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A Nine-Codon Deletion Mutation in the Cytomegalovirus UL97 Phosphotransferase Gene Confers Resistance to Ganciclovir

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A deletion mutation (codons 595 to 603) in the cytomegalovirus (CMV) UL97 gene was recently reported after sequence analysis of leukocyte DNA from a patient receiving ganciclovir. The corresponding viral phenotype was examined by transfer of this mutation to a laboratory CMV strain (strain Towne). The recombinant virus was resistant to ganciclovir (8.4-fold increase in the 50% inhibitory concentration), was sensitive to foscarnet, and replicated normally in cell culture.

Drug resistance that develops after prolonged ganciclovir therapy for human cytomegalovirus (CMV) infection usually involves a mutation in the viral UL97 phosphotransferase gene (3). UL97 is responsible for the initial phosphorylation of the drug, which is necessary for its antiviral activity (15). Mutations in the CMV DNA polymerase gene may also contribute to ganciclovir resistance (6, 7, 9).

Previous studies have documented that mutations at UL97 codons 460, 520, and 591 to 607 are associated with ganciclovir resistance in clinical CMV isolates (1–6, 10), with amino acid substitutions at codons 460, 594, and 595 being the most common (4, 5). Deletion mutations conferring ganciclovir resistance are relatively unusual. Reported cases include a one-codon deletion at codon 595 (2) and a four-codon deletion (codons 590 to 593 or 591 to 594) (5, 15). Recently, a nine-codon deletion (UL97 codons 595 to 603) was detected in DNA extracted from leukocytes and colon tissue of a renal transplant recipient after prolonged exposure to ganciclovir (12). Although this mutation was associated with treatment-resistant CMV disease, no viral isolate was available from this patient to confirm the drug resistance phenotype that was suspected to be conferred by this mutation. Additionally, because this was the largest UL97 deletion that had been reported in a clinical specimen, the question of its replication competence arose, since UL97 activity appears to be necessary for normal CMV replication and laboratory mutants containing large deletions in UL97 are either difficult to make or are very much attenuated (13, 14). We therefore performed experiments to transfer the deletion from codons 595 to 603 into a laboratory CMV strain (strain Towne) in order to examine its associated phenotype.

The transfer of the deletion into CMV Towne (VR-977; American Type Culture Collection, Manassas, Va.) was performed by homologous recombination as published previously (4). The virus was propagated in human fibroblasts, and Towne DNA was extracted from culture supernatants by ultracentrifugation and lysis of the viral pellet with sodium dodecyl sulfate and proteinase K, followed by phenol-chloroform extraction and ethanol precipitation (4). A PCR product (PCR41999) containing a segment of the CMV UL97 gene (from codon 510 onward) was amplified from a leukocyte extract of the patient previously reported to have the UL97 deletion from codons 595 to 603 (12). The PCR primers were CPT1528 (5′-AACT GCTCGCACCGTCTGCGC-3′) and CPTY (5′-GGTGAGCT CCTCATCGTCGTCG-3′). PCR41999 was digested with restriction enzymes *Pst*I and *Xho*I, taking advantage of naturally occurring restriction sites within PCR41999 corresponding to nucleotides 142070 and 142713, respectively, of the published complete CMV sequence (GenBank accession no. X17403). The resulting 0.64-kb fragment was isolated by agarose gel electrophoresis and was cloned into Bluescript vector pBS2KS+ (Stratagene, La Jolla, Calif.), forming plasmid pZC32 (Fig. 1). A 4.7-kb segment of the Towne genome, corresponding to nucleotides 139690 to 144436 of the complete CMV sequence (Genbank accession no. X17403) and incorporating the UL97-coding sequence, was subcloned from cosmid Tn*26* (11) by digestion with restriction enzymes *Not*I and *Kpn*I, followed by preparative agarose gel electrophoresis and ligation into vector pBS2KS+, forming plasmid pZC33 (Fig. 1). The UL97 segment of pZC32 containing the deletion from codons 595 to 603 was then swapped into pZC33 (Fig. 1), forming pZC34. Homologous recombination was achieved by cotransfection of 20 μg of Towne DNA and 2 μg of pZC34 digested with *Not*I and *Kpn*I into subconfluent human foreskin fibroblast monolayers by the calcium phosphate precipitation method (4). A viral cytopathic effect was noted at 9 days posttransfection, and the resulting extracellular virus was propagated twice in the presence of 20 μM ganciclovir and was then plaque purified once in the presence of 20 μM ganciclovir and twice in the presence of no drug.

The entire 2.1-kb UL97-coding sequence and the 3.7-kb DNA polymerase-coding sequence of plaque-purified (three times) virus T963-5-6-2 were sequenced by PCR amplification and multiple fluorescent dideoxy sequencing reactions (6). The DNA polymerase-coding sequence was identical to that of the Towne strain (GenBank accession no. D14980), and the UL97-coding sequence had no amino acid changes compared with the sequence of the Towne strain (GenBank accession no. U07355) other than the deletion from codons 595 to 603. The presence of the same deletion as in pZC34, along with absence of any other mutations in UL97 or DNA polymerase, the only known CMV genes that affect ganciclovir susceptibility, was interpreted as evidence that the deletion confers ganciclovir resistance, similar to conclusions drawn from results of studies of other UL97 marker transfers (1–4, 6, 10).

