Viral DNA Polymerase Mutations Associated with Drug Resistance in Human Cytomegalovirus

Sunwen Chou, 1 Nell S. Lurain, 2 Kenneth D. Thompson, 2 Richard C. Miner, 4 and W. Lawrence Drew 4

1Medical and Research Services, Veterans Affairs Medical Center, and Division of Infectious Diseases, Oregon Health & Science University, Portland, Oregon; 2Department of Immunology/Microbiology, Rush-Presbyterian-St. Luke’s Medical Center, and 3Department of Pathology, University of Chicago, Chicago, Illinois; 4Departments of Laboratory Medicine and Medicine, University of California, San Francisco, Mount Zion Medical Center, San Francisco, California

Certain mutations in the viral DNA polymerase (pol) gene are known to confer drug resistance when transferred to susceptible human cytomegalovirus (CMV) strains, whereas other putative resistance mutations remain unproven. A new marker-transfer technique was used to produce recombinant CMV strains, to determine the drug susceptibility phenotypes conferred by 10 pol mutations (9 observed in clinical isolates). Various degrees of resistance to ganciclovir and cidofovir were conferred by mutations D301N, N410K, D413E, T503I, and L516R, which are located within exonuclease functional domains where D301N and D413E affect highly conserved residues. Mutations A692S, E756K, and E756D, which are not located within recognized functional domains, each conferred foscarnet resistance. This study significantly increases the number of confirmed CMV pol resistance mutations, at both conserved and nonconserved loci, with implications for molecular mechanisms and the genotypic diagnosis of antiviral resistance.

Ganciclovir, cidofovir, and foscarnet are the systemic antiviral drugs that are currently licensed for treatment of human cytomegalovirus (CMV) infection. All have been in use for some years, and all 3 drugs, in their active forms, target the viral DNA polymerase (pol) (CMV gene UL54). Viral resistance to these drugs has been observed both after selection in cell culture and in clinical isolates (reviewed in [1]). Until the advent of combination antiretroviral therapy, most instances of CMV drug resistance occurred in patients with AIDS receiving long-term treatment for invasive CMV disease, such as retinitis. More recent trends in antiviral drug therapy have been accompanied by increasing reports of resistant CMV in transplant recipients [1–3].

Resistance to ganciclovir, the most widely used antiviral drug, is most commonly due to a mutation in the viral UL97 phosphotransferase gene, a mutation that reduces the initial phosphorylation of ganciclovir and thereby impairs its conversion to the triphosphate compound that inhibits the viral DNA pol [4]. However, mutations in the viral pol gene can also result in ganciclovir resistance that is generally accompanied by cidofovir cross-resistance. Pol mutations usually appear after prolonged therapy and may increase the drug resistance beyond that conferred by a preexisting UL97 mutation [5]. Foscarnet-resistance mutations have also been mapped to the viral pol gene [1]. Mutations conferring foscarnet resistance are largely distinct from those conferring ganciclovir-cidofovir resistance, but single pol mutations conferring multidrug resistance have been reported [6].

Because of the slow growth of CMV in cell culture, the most practical means of laboratory diagnosis of drug-resistant virus is genotypic testing for resistance-related mutations, supported by information on the interstrain pol sequence variation among drug-susceptible isolates [7]. Because multiple mutations may occur in a genome as large as that of CMV (230 kb), individual mutations suspected of conferring drug resistance are validated by a process of marker transfer whereby the mutation is transferred to a drug-susceptible...
tible CMV laboratory strain. Genotypic and phenotypic assays of the resulting mutant virus are then used to determine the properties conferred by the mutation in question. Traditional techniques of homologous recombination, followed by selection under drug in cell culture, were used to produce mutant viruses for the study of several CMV pol resistance mutations [1]. However, these procedures were inefficient, in that a very small fraction of mutant virus had to be isolated from a population dominated by wild-type virus. In a single study, a cosmid-based recombination system [8] was used to overcome this difficulty and to characterize several additional CMV pol mutations. However, we have only occasionally been able to produce viable recombinants by use of this method, probably because multiple recombination events and error-free cosmids are required to reconstitute a single complete genome.

