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# Single Amino Acid Changes in the DNA Polymerase Confer Foscarnet Resistance and Slow-Growth Phenotype, while Mutations in the UL97-Encoded Phosphotransferase Confer Ganciclovir Resistance in Three Double-Resistant Human Cytomegalovirus Strains Recovered from Patients with AIDS

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**Three human cytomegalovirus (HCMV) strains (VR4760, VR4955, and VR5120) showing double resistance to ganciclovir (GCV) and foscarnet (PFA) were isolated from three patients with AIDS who underwent multiple sequential courses of therapy with GCV and PFA (A. Sarasini, F. Baldanti, M. Furione, E. Percivalle, R. Brerra, M. Barbi, and G. Gerna, J. Med. Virol., 47:237–244, 1995). We previously demonstrated that the three strains were genetically unrelated and that each of them was present as a single viral population in vivo. Thus, in each of the three cases, a single viral strain was resistant to both GCV and PFA. In the present paper, we report the characterization of the molecular bases of the double resistance and demonstrate that the PFA resistance is associated with a slower replication of HCMV strains in cell cultures. Sequencing of the UL97 and UL54 genes, GCV anabolism assays, and marker transfer experiments showed that GCV resistance was due to single amino acid changes in the UL97 gene product (VR4760, Met-460→Ile; VR4955, Ala-594→Val; VR5120, Leu-595→Ser), while single amino acid changes in domain II of the DNA polymerase (VR4760 and VR5120, Val-715→Met; VR4955, Thr-700→Ala) were responsible for both the PFA resistance and the slow-growth phenotype. Thus, in these three cases, double resistance to GCV and PFA was not due to a single mutation conferring cross-resistance or to the presence of a mixture of strains with different drug susceptibilities. The HCMV DNA polymerase recombinant strains carrying the mutations conferring PFA resistance were sensitive to GCV and (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine (HPMPC). In addition, the same UL54 mutations were responsible for the slow growth of the clinical isolates, since the recombinant strains showed a marked delay in immediate-early antigen plaque formation and a reduction of infectious virus yield compared with AD169, from which they were derived. These results may have some important implications for the successful isolation, propagation, and characterization of PFA-resistant strains from clinical samples containing mixed viral populations.**

Ganciclovir (GCV) and foscarnet (PFA) are the only two drugs currently available in clinical practice for management of human cytomegalovirus (HCMV) disseminated infections in patients with AIDS. Both drugs are effective inhibitors of viral DNA replication, acting by different mechanisms. