Cytomegalovirus UL97 Phosphotransferase Mutations That Affect Susceptibility to Ganciclovir

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Most ganciclovir (GCV)–resistant cytomegalovirus (CMV) isolates contain UL97 gene mutations at codon 460 or 520 or between codons 590 and 607, where an increasing variety of mutations have been detected, including deletions. To determine their phenotypic effect, 9 UL97 mutations not previously studied were transferred to drug-sensitive laboratory CMV strains that contained unique restriction sites developed for this purpose. Deletion of the entire codon range 591–607 conferred a 6-fold increase in GCV resistance, with little effect on viral replication. Some mutations found in clinical isolates, including C592G and A594T, conferred only 2–3-fold decreases in GCV susceptibility. For C592G, this phenotype was confirmed by transfer to different CMV strains and by restoration of full drug susceptibility after removal of the mutation. Low drug levels resulting from oral GCV therapy may predispose the virus to the initial selection of these low-grade UL97 resistance mutations and to later accumulation of other mutations and greater resistance.

Ganciclovir (GCV) is currently the usual treatment for cytomegalovirus (CMV) disease in persons with AIDS and in transplant recipients [1, 2]. Prolonged therapy may result in the emergence of drug-resistant virus [3]. Most GCV-resistant clinical CMV isolates contain a mutation in the UL97 phosphotransferase gene [3–5]. UL97 mediates the initial phosphorylation of GCV, which is needed for its antiviral action [6–8]. Resistance mutations decrease GCV phosphorylation, apparently without impairing the poorly understood but important function of this enzyme in viral replication [9, 10]. Mutations in the viral DNA polymerase gene (UL54, pol) can also confer increased GCV resistance and cross-resistance to other drugs [11–14].

GCV resistance mutations in UL97 have been mapped to codons 460, 520, and 590–607 [3–5, 7, 15, 16]. Historically, the most common mutations in clinical isolates have been at codons 460 (M460V/I), 594 (A594V), and 595 (L595S) [3–5]. An increasing variety of GCV resistance mutations have now been reported at codons 460, 520, and 590–607, including deletions [17–19]. The recent widespread use of oral GCV appears to have altered the relative frequency of some mutations [20]. In the current study, we present an updated frequency distribution of UL97 GCV resistance mutations and report marker transfer studies on mutations that have not been characterized elsewhere.

The phenotypic effects of putative resistance mutations are confirmed by transfer of the mutation to a reference CMV strain (e.g., AD169 or Towne) by homologous recombination. Traditionally, this marker transfer has been accomplished by cotransfecting viral DNA from the reference strain with a DNA fragment (polymerase chain reaction [PCR] product or clone) that contains the desired mutation [4, 7] and then selecting with GCV to isolate the very small fraction of mutant recombinant virus that may result. This process is inefficient and unproductive for mutations that confer little or no drug resistance. Propagation in the presence of drug could also lead to unintended genetic changes elsewhere in the viral genome. To overcome these problems, we produced derivatives of CMV reference strains that contained unique restriction sites in UL97. Transfection of a mixture of restriction-digested viral genomic DNA and mutant UL97 DNA allowed the isolation of desired mutant viruses with much higher frequency and without the need for drug selection. Fifteen recombinant viruses were generated to examine the phenotypic effects of UL97 mutations observed in clinical CMV isolates.

Materials and Methods

Viruses. CMV strains Towne (ATCC VR-977) and AD169 (ATCC VR-538) were obtained from the American Type Culture Collection. Clinical isolates were obtained from subjects who had received GCV therapy and were referred from a variety of sources. Isolates studied in our laboratory that have been the subject of reports published elsewhere were included, so that the cumulative

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frequency of various UL97 mutations could be assessed. Multiple isolates from the same individual were not counted unless they contained different mutations, and mutations detected directly in clinical specimens without virus isolation were also excluded. Isolates were phenotypically resistant to GCV, as judged by an IC50 in plaque reduction assays of \( > 5 \mu M \) if accompanied by a UL97 or pol mutation or \( > 8 \mu M \) without a detectable mutation. A mutation was defined as an amino acid change not known to occur as normal strain variation in baseline, drug-susceptible CMV isolates [4, 5].