After the recent successful application of laboratory CMV strains modified to contain a unique restriction site in the UL97 gene [9], we used a similar approach for the marker transfer of pol mutations. We describe here the introduction of Pmel restriction-recognition sites into the pol gene of the Towne strain of CMV, sites that do not otherwise exist in the viral genome. Pmel digestion of viral genomic DNA then results in a targeted break in the pol gene, a break that can be repaired by homologous recombination with cloned mutant pol DNA. This method enabled both the rapid isolation of mutant recombinant viruses, without drug selection, in cell culture and the phenotypic characterization of a number of mutations in the pol gene.

**MATERIALS AND METHODS**

**Viruses.** Clinical CMV isolates, including several previously reported isolates that had not been analyzed by marker-transfer analysis, were obtained from transplant recipients and patients with AIDS [10, 11]. The laboratory Towne strain of CMV was obtained from American Type Culture Collection (VR977). A ganciclovir-resistant mutant (L516R) was selected CMV was obtained from American Type Culture Collection patients with AIDS [10, 11]. The laboratory Towne strain of CMV, sites that do not otherwise exist in the viral genome. Pmel digestion of viral genomic DNA then results in a targeted break in the pol gene, a break that can be repaired by homologous recombination with cloned mutant pol DNA. This method enabled both the rapid isolation of mutant recombinant viruses, without drug selection, in cell culture and the phenotypic characterization of a number of mutations in the pol gene.

**Mutant CMV DNA.** Bluescript plasmid clones of the Towne strain CMV pol gene region containing introduced Pmel restriction sites, with or without amino acid changes to be analyzed by marker transfer, were derived by polymerase chain reaction mutagenesis [9] of a subclone of cosmid Tn47 [8]. This subclone of Towne strain corresponds to nt 74828–82550 of the strain AD169 numbering scheme (GenBank accession no. X17403) and is bounded by XbaI and EcoRI sites, respectively (figure 1). Pmel restriction sites were created by mutagenesis at codons 453 and 897 of the pol coding sequence, resulting in amino acid changes at codons 897 and 898 (figure 1), changes that are common polymorphisms of the pol gene present in many CMV isolates [7]. A further derivative, containing the dual Pmel restriction sites and the resistance mutations P522S and V781I [8], was used initially for selection of recombinant virus in cell culture. An 8-kb EcoRI fragment of the clinical strain D16 [12] (codons 74118–82550) was also cloned, a fragment that contained several amino acid differences (from the Towne strain) in the pol sequence.

**Marker transfer.** Laboratory CMV strains (Towne and derivatives) grown from extracellular virus were propagated in human foreskin fibroblast cultures, and viral genomic DNA was extracted from infected cell cultures, by use of detergent and proteinase K, followed by phenol extraction, as described elsewhere [9]. DNA from derived strains containing Pmel restriction sites was digested by Pmel (4 U/μg of DNA) (New England Biolabs), at 37°C overnight, in the buffer specified by the manufacturer. Cotransfection of fibroblast cultures with 15 μg of viral genomic DNA and 2 μg of plasmid (linearized with restriction enzymes XbaI and EcoRI) containing the desired pol mutations was performed by the calcium phosphate precipitation method [9]. After trypsinization and passage of the cells, at 4–7-day intervals, viral cytopathology was generally evident at 10–14 days. A sample of the infected cell culture was screened for the desired pol mutation, and supernatant virus was plaque purified at least 2 times after the stage at which DNA sequencing revealed both the presence of mutant virus and the absence of the parental sequence. No drug selection was used in cell culture, except in the initial experiments performed to create the Pmel restriction sites in strain T1330.

DNA sequencing was performed by fluorescent dideoxy chain-termination, on polymerase chain reaction–generated templates, by use of both an automated sequencer (ABI 377) and the kit supplied by the manufacturer (Big Dye Terminator; Applied Biosystems). Multiple primers within the pol gene were used to obtain overlapping sequences, which were assembled and aligned with the AD169 reference sequence [7]. Recombinant viruses resulting from marker transfer were checked, in their entire pol and UL97 coding sequences, for both the presence of the desired mutations and the absence of unintended changes.

