have had prolonged exposure to ganciclovir (4, 14, 18). Recently, we described the early emergence of ganciclovir-resistant virus in children with primary combined immunodeficiency (22). Here, we report the unusual isolation of multiple drug-resistant variants, containing point mutations and different deletions of the UL97 gene, from one of these patients after 3 weeks of ganciclovir therapy.

The patient (patient 4) was a 5-month-old girl with B− severe combined immunodeficiency (SCID) who received haploidentical T-cell-depleted bone marrow transplantation. She was HCMV seropositive before transplantation and received a bone marrow graft from a seronegative donor. She had been treated with acyclovir for 3 weeks before secondary HCMV infection with HCMV pneumonia developed. Intravenous ganciclovir therapy (5 mg/kg of body weight twice daily) was initiated; however, the patient’s condition deteriorated, and a leukocyte culture 3 weeks after initiation of therapy was positive for HCMV.

Viral isolate propagation, plaque purification, and antiviral susceptibility assays of the isolate and the plaque-purified viruses were done as previously described (21). The laboratory-adapted ganciclovir-sensitive HCMV strain AD169 was included as a sensitive control with each assay. HCMV strains were considered sensitive to ganciclovir if their 50% effective doses (ED50) were ≤6 μM. The leukocyte isolate, the corresponding plasma specimen, and the plaque-purified viruses were subjected to direct PCR sequencing of the UL97 gene (21, 22), using primers encompassing nucleotides 1207 to 1979. The PCRs were performed under stringent conditions. Buffer controls and control samples were run along with test samples in each reaction. The positive control samples did not include ganciclovir-resistant strains. Sequence changes were confirmed by sequencing both strands of at least two independent PCR products, amplified in different PCR runs. To assess the genetic relatedness of the plaque-purified viruses and to determine the presence of one or a mixture of strains in the patient’s isolate, glycoprotein B (gB) genotype analysis of the isolate and of the plaque-purified viruses was carried out. For gB analysis, PCR products amplified with primers gB 1319 and gB 1604 (6) were digested with the restriction enzymes HinfI and Rsal, and the pattern that was obtained was classified on the basis of comparisons with control sequences.

The ganciclovir-resistant leukocyte isolate demonstrated mixed wild-type and mutant sequences in both methionine 460 and the proposed substrate binding site. The same sequence mixture found in the low-passage isolate was detected by direct sequence analysis of the corresponding plasma specimen, suggesting that the virus population isolated in vitro reflected the in vivo population. Since the mixed isolate demonstrated an unusual multiplicity of mutations, we examined the different variants composing the mixed population to identify the individual mutations and their susceptibility phenotypes. The UL97 gene sequences of 50 plaque-purified viruses, purified and propagated in the absence of ganciclovir, were analyzed. Eleven plaque-purified viruses contained mutations in the UL97 gene, and 39 had wild-type UL97 sequences. Six of the 11 mutated viruses (55% of the mutated viruses and 12% of the total population of plaque-purified viruses) contained single-nucleotide mutations resulting in single-amino-acid substi-
TABLE 1. HCMV UL97 amino acid alterations in plaque-purified viruses from a leukocyte isolate of a SCID patient

<table>
<thead>
<tr>
<th>UL97 gene mutation</th>
<th>No. of plaque-purified viruses (n = 50)</th>
<th>Ganciclovir ED50 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single-codon substitutions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Met-460-Val</td>
<td>3</td>
<td>12.2, 11.8, 18.7</td>
</tr>
<tr>
<td>Ala-594-Val</td>
<td>2</td>
<td>10.8, 15.6</td>
</tr>
<tr>
<td>Leu-595-Ser</td>
<td>1</td>
<td>15.7</td>
</tr>
<tr>
<td>Total</td>
<td>6 (12%)</td>
<td></td>
</tr>
<tr>
<td>Codon deletions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>590–600 (33 bp)</td>
<td>3</td>
<td>16.7, 23.4, 14.8</td>
</tr>
<tr>
<td>595 (3 bp)</td>
<td>2</td>
<td>14.2, 10.3</td>
</tr>
<tr>
<td>Total</td>
<td>5 (10%)</td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>39 (78%)</td>
<td>1.7–5.0; 2.9 ± 0.9°</td>
</tr>
</tbody>
</table>