Mutant CMV DNA for marker transfer. A series of plasmid clones representing the UL97 region and containing desired genetic markers (restriction sites or mutations) were derived from plasmid ZC33 or ZC34, published elsewhere [19], each of which contains a 4.7-kb segment of the CMV Towne genome that corresponds to nt 139690–144436 of the AD169 sequence (EMBL X17403). A \( Pme \)I recognition site (GTTTAAAC) was introduced at codons 584–586 of UL97 in several clones. This modification had no effect on UL97 amino acid encoding (figure 1) but was expected to create a unique restriction site, because no \( Pme \)I sites exist in the published AD169 sequence.

PCR products containing \( Pme \)I recognition sites were prepared by use of mutant primer CPT1743M (5′-GGCGTTGCTGTTTAAACACGCCGGCGC-3′) paired with CPTT (5′-AAACAGACTGAGGGGGCTCATCGTC-3′) and CPT1457 (5′-AGCCCTATCCGGATTAACAACGCA-3′) paired with CPT1769M (5′-GCGCCGGCGTGTTTAAACAGCAACGCC-3′). The 2 separate PCR products were amplified with a \( Pwo \)-Taq polymerase mixture (Expand; Roche Molecular Biochemicals) from Towne strain DNA or from clinical isolate DNA that contained either the C592G or the L634Q mutation. Then the primers were removed from the PCR products, the 2 products were combined and were reamplified with CPT1528 and CPTY [19], which resulted in a UL97 PCR product that contained the \( Pme \)I site, with or without C592G or L634Q also present. The product was digested with restriction enzymes \( Pst \)I and \( Xho \)I at naturally existing sites and was cloned into ZC33 or ZC34 [19], which had been digested with the same enzymes. The resulting DNA clones were ZC40 (figure 1), containing the \( Pme \)I site; SC49 (figure 2), containing the \( Pme \)I site; and L634Q and SC55 (figure 1), containing the \( Pme \)I site and C592G.

Several other PCR products, which contained mutations targeted for marker transfer (table 1), were amplified from corresponding clinical isolates by use of CPT1528 and CPTY, were digested with \( Pst \)I and \( Xho \)I, and were used to create UL97 mutant plasmid clones from ZC34, by exchange of the corresponding restriction fragment. Finally, codons 591–607 (del591–607) were artificially deleted by use of forward and reverse primers (5′-CTCTTTAAGCCACGCCCGCGGTCTCTTCTTGGCGCCGGA-3′ and 5′-TTGCGCCCGCCAGAATGTAGACGCCGGCGCGGTCTTTAAGCC-3′) and Towne strain DNA and then were cloned into ZC33 by use of the same mutagenesis approach used for the \( Pme \)I site, as described above, producing clone ZC38 (containing del591–607).

Following a similar site-directed mutagenesis approach, a PCR product (PCR 43362) that contained both the \( Pme \)I recognition site at codon 584–586 and the L595S mutation was amplified by use of primers CPT1088 [4] and CPTY [19], starting from an AD169 recombinant virus that contained L595S [4]. This product (PCR 43362) was used for introduction of the 2 markers into strain AD169 (figure 3). All the mutant plasmids we used to derive recombinant viruses were checked, by sequencing the inserted DNA, for the presence of the intended mutations and the absence of errors.

Figure 1. A, Overlapping Towne-derived cosmids used to construct recombinant virus T1141. Cosmids cover the entire Towne cytomegalovirus (CMV) genome, except for a gap between Tn42 and Tn39. B, Gap between Tn42 and Tn39 is filled with plasmid ZC40, containing the desired UL97 sequence change. Nucleotide numbering is based on a published AD169 sequence (EMBL X17403). C, UL97 codon 581–595 sequences showing change from wild-type sequence to one that contains the \( Pme \)I recognition site in plasmids and recombinant CMV strains.
Towne strain cosmid recombinant virus. Cosmid clones (Tn15, Tn24, Tn45, Tn47, Tn42, Tn39, and Tn46) obtained from CMV Towne (non-ATCC source), as described elsewhere [21], were propagated in *Escherichia coli* strain Top10F, and their DNA was extracted by use of a commercial kit (Tip-500; Qiagen). These overlapping clones cover the entire Towne genome, except for the segment corresponding to AD169 nt 141,452–143,824 (figure 1). Each cosmid (2 μg) was digested with *Pac*I, and all were blended with ZC40 (0.3 μg) containing the *Pme*I restriction site in UL97, which had been digested with *Kpn*I and *Not*I. The mixture was ethanol precipitated and redissolved, and a calcium phosphate precipitate was made, followed by transfection into human embryonic lung fibroblasts, as described elsewhere [21]. After 2 weeks in cell culture, CMV cytopathology was observed. Extracellular virus (T1141) was prepared and characterized by sequencing of the entire UL97 and *pol* coding regions and by drug sensitivity phenotyping.