**Drug-susceptibility assays by plaque reduction.** Plaque-reduction assays for clinical isolates and for recombinants were performed as described elsewhere [13]. The IC_{50} was determined in fibroblast monolayers growing in 24-well culture plates that had been inoculated with standardized amounts of virus and were then covered with agarose-containing media containing various concentrations of drugs (ganciclovir, cidofovir, foscarnet). After 7–10 days, plates were fixed and were stained, and plaques were counted visually. Assays were performed at least in triplicate and were averaged. Similar to those of a previous study [8], IC_{50} values that were increased >2-fold.
Figure 1. Construction of cytomegalovirus recombinant T1472 containing Pmel sites in polymerase (pol) gene. FOS, foscarnet; GCV, ganciclovir.

above the value obtained simultaneously from a susceptible parental virus were regarded as evidence of resistance.

Drug-susceptibility assays by yield reduction. To confirm the results of plaque-reduction assays, a second phenotyping method was developed. Fibroblast monolayers in 24-well plates were inoculated with cell-free virus stock, at an MOI of ~0.01, and were incubated for 1 week under a fixed concentration of drug (ganciclovir, 4 μmol/L; foscarnet, 150 μmol/L; or cidofovir, 0.8 μmol/L). A control monolayer was inoculated similarly but was grown without added drug. Supernatant virus was collected and was frozen in aliquots for subsequent quantitation. Virus was quantitated by spin inoculation of diluted supernatant, onto separate fibroblast monolayers, in 24-well plates, and was assayed the following day by staining for the CMV immediate-early antigen, by use of monoclonal antibody L-14 in an immunoperoxidase procedure, as described elsewhere [14]. The number of nuclei staining positive for the CMV antigen was counted by both digital photomicroscopy and
age analysis software (ImageJ; National Institutes of Health). This measure of the infectivity of the supernatant virus was used to compare the virus yield obtained without drug to that obtained with drug. The ratio was calculated as the yield-reduction factor; lower yield-reduction factors indicate greater drug resistance. For baseline drug-susceptible strains, drug concentrations were empirically selected to give 20–40-fold yield reductions. On the basis of results from both control strains without mutations and resistant viruses, as documented by plaque reduction, yield-reduction factors of either ≤15 for both ganciclovir and cidofovir or ≤10 for foscarnet were interpreted as evidence of resistance. Assays were performed at least in triplicate and were averaged.

Viral-growth curve. Replicate monolayers of human foreskin fibroblast cultures, in 12-well plates, were inoculated with either Towne strain viruses or derived mutant recombinants, at an MOI of 0.01. The virus yield in the supernatant was monitored at intervals, over multiple cycles of viral replication, during a 7-day period. Cultures were maintained with 4 mL of modified Eagle medium with 10% fetal calf serum. Each day, half of the culture medium was removed and was frozen at −80°C, for later titration. Fresh medium was added to replace the aliquot that had been removed. At the end of the experiment, dilutions of the frozen supernatants were spin inoculated onto 24-well fibroblast cultures, and, on the following day, the virus titer was determined by immediate-early antigen staining [14] of infected cell nuclei. Positively stained cells were enumerated by digital photomicroscopy and image analysis software, as in the yield-reduction assays above.

RESULTS

Mutations and sources of isolates containing mutations. After plaque purification under ganciclovir, 2 ganciclovir-cidofovir double-resistant strains (D16 and D19) were recovered as minor components of a single bronchoalveolar lavage isolate, from a heart-transplant recipient [12] who had not received antiviral therapy. D16 contained the pol mutation D301N, and D19 contained the mutation T503I. Both strains, as well as the drug-susceptible component of the isolate, showed 6 other amino acid changes (from Towne strain) in the pol gene. D16 was also suspected to be slightly resistant to foscarnet [12].