Cell culture susceptibility testing by plaque reduction (8) of recombinant virus T963-5-6-2, simultaneously performed three

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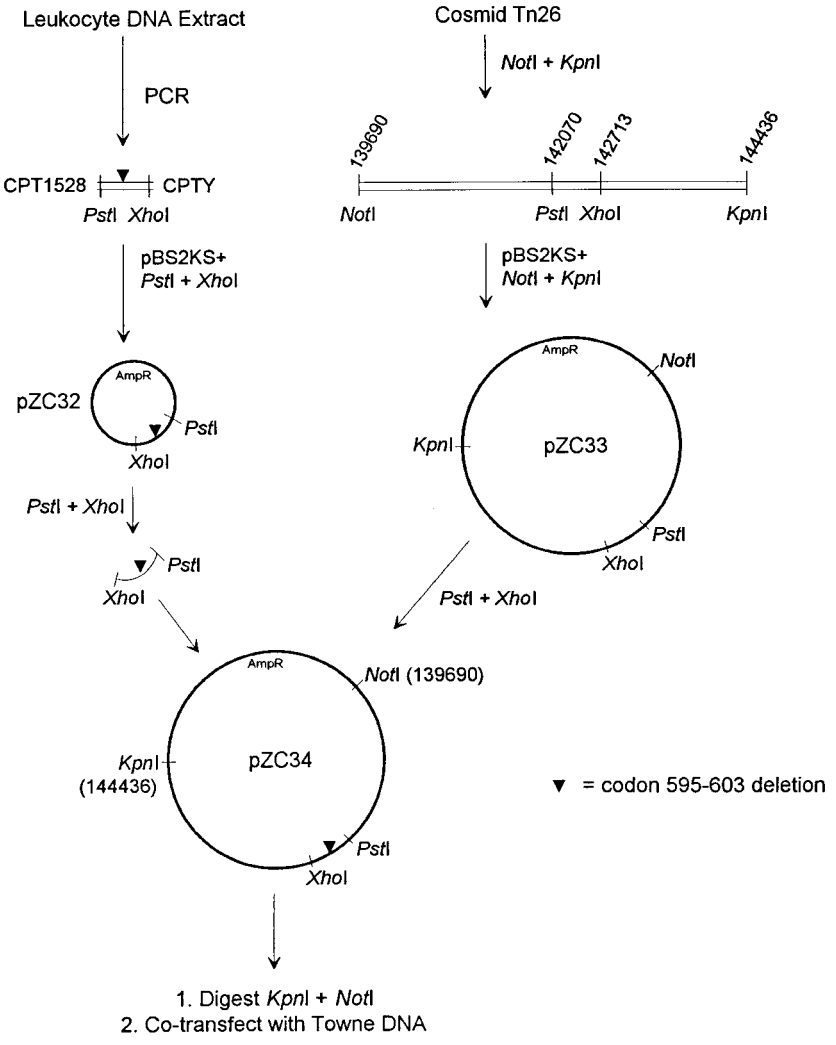

FIG. 1. Strategy for construction of CMV recombinant containing the deletion from codons 595 to 603.

times with a wild-type Towne strain as a control, gave a mean ganciclovir 50% inhibitory concentration (IC_{50}) of 24.4 μM (standard deviation [SD], 2.9 μM) and a mean foscarnet IC_{50} of 119 μM (SD, 31.5 μM). The mean ganciclovir IC_{50} for the Towne control virus was 3.1 μM (SD, 1.2 μM), and the mean foscarnet IC_{50} was 91 μM (SD, 9.6 μM). The ganciclovir IC_{50} for the recombinant virus was 8.4-fold (SD, 2.7-fold) increased over that for the Towne control strain, whereas the foscarnet IC_{50} was 1.3-fold that for the Towne control strain; the difference was considered to be not statistically significant. The degree of ganciclovir resistance is similar to the 7- to 10-fold increases reported previously for UL97 mutants (4, 6) and higher than the 3.5- to 5.3-fold increases reported for DNA polymerase mutants (6, 7).

The recombinant virus T963-5-6-2 was observed to form normal-sized plaques and a cytopathic effect in fibroblast culture; the plaque sizes and cytopathic effects are comparable to those of the Towne strain at the same intervals postinfection. After inoculation of 7×10^5 fibroblasts with the same number of PFU of T963-5-6-2 or the Towne strain control, the virus titers in the supernatant were comparable at several time points afterward, as assayed by plaque titration on fibroblast monolayers (Fig. 2). We therefore conclude that the recombi-

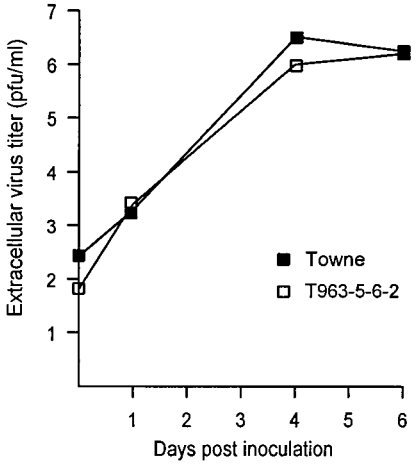

FIG. 2. Comparative growth curves of recombinant virus and Towne strain control. The day 0 value was determined 4 h after removal of the original inoculum.

nant virus retains full replication competence.

Results of this study strengthen the current impression (3) that mutations at codons 460, 520, and 591 to 607 (or possibly slightly beyond codon 607) confer ganciclovir resistance and that genotypic screening of UL97 for ganciclovir resistance should focus on this relatively limited range of codons. Furthermore, since CMV strains with deletions of either codons 591 to 594 (15) or codons 595 to 603 appear to be fully replication competent, the range of codons from codons 591 to 607 seems to be dispensable for the normal kinase function of UL97, while it strongly affects ganciclovir phosphorylation. We may expect the continued occasional appearance of clinical isolates with various deletions over the range from codons 591 to 607. The frequency of such mutations cannot be high given the number of resistant isolates that have been analyzed (3), but their existence should be taken into account in the design of genotypic assays.

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