**Figure 2.** Relationships and properties of Towne cosmid–derived recombinant cytomegaloviruses (CMVs). For each recombinant, UL97 mutations are listed, along with the ganciclovir (GCV) IC₅₀ value and its ratio to that of the parental virus (T1141 or T1432). *Pme*I, restriction site at codons 584–586.

Towne strain cosmid recombinant virus. Cosmid clones (Tn15, Tn24, Tn45, Tn47, Tn42, Tn39, and Tn46) obtained from CMV Towne (non-ATCC source), as described elsewhere [21], were propagated in *Escherichia coli* strain Top10F, and their DNA was extracted by use of a commercial kit (Tip-500; Qiagen). These overlapping clones cover the entire Towne genome, except for the segment corresponding to AD169 nt 141,452–143,824 (figure 1). Each cosmid (2 μg) was digested with *Pac*I, and all were blended with ZC40 (0.3 μg) containing the *Pme*I restriction site in UL97, which had been digested with *Kpn*I and *Not*I. The mixture was ethanol precipitated and redissolved, and a calcium phosphate precipitate was made, followed by transfection into human embryonic lung fibroblasts, as described elsewhere [21]. After 2 weeks in cell culture, CMV cytopathology was observed. Extracellular virus (T1141) was prepared and characterized by sequencing of the entire UL97 and *pol* coding regions and by drug sensitivity phenotyping.

**CMV genomic DNA.** Viral DNA for transfection was extracted from fibroblast cultures that showed 100% cytopathology at 1 week after inoculation. Infected cells were dislodged into the medium, sonicated with a microtip for 15 s, and then centrifuged at 5000 g for 15 min. The supernatant was ultracentrifuged for 30 min at 85,000 g in a Ti70 rotor (Beckman), and the pellet was resuspended in PBS and was ultracentrifuged once again. The pellet was resuspended in 50 mM Tris (pH 8.0) and 10 mM EDTA. Proteinase K (final concentration, 50 μg/mL) and SDS (final concentration, 0.6%) were added, and the mixture was incubated at 37°C for 1 h. The digest then was extracted with 1:1 phenol:chloroform, then butanol, and the DNA was ethanol precipitated.

**AD169 strain recombinants.** Recombinants containing the *Pme*I recognition site were derived from virus T1078 (figure 3), which was obtained by traditional cotransfection methods [4] that used wild-type AD169 genomic DNA and PCR 43362. Strain T1078, which contained both the *Pme*I site and mutation L595S, was isolated after 2 passages under 30 μM GCV and was plaque purified 3 times, once under 30 μM GCV and twice without drug. The triply plaque-purified virus then was sequenced in all the UL97 and *pol* coding regions, was phenotyped for GCV sensitivity, and was used for derivation of other AD169 recombinant viruses.

**UL97 mutagenesis.** CMV genomic DNA was extracted from recombinant virus T1141 or T1078; 20 μg of the DNA was digested overnight with 4 U/μg of restriction enzyme *Pme*I and was mixed with 2 μg of cloned DNA (figures 2 and 3) that contained the desired mutations or restriction sites, which had been digested with *Kpn*I and *Not*I to release the CMV sequences. This DNA mixture was coprecipitated with calcium phosphate and was transfected into fibroblasts, as described elsewhere [4]. CMV cytopathology was generally visible at ~10 days, and the infection was allowed to proceed to involve the entire monolayer. In the first experiment, in
which the del591–607 mutation was transferred, virus was propagated 3 times under low concentrations of GCV (5 \( \mu M \)), to select for recombinant virus (as with the traditional marker transfer technique), but this GCV selection was found to be unnecessary and was omitted entirely in subsequent experiments. Supernatant virus then was plaque purified without drug, and extracellular virus stock was prepared, titered, and characterized by phenotype and by sequencing.