Other mutations occurring in or near regions linked to ganciclovir-cidofovir resistance are N410K, D413E, D515G, L516R, and G971D. Mutation N410K was found in a ganciclovir-resistant blood isolate obtained from the same patient with AIDS who had provided the semen isolate reported elsewhere [6]. Like the semen isolate, the blood isolate also contained UL97 mutation L595S, although the pol mutations differed. D413E was found in a ganciclovir-cidofovir–resistant isolate that also contained UL97 mutation H520Q [10]. D515G was found in a drug-susceptible clinical isolate [7] but was chosen for marker transfer because it is adjacent to L516R, which was found in a ganciclovir-resistant virus derived from laboratory strain AD169. G971D was found in a ganciclovir-resistant isolate obtained from a heart-transplant recipient who had received antiviral therapy [2]; the isolate also contained UL97 mutation L595S.

Mutations A692S, E756K, and E756D were possibly linked to foscarnet resistance. A692S was reported in an isolate of unknown foscarnet susceptibility obtained from a subject who had received ganciclovir, foscarnet, and cidofovir [15]. We also observed this mutation in 2 other isolates, which had slightly elevated foscarnet IC_{50} values (470 μmol/L and 530 μmol/L). E756D was found in both an isolate obtained from a subject treated with ganciclovir and foscarnet [10] and an independent foscarnet-resistant isolate. E756K was identified in a foscarnet-resistant, ganciclovir-susceptible isolate [11].

Towne strain derivative containing unique restriction sites. A reference drug-susceptible Towne strain derivative was created in 2 stages (figure 1). In the first stage, a recombinant virus (T1330) containing Pmel restriction sites, along with mutations P522S and V781I conferring ganciclovir and foscarnet resistance, was created by cotransfecting Towne strain genomic DNA with a mutant plasmid, followed by 3 cycles of drug selection, the last of which comprised 20 μmol/L ganciclovir and 400 μmol/L foscarnet. In the next stage, T1330 DNA was digested by Pmel and was cotransfected with a Towne-derived plasmid containing the Pmel restriction sites but without the mutations P522S and V781I. The resulting infectious CMV (T1472), which was isolated without the use of any selective medium, was plaque purified 3 times and was designated for use as a reference strain. Sequencing of T1472 showed no changes (from Towne strain) in UL97. In the pol region, the Pmel sites and associated amino acid changes S897L and D898N were verified (figure 1), and no other changes were found. Susceptibility testing by plaque reduction and yield reduction showed the virus to be susceptible to all 3 drugs—ganciclovir, cidofovir, and foscarnet—as had been expected (table 1).

Marker-transfer analysis of pol mutations. Genomic viral DNA from strain T1472 was digested by Pmel and was cotransfected, into fibroblast cultures, with linearized plasmids containing 1 of the various mutations studied. Recombinant viruses containing the desired mutations were routinely identified in the infectious CMV initially recovered from the transfected cultures. After plaque purification, extracellular virus stocks were prepared, were titrated, and were phenotyped by plaque-reduction and yield-reduction assays; results are shown in table 1. Mutation D301N conferred ganciclovir-cidofovir resistance and did not confer foscarnet resistance. The degree of ganciclovir-cidofovir resistance was less when transferred as a single mutation (T1891) and was greater in the pol sequence.
context of its clinical isolate (T1636), which contains 6 other amino acid changes (from Towne strain). Mutations N410K and D413E conferred moderate ganciclovir-cidofovir resistance. The reproducible >800-fold yield reduction of the D413E mutant, under 150 μmol/L foscarnet, indicates hypersusceptibility to this drug. This was confirmed by assaying under varying concentrations of foscarnet, resulting in an IC50 of 52 μmol/L for this mutant, compared with an IC50 of 106 μmol/L for the parental T1472 control virus. The amino acid change D515G, which was not associated with phenotypic resistance in a clinical isolate, was confirmed to not confer resistance, despite its being located adjacent to L516R, which was shown to confer ganciclovir-cidofovir resistance. Mutation T503I also conferred ganciclovir-cidofovir resistance without effect on foscarnet susceptibility. The mutations A692S, E756D, and E756K each conferred similar levels of foscarnet resistance, with E756K also showing, by plaque reduction, some ganciclovir resistance and borderline cidofovir resistance. Mutation G971D did not confer detectable resistance to any of the 3 drugs.