* Mean values derived from three independent susceptibility assays for each virus. The laboratory strain AD169 (ED50, 3.1 ± 1.3 µM) was included as a control in each assay.

b Range and mean ± standard deviation of ED50s obtained for 21 randomly selected plaque-purified viruses.

tutions (Table 1). Five of the 11 strains (45% of the mutated viruses and 10% of the total) demonstrated different deletions of the UL97 gene: a novel deletion of 33 bp resulting in an 11-codon deletion at positions 590 to 600 was found in three independently plaque-purified ganciclovir-resistant viruses. All three viruses were replication competent, and their plaque sizes, morphologies, rates of spread, and cytopathic effects could not be distinguished from those of wild-type viruses. A 3-bp deletion, resulting in a deletion of residue 595, was found in two plaque-purified viruses. In contrast to the multiple UL97 variants, gB analysis revealed the presence of a single genotype (genotype 3) in the isolate and the plaque-purified viruses. This indicated that the patient was infected with a single strain from which several drug-resistant mutants developed under ganciclovir selection.

The majority of UL97 mutations which evolve in the clinical setting are single-nucleotide substitutions, suggesting a critical function for the protein in the virus life cycle. Although ganciclovir serves as a substrate, the UL97 protein is predicted to function as a protein kinase (5, 13), yet the role of the UL97 protein and its natural substrate in HCMV replication has remained undefined. Recently, it has been shown that the UL97 kinase is the target of a novel, highly effective antiviral benzimidazole compound (23). Moreover, a recombinant virus with a deletion of the entire UL97 catalytic domain exhibited a severe replication deficiency, further indicating that the UL97 gene plays a highly important role in HCMV replication (17). The clustering of ganciclovir resistance mutations at specific sites may identify active domains with distinct substrate specificities. The majority of the resistance-conferring mutations cluster in residues 460, 594, and 595 (7, 8, 21). Additional single-amino-acid substitutions at position 520 and between positions 590 and 607 have been described (3, 8, 12). Interestingly, we have detected all three prevalent mutations in the ganciclovir-resistant viruses isolated from the patient’s leukocytes. In addition, plaque-purified mutants containing a deletion of residue 595 and a deletion of 11 residues at positions 590 to 600 were identified. Deletions in the UL97 gene are not commonly found in clinical strains. Thus far, deletions at positions 595, 591 to 594, and, recently, 595 to 603 have been reported (2, 8, 9, 15, 19). The deletion of amino acids 590 to 600 described here is the largest identified in a clinical strain. The reports that smaller deletions within the 590-to-600 region, as well as a partially overlapping 9 codon deletion, confer ganciclovir resistance without affecting viral replication and the finding of the 11-codon deletion in replication-competent ganciclovir-resistant viruses which were independently purified suggest that the deletion confers ganciclovir resistance without impairing viral replication. Experiments are under way to confirm the impact of the deletion of amino acids 590 to 600 directly in a wild-type background by marker transfer. It is important to note that all the deletions occurring in the clinical setting are in-frame deletions which preserve the UL97 carboxy-terminal domain, suggesting a possible role for this domain in the recognition of the natural kinase substrate. Indeed, it has recently been shown that truncation of a recombinant UL97 protein at position 617 resulted in impaired protein kinase activity, as reflected in its impaired autophosphorylation (16).

The emergence of a mixed virus population during drug treatment has been described in immunocompromised individuals. Yet the finding of two different deletions along with multiple-amino-acid substitutions in one patient early during ganciclovir therapy is striking and may be related to the nature of the immunodeficiency in the SCID patient. The prior treatment with acyclovir could have contributed to the selection of UL97 variants, since acyclovir is also a substrate for the UL97 kinase (20). The identification of a previously uncharacterized UL97 mutation underscores the need for a versatile genotypic assay in the monitoring of patients receiving ganciclovir.

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REFERENCES