**Sequence analysis.** DNA sequencing of UL97 and pol was performed by fluorescent dideoxy chain termination PCR on products of viral DNA extracts, using a standard kit (ABI Big Dye) and an automated sequencer (ABI 377). Multiple sequencing primers representing both strands of UL97 and pol were used, spaced ~300 bases apart [4]. Clinical isolates first were sequenced to check for mutations in 79 cytomegalovirus isolates. For recombinant viruses representing both strands of UL97 and was omitted entirely in subsequent experiments. Supernatant virus then was plaque purified without drug, and extracellular virus stock was prepared, titered, and characterized by phenotype and by sequencing.

**Drug sensitivity phenotyping.** The IC\(_{50}\) value was determined in 24-well fibroblast monolayer cultures, as described elsewhere [22]. In brief, the same quantity (~80 pfu) of virus was inoculated into each of multiple wells, using cell-associated virus for clinical isolates and cell-free virus stock for laboratory strains and recombinants. After a 90-min adsorption period, the inocula were removed, and the wells were overlaid with a range of 2-fold GCV dilutions, starting with a no-drug control well and ending at 24 or 48 \( \mu M \), depending on the level of resistance of the virus being tested. The drug overlays contained agarose, to prevent secondary plaque formation. After 1 week, the plates were fixed and stained, and the plaques in each well were counted visually. The IC\(_{50}\) value was calculated by interpolation after fitting an exponential curve to the graph of the plaque count versus drug concentration. Because of some interassay variability in IC\(_{50}\) that resulted from the growth condition of cell cultures and infection conditions, testing of recombinants was done \( \geq 3 \) times, simultaneously with testing of the parental strain. The mean and SD of the IC\(_{50}\) values, along with the ratio of IC\(_{50}\) values to a simultaneous control, were used to judge the phenotypic effects of transferred mutations.

**Viral growth kinetics.** Replicate 25-cm\(^2\) cell culture flasks that contained 9 \( \times 10^5 \) human foreskin fibroblasts were inoculated at an MOI of 0.05 pfu/cell with extracellular virus stocks of Towne strain or derivative recombinants, including one with a deletion of codons 591–607 (T1219). The culture was maintained with 5 mL of Eagle MEM with 10% fetal calf serum. On each of the following 6 days, a 1-mL aliquot was removed and was frozen at ~80°C in 10% dimethyl sulfoxide for later virus titration, and 1 mL of fresh medium was replaced. At the end of the experiment, serial 10-fold dilutions were made of each thawed aliquot and were inoculated into 24-well cluster plates that contained confluent fibroblasts, then were overlaid with medium that contained 0.4% agarose. After 1 week of incubation, wells that contained well-separated plaques were counted to determine the titer of infectious virus in each aliquot of frozen medium.

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### Table 1. Frequency of specific UL97 ganciclovir (GCV) resistance mutations in 79 cytomegalovirus isolates.