**Effects on viral growth.** Infectious virus yields were determined from supernatants obtained at intervals after inoculation, at low MOIs, with the parental virus T1472 and the various resistant mutant recombinants. Figure 2 shows that the most attenuated virus, T1891 containing the single pol mutation D301N, had virus yields at days 2–6 that were 1–2 log10 lower than those of parental viruses T1472 and Towne, even allowing for variation in virus inocula (day 0). Other pol amino acid changes in the corresponding clinical isolate, as contained in virus T1636, appeared to compensate for this growth attenuation, because T1636 was consistently found to outgrow T1891. Other resistant mutants (D413E, T503I, L516R, E756K, E756D, and A692S) were modestly attenuated, a result that is similar to the curve for E756K shown in figure 2.

**Resequencing of mutant virus propagated under ganciclovir.** The mutation D301N is located in a highly conserved

### Table 1. Drug susceptibility of cytomegalovirus (CMV) recombinants resulting from marker transfer.

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>pol mutation</th>
<th>GCV μmol/L</th>
<th>Ratioa</th>
<th>FOS μmol/L</th>
<th>Ratioa</th>
<th>CDV μmol/L</th>
<th>Ratioa</th>
<th>Yield-reduction factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Towne (ATCC)</td>
<td></td>
<td>3.2</td>
<td>120</td>
<td>0.32</td>
<td></td>
<td>37</td>
<td>41</td>
<td>27</td>
</tr>
<tr>
<td>T1472</td>
<td></td>
<td>2.3</td>
<td>128</td>
<td>0.19</td>
<td></td>
<td>30</td>
<td>22</td>
<td>23</td>
</tr>
<tr>
<td>T1299</td>
<td>981–982 deletion</td>
<td>48</td>
<td>8.3</td>
<td>298</td>
<td>3.6</td>
<td>1.7</td>
<td>2.8</td>
<td>2</td>
</tr>
<tr>
<td>T1636</td>
<td>D301N</td>
<td>32</td>
<td>5.6</td>
<td>166</td>
<td>1</td>
<td>6.6</td>
<td>7.3</td>
<td>4</td>
</tr>
<tr>
<td>T1891</td>
<td>D301N</td>
<td>14.7</td>
<td>2.6</td>
<td>88</td>
<td>0.5</td>
<td>2.7</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>T1912</td>
<td>N410K</td>
<td>6.7</td>
<td>2.9</td>
<td>108</td>
<td>0.8</td>
<td>0.57</td>
<td>3</td>
<td>14</td>
</tr>
<tr>
<td>T1819</td>
<td>D413E</td>
<td>11</td>
<td>4.8</td>
<td>105</td>
<td>0.8</td>
<td>0.81</td>
<td>4.3</td>
<td>10</td>
</tr>
<tr>
<td>T1910</td>
<td>T503I</td>
<td>16.7</td>
<td>2.9</td>
<td>91</td>
<td>0.5</td>
<td>5.5</td>
<td>6.1</td>
<td>4</td>
</tr>
<tr>
<td>T2020</td>
<td>D515G</td>
<td>7.9</td>
<td>1</td>
<td>230</td>
<td>1.2</td>
<td>0.8</td>
<td>1</td>
<td>26</td>
</tr>
<tr>
<td>T2027</td>
<td>L516R</td>
<td>16</td>
<td>2.1</td>
<td>150</td>
<td>0.8</td>
<td>4.1</td>
<td>5.1</td>
<td>11</td>
</tr>
<tr>
<td>T2005</td>
<td>A692S</td>
<td>5.7</td>
<td>1.6</td>
<td>476</td>
<td>3.3</td>
<td>1.7</td>
<td>1.7</td>
<td>16</td>
</tr>
<tr>
<td>T1674</td>
<td>E756D</td>
<td>6.9</td>
<td>1.2</td>
<td>575</td>
<td>3.4</td>
<td>0.6</td>
<td>0.7</td>
<td>18</td>
</tr>
<tr>
<td>T1671</td>
<td>E756K</td>
<td>8.2</td>
<td>3.5</td>
<td>&gt;1000</td>
<td>&gt;8</td>
<td>0.41</td>
<td>2.2</td>
<td>13</td>
</tr>
<tr>
<td>T1974</td>
<td>G971D</td>
<td>6.9</td>
<td>1.4</td>
<td>150</td>
<td>1.2</td>
<td>0.5</td>
<td>1</td>
<td>30</td>
</tr>
</tbody>
</table>

**NOTE.** Values indicating drug resistance are shown in bold type. CDV, cidofovir; FOS, foscarnet; GCV, ganciclovir.

---

Figure 2. Growth curve of recombinant viruses. Day 0 value is based on quantitation of virus inoculum.
To look for evidence of a hypermutator phenotype possibly resulting from such a mutation, recombinant virus T1891 was passaged 1 time under 3 μmol/L ganciclovir and 7 times (2 times as infected cells and 5 times as extracellular virus) under 5 μmol/L ganciclovir. The extracted viral DNA was then resequenced in the UL97 and pol gene regions. Results show the continued presence of D301N in the pol gene, and no other mutations during replication under drug were found.

**DISCUSSION**

An improved marker-transfer method was developed to determine the drug-resistance phenotypes of previously uncharacterized CMV pol mutations found in either drug-resistant clinical isolates or regions of the gene associated with resistance. These phenotypes confirmed the existence of several ganciclovir-cidofovir–resistance conferring mutations within pol exonuclease domains, including the highly conserved codons 301 and 413. Mutations conferring foscarnet resistance were confirmed at nonconserved codons 692 and 756. The number of proven drug-resistance mutations in CMV pol has now been increased by ~30%.

The locations of these mutations, combined with the mutations previously validated by marker transfer (figure 3), provide insight into the molecular mechanisms by which pol mutations confer resistance to current anti-CMV drugs. Although no direct crystallographic data are available to place the mutations within a 3-dimensional context, the CMV polymerase belongs in the large DNA polymerase B family [16]. Sequence homologies were initially used to identify conserved regions of probable functional significance [17], as shown in figure 3. Crystallographic findings concerning other members of the polymerase B family, including the DNA polymerase of the T4 bacteriophage RB69 [18–20], may be used to deduce the structural significance of CMV pol mutations.

Most of the known ganciclovir-cidofovir resistance mutations (figure 3) map to exonuclease domains located between codons 300 and 545, a finding suggesting that relative enhancement of exonuclease activity, to facilitate the excision of misincorporated nucleotides, could be a favored mechanism of resistance to these drugs. Two mutations confirmed by the current study (D301N and D413E) are remarkable for their direct correspondences to highly conserved aspartate residues (D114 and D222) in the RB69 polymerase located at the center of the exonuclease active site [19]. D301N involves the nearly invariant “FDIE” ExoI motif that is present in both the RB69 [18] and herpesvirus polymerases [17]. The free carboxyl groups of the D and E residues in this motif were considered to be essential for magnesium binding, exonuclease activity, and viral replication [21–23]. The D301N mutation observed here does not appear to result in a severe exonuclease deficit, because the virus was viable, and multiple passages under ganciclovir did not yield detectable new mutations in either UL97 or pol, as might be expected of a virus with a high replication error rate. The free amide group of asparagine (N) may be an adequate functional substitute for the aspartate (D) that is normally present, although the D301N mutant virus T1891 is attenuated in growth by 1–2 log_{10} at various sampling intervals (figure 3). The growth defect was less, and the ganciclovir-cidofovir resistance was increased, in the D301N mutant T1636, which also contained several other pol amino acid changes that were present in the clinical isolate. The effect of background

---

**Figure 3.** Map of cytomegalovirus DNA polymerase functional domains, resistance mutations, and associated phenotypes. All listed mutations have been found in clinical isolates and have been validated by marker transfer (italics = this study). Shaded regions indicate where resistance mutations are clustered, with associated phenotypes indicated below.
sequence variation in pol on the growth and resistance phenotype of specific point mutations has not been adequately studied. Other mutations in the exonuclease domains (figure 3) are located at varying distances from the active site and often affect nonconserved residues, such as N410K, T503I, and L516R, which have been shown here to confer varying degrees of ganciclovir-cidofovir resistance.