<table>
<thead>
<tr>
<th>WT AA</th>
<th>Mut AA</th>
<th>Codon</th>
<th>No. (%) of isolates*</th>
<th>Mean GCV IC(_{50}) (range), ( \mu M )*</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>V</td>
<td>460</td>
<td>11 (14)</td>
<td>16 (9.8–29)</td>
</tr>
<tr>
<td>M</td>
<td>I</td>
<td>460</td>
<td>7 (9)</td>
<td>11 (8.4–13)</td>
</tr>
<tr>
<td>H</td>
<td>Q</td>
<td>520</td>
<td>4 (5)</td>
<td>18 (6.7–45)</td>
</tr>
<tr>
<td>A</td>
<td>V</td>
<td>591</td>
<td>2 (3)</td>
<td>4</td>
</tr>
<tr>
<td>C</td>
<td>G</td>
<td>592</td>
<td>10 (13)</td>
<td>7.5 (5.0–11)</td>
</tr>
<tr>
<td>A</td>
<td>T</td>
<td>594</td>
<td>1 (1)</td>
<td>7.9</td>
</tr>
<tr>
<td>A</td>
<td>V</td>
<td>594</td>
<td>23 (29)</td>
<td>19 (7.8–50)</td>
</tr>
<tr>
<td>L</td>
<td>S</td>
<td>595</td>
<td>18 (23)</td>
<td>21 (7.8–50)</td>
</tr>
<tr>
<td>L</td>
<td>F</td>
<td>595</td>
<td>3 (4)</td>
<td>21</td>
</tr>
<tr>
<td>L</td>
<td>W</td>
<td>595</td>
<td>2 (3)</td>
<td>15</td>
</tr>
<tr>
<td>E</td>
<td>G</td>
<td>596</td>
<td>1 (1)</td>
<td>7.4</td>
</tr>
<tr>
<td>L</td>
<td>Del</td>
<td>600</td>
<td>1 (1)</td>
<td>11</td>
</tr>
<tr>
<td>C</td>
<td>W</td>
<td>603</td>
<td>2 (3)</td>
<td>48</td>
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<tr>
<td>C</td>
<td>F</td>
<td>607</td>
<td>1 (1)</td>
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<tr>
<td>C</td>
<td>Y</td>
<td>607</td>
<td>1 (1)</td>
<td></td>
</tr>
<tr>
<td>Del</td>
<td>591–594</td>
<td>1 (1)</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

Any UL97 mutation 76 (96)

*Isolates containing indicated mutation by itself or in combination with other UL97 or pol mutations.

**NOTE.** Underlined mutations were examined by marker transfer (present study). Marker transfers have been published on all other mutations shown. Del, deletion at indicated codon(s); Mut AA, mutation amino acid; WT AA, wild-type amino acid.

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**Figure 3.** Relationships and properties of AD169-derived recombinants. Ganciclovir (GCV) IC\(_{50}\) values are listed; ratios are based on T1195 tested simultaneously. PCR, polymerase chain reaction.
Results

Relative frequency of UL97 resistance mutations. The cumulative frequencies of detection of various UL97 mutations in 79 GCV-resistant clinical isolates referred to our laboratory are shown in table 1. Consistent with earlier reports [4, 5], the data confirm that mutations at codons 460, 594, and 595 are the most common, but the occurrence of mutations at codon 592 (C592G) has increased since the last tally [5], such that it is now the fourth most common resistance mutation (13% of isolates). Ten isolates had >1 UL97 mutation.

GCV resistance phenotypes of clinical isolates. GCV-resistant clinical isolates had IC50 values, as determined by plaque reduction, that ranged widely but were usually 10–20 μM when a single mutation was present (table 1). Four independent clinical CMV isolates that contained only the C592G mutation and no other known UL97 or pol resistance mutations had a lower mean GCV IC50 value of 7.5 ± 2.6 μM. In contrast, 6 other isolates that contained C592G in combination with other mutations in UL97 or pol had a mean IC50 value of 32 ± 15 μM. Three isolates that were phenotypically resistant to GCV had no UL97 mutations; 1 had a DNA polymerase mutation (reported elsewhere [11]), and 2 others remain genetically undiagnosed, with IC50 values of 12 and 18 μM. One isolate, not included in table 1, had a borderline GCV IC50 of 6.8 μM, no mutations previously associated with GCV resistance, and an unusual amino acid change at codon 634 (L634Q), which was examined by marker transfer. The mutation A591V was first noted in a clinical isolate with a GCV IC50 of 14.5 μM. Further analysis showed that the isolate also had the pol mutation P522S, a known GCV resistance marker [14]. An earlier isolate from the same subject with only the A591V mutation, and not P522S, had a GCV IC50 of 4 μM, which is generally considered to be within the susceptible range.

Characterization of Towne cosmid recombinant virus. Recombinant virus T1141, which resulted from transfection of the cosmid set and ZC40 into human fibroblasts, was sequenced in UL97 and was confirmed to have the intended PmeI site at codons 584–586 (figure 1). Otherwise, the UL97 amino acid sequence was the same as that of the Towne strain, except for 1 codon (329) where a mutation (D329H) was observed. This mutation occurred at a location corresponding to the end of cosmid Tn42 but was not present in Tn42 itself or in ZC40, the overlapping DNA clones representing this region (figure 1) that were used to make T1141. The mutation may have occurred, therefore, during cosmid recombination. The pol sequence of T1141 was unchanged from that of the Towne (ATCC) strain. T1141 appeared to have the same cytopathology and growth properties as the Towne strain; 8 days after inoculation of fibroblast cultures at an MOI of 0.1, extracellular virus stock was produced that had an infectivity titer of 4.9 × 106 pfu/mL. Plaque reduction susceptibility testing against GCV showed an IC50 of 4.5 ± 0.9 μM (figure 2). The Towne (ATCC) strain had an IC50 of 6.4 ± 1.3 μM.

Towne strain recombinant viruses from T1141. To produce recombinant viruses that contained specific UL97 mutations, viral genomic DNA from T1141 was digested with PmeI and was cotransfected into fibroblasts with cloned DNA ZC38, SC49, and SC55, representing mutations del591–607, L634Q, and C592G, respectively (figure 2). Recombinant virus T1219 (figure 2) was selected under 5 μM GCV and then was triply plaque purified without drug. Sequence analysis of UL97 revealed the desired deletion of 17 codons (del591–607) and reversion of codon 329 to wild type (D329). There were no other amino acid changes in UL97 or in pol. T1219 had cytopathology that appeared to be normal in plaque size and morphology. Despite the 17-codon deletion, the virus had the same growth properties as wild-type virus (figure 4). GCV susceptibility testing by plaque reduction (figure 2) showed a 6.2-fold increase in IC50 values over those of parental strain T1141. This amount of increase in the GCV IC50 is similar to that observed with the common UL97 resistance mutations examined elsewhere by marker transfer [4].

Recombinant virus T1220 (figure 2) was isolated without any drug selection. At the initial plaque purification of virus resulting from cotransfection, 6 plaques were sequenced, and the major species present in all 6 was found to be the L634Q mutation, with some minor mixture of wild-type sequence also present. After 3 rounds of plaque purification, sequencing showed only the L634Q amino acid change, the presence of the PmeI restriction site, and reversion of codon 329 to wild type. There were no other amino acid changes in UL97 or in pol. The virus had the same plaque properties as the parental strain, and GCV susceptibility testing (figure 2) showed no drug resistance.

Recombinant virus T1308 (figure 2) was isolated without any drug selection. At initial plaque purification, sequencing showed 4 of 6 plaques to be positive for the C592G mutation and the other
2 to be mixtures of C592G and wild type (C592). After 2 further rounds of plaque purification, sequence analysis of UL97 revealed the desired C592G amino acid change, the presence of the PmeI restriction site (figure 1), and reversal of codon 329 to wild type (D329). There were no other amino acid changes in UL97 or in pol. The virus produced normal-appearing plaques, and GCV susceptibility testing showed a modest but reproducible 2.5-fold increase in GCV resistance (figure 2).

Although no drug selection had been used to produce the recombinant T1308 and no selection for extraneous mutations was expected, the introduced C592G mutation was removed to confirm the predicted reversion from low-grade GCV resistance to normal susceptibility. Viral genomic DNA was extracted from T1308, digested with PmeI, and cotransfected with ZC40 (figure 2). A new recombinant virus (T1432) was isolated without drug selection and was plaque purified 3 times. Sequence analysis of UL97 revealed no amino acid changes from the Towne sequence (absence of C592G and D329H mutations) and the presence of the PmeI site (codons 584–586). Plaque reduction testing showed normal GCV susceptibility similar to that of T1141 (figure 2). The virus also grew in the same manner as wild-type Towne strain (figure 4). Because of these features, T1432 was adopted as the parental virus for subsequent marker transfers into Towne strain.