The confirmed ganciclovir-cidofovir resistance mutations in region V (figure 3) are A987G and a deletion of codons 981–982. The homologous region in the RB69 polymerase is located at the “thumb” region of the enzyme and not at the polymerase catalytic center. In T4 phage polymerase, a mutation in this vicinity (A737V) increased the relative exonuclease activity of the enzyme, as shown by increased deoxynucleoside triphosphate (dNTP) utilization [20, 24]. The G971D mutation at a nonconserved residue just outside region V, although newly appearing in an isolate obtained after antiviral therapy, did not confer appreciable drug resistance when transferred in isolation (table 1).

Crystallographic data from the RB69 polymerase also helped in the interpretation of foscarnet-resistance mutations now validated at diverse codons (588, 692–821, 981–982) (figure 3). On the basis of its structure, foscarnet is expected to inhibit the pyrophosphate exchange that occurs during attachment of new bases to the growing DNA strand. The RB69 “fingers” residues involved in this process include R482, K486, and K560, which interact with the phosphate groups of the incoming nucleotide pol [20]; the corresponding CMV residues are R784, R788, and K811. RB69 residues D411, L415, Y416, L561, Y567, G568, and D623, which are involved in the recognition of the incoming nucleotide pol [20], correspond to residues 717, 721, 722, 812, 818, 819, and 912 of the CMV enzyme. Many known foscarnet-resistance mutations in CMV are located quite close to these 2 sets of residues, a finding supporting the hypothesis that foscarnet would interfere with the addition of incoming nucleotides. The function of these residues in attaching the incoming base could also explain why a moderate degree of ganciclovir resistance has been reported in connection with some foscarnet mutations in region III [1]. In the present study, other foscarnet mutations were confirmed at more distant nonconserved codons (692 and 756), a finding showing that not all foscarnet mutations can be mapped to phosphate-binding sites, and some may act through more distant conformational effects or at other functional sites. We also noted foscarnet hypersusceptibility in the D413E mutant, by yield-reduction assay, that is possibly related to increased dNTP utilization by the mutant. Foscarnet hypersusceptibility has been observed in a T4 polymerase [25] mutated at a structurally similar position (R226H).

Because the overall rate of DNA replication is dependent on the rate of addition of new bases, offsetting any losses due to exonuclease and editing activity, resistance mutations at both the exonuclease and polymerase domains can be expected to have some effect on the growth rate of the virus. Growth attenuation has been reported elsewhere for mutations at codons 513 [26], 700, and 715 [27] and has been observed here for mutations at codon 301 and, to a lesser extent, at codons 413, 503, 516, 692, and 756. Slowly growing mutants may affect some phenotypic assays; for example, the delayed appearance of plaques may be misinterpreted as drug susceptibility in plaque-reduction assays.

The increasing diversity of pol resistance mutations suggests that, for genotypic-resistance testing, the most reliable approach is sequencing of codons 300–1000, with special attention to the shaded areas in figure 2. There are insufficient data to document the relative frequency of the various pol resistance mutations, but it is unlikely that a few mutations are dominant, as is seen in the UL97 gene [9]. Resistance mutations are not tightly localized, and not all mutations located near other resistance mutations will confer significant resistance. These findings are illustrated by the discordant phenotypes shown by D515G (no resistance) versus L516R (ganciclovir-cidofovir resistance), E756D/K (foscarnet resistance) versus V759M (no resistance [8]), and G971D (no resistance) versus del981–982 (multiresistant [6]).

As with the UL97 gene [9], the development of laboratory CMV strains modified with unique restriction sites in the pol gene facilitates marker transfer or recombinant phenotyping, with advantages of rapid isolation of the desired mutants and avoidance of artifacts resulting from drug selection in cell culture. Mutations can be added (and subsequently removed, if desired) regardless of the degree of drug resistance conferred. This will enable a wider variety of both pol mutations and combinations of mutations to be studied.

Acknowledgments

We thank Gail Marousek, Matthew Shiveley, Barbara Sisson, Kevin Michels, Rachel Waldemer, and Anne Senters, for technical assistance.

References

SA. High-level resistance of cytomegalovirus to ganciclovir is associated with alterations in both the UL97 and DNA polymerase genes. J Infect Dis 1997; 176:69–77.