Additional marker transfers into strain T1432. After digestion of T1432 genomic DNA with PmeI and cotransfection with digested plasmids that contained various UL97 mutations that had not previously been transferred (table 1), recombinant viruses were isolated. For each of the mutations A591V, A594T, L595W, E596G, del600, and C607F, sequencing of the unpurified recombinant virus resulting from transfection showed only the intended mutation and no evidence of the wild-type configuration. After plaque purification and resequencing of the entire UL97 and pol coding sequences to confirm the UL97 mutation and the absence of other UL97 or pol mutations, the GCV resistance phenotype was determined. As shown in figure 2, most of these mutations conferred only slight decreases in GCV susceptibility (GCV IC₅₀ increased 1.3–2.7-fold over a simultaneous T1432 control). The mutation L595W conferred a higher (5-fold) level of GCV resistance. These results are consistent with available IC₅₀ data from clinical isolates that contained these mutations in isolation (table 1).

AD169-based recombinants. Selection under GCV resulted in a recombinant virus T1078 that was shown by sequencing to contain both the PmeI restriction site at codons 584–586 and the GCV resistance mutation L595S (figure 3). There were no other UL97 or pol amino acid changes from strain AD169. As expected, the L595S mutation in T1078 increased the GCV IC₅₀ 5-fold (figure 3). PmeI-digested T1078 DNA was cotransfected with plasmids that contained a PmeI site in UL97, with or without mutation C592G also present (figure 3). This resulted in the derivation of AD169-based viruses that contained the desired UL97 sequence changes. Recombinant virus T1195 resulted from cotransfection with plasmid ZC40. UL97 sequence analysis showed the PmeI site at codons 584–586 and the amino acid change I244V, the latter of which is a Towne strain variation introduced by plasmid ZC40. Recombinant T1337 contained the same PmeI site and the mutation C592G in UL97; there were no other UL97 amino acid changes from strain AD169. Plaque reduction testing (figure 3) showed GCV IC₅₀ to be elevated 2.6-fold, similar to that of the corresponding Towne-based mutant. Genomic DNA was extracted from T1337, digested with PmeI, and cotransfected with plasmid ZC40 to remove the C592G mutation, resulting in recombinant virus T1425, which had GCV susceptibility restored to the wild-type level shown by AD169 and T1195. The UL97 sequence of T1425 contained the PmeI site at codons 584–586 and the amino acid changes N108S and I244V (introduced by plasmid ZC40).

Discussion

The cumulative data presented here confirm and extend our earlier findings on the frequency of specific mutations in the UL97 phosphotransferase gene of clinical CMV isolates resistant to GCV. On the basis of analysis of ~3 times as many GCV-resistant isolates as before [4, 5], mutations at codons 460 (M460V and M460I), 594 (A594V), and 595 (L595S) remain the most common, but the mutation C592G has become more frequent and now occurs in 13% of all isolates examined. Recent reports have shown an increasing variety of mutations in the codon range 590–607, including deletions [17–19, 23–25], that have been found in resistant isolates. The increased frequency of C592G and some other UL97 mutations that confer only low-grade GCV resistance is probably due to the lower drug levels attained with use of oral GCV.

Marker transfer studies, in which putative resistance mutations are transferred to laboratory strains of CMV to examine their phenotypic effect, have now been performed for most of the known UL97 resistance mutations. Earlier work has shown that mutations at codons 460 [4, 26], 520 [15], 594 [4], 595 [4, 17, 27], 599 [28], 603 [13], and 607 [16], along with deletions of codons 590–593 [7] and 595–603 [19], all conferred GCV resistance on laboratory CMV strains, usually resulting in a 5–10-fold elevation of IC₅₀ values. In this article, we have shown that the entire codon 591–607 range can be deleted without major effects on viral growth, while conferring 6-fold increased GCV resistance. This finding may explain the tendency of many different resistance mutations to cluster in this area and is consistent with the hypothesis that this region of UL97 is involved with GCV substrate recognition and not with the kinase function of UL97 that is important for viral replication [9].

New marker transfers were performed for 8 UL97 mutations detected in clinical isolates. Other than L595W, which conferred as much GCV resistance as the more common mutation L595S, the mutations studied conferred no more than a low-grade GCV resistance. Notable in this regard was C592G, which was the
most common mutation detected in subjects who received oral GCV (37% of subjects showing genotypic resistance [20]). The low level of resistance conferred (2–3-fold increase in IC50) was confirmed by transfer of C592G to different strains of CMV (Towne and AD169) without in vitro drug selection and by removal of the mutation to restore wild-type susceptibility. Clinical isolates that contained only C592G likewise displayed low-grade GCV resistance, and isolates often contained C592G in combination with other mutations that increased the overall level of drug resistance. These data suggest that the low blood levels achieved with oral GCV permit the selection of UL97 mutations that initially confer low-grade resistance, which then predispose the virus to mutations with greater levels of resistance as additional mutations accumulate or are substituted. Mutation A594T, which was also relatively frequent among oral GCV recipients (15% of subjects with genotypic resistance [20]), may play a similar role, along with A591V and other less common mutations at codons 590–607. Mutations in this codon range confer varying degrees of GCV resistance, ranging from insignificant to ~10-fold. The lesser degrees of resistance may escape detection by phenotypic assays [22, 29], because some clinical isolates that contained C592G were found to have GCV IC50 values < 6 μM, and one isolate that contained A591V had an IC50 value of 4 μM. Isolates are not generally considered to be GCV resistant at IC50 values < 6 μM. Although mutations such as A591V confer little GCV resistance, they have not been observed in baseline isolates from untreated subjects [30] and may, therefore, be considered to result from drug selection pressure. The only amino acid variation noted so far in baseline isolates in the codon range 590–607 is at codon 605 (D605E) [30].

An open question at this time is whether UL97 mutations outside codons 460, 520, or 590–607 can confer significant GCV resistance. Because the UL97 sequence is highly conserved among clinical isolates, changes at codons outside these ranges in isolates from patients who receive GCV might represent additional instances of resistance mutations. However, our experience with L634Q suggests that the phenotypic effects of these mutations need to be investigated by marker transfer. We showed that mutation L634Q conferred no appreciable GCV resistance, even though the corresponding clinical isolate had a borderline GCV IC50 unexplained by any other UL97 or pol mutation. It remains a possibility that some mutations confer GCV resistance only in certain CMV strains or viral genetic backgrounds, although this has not been demonstrated.

Construction of well-defined mutant CMV recombinants is the best way to confirm the significance of a mutation found in a resistant isolate. Ideally, to avoid selection of unintended mutations, these marker transfers should be performed without in vitro drug selection and should be accompanied by rescue experiments in which the introduced mutation is removed and the original phenotype restored. This rigorous standard has not been met in prior CMV resistance work. Newer approaches to the construction of CMV recombinants, in which cosmid clones [14, 21] or bacterial artificial chromosomes [31] have been used as sources of the viral genome, are facilitating this work, although it remains a challenge to create recombinants that contain only the desired point mutation, with the rest of the viral genome entirely unaltered. Unintended mutations may sometimes occur during recombination, as shown in Towne cosmid recombinant T1141 by the change at UL97 codon 329 (D329H). Fortunately, this mutation did not affect the GCV susceptibility phenotype (T1141 vs. T1432), and codon 329 reverted to wild type in all 3 recombinants further derived from T1141. Misleading results caused by artificial genetic changes can be reduced by minimizing the number of recombination events used to create the mutant virus and by independent transfers to different viral strains.

When the marker transfers reported here were initially attempted by classical methods that used GCV selection in cell culture, no recombinant viruses were isolated. This is now explainable by the low level of resistance conferred and the low recombination frequency. We used 2 technical approaches, therefore, to create derivatives of Towne and AD169 CMV strains that contained a unique PmeI site in the UL97 coding region, with no effect on amino acid encoding. There are no natural PmeI recognition sites anywhere in the published sequence of CMV strain AD169 (EMBL X17403) and in the available sequences for Towne strain. Digestion of viral genomic DNA with PmeI results in a well-defined break that can be repaired by homologous recombination with a single overlapping mutant DNA segment. This made the targeting of a single locus more efficient, provided a parental virus for phenotypic comparison, and enabled the removal of inserted mutations to rescue the original phenotype. These PmeI-modified viral strains (such as T1432 or T1195) are useful for the construction of other mutants to explore the biological function of UL97 and for the recombinant phenotyping assays that have taken on an increasing role in molecular viral diagnostics, because conventional virus isolation is now often abandoned in favor of rapid assays that are dependent on detecting viral sequences directly in clinical specimens (e.g., leukocytes or plasma).

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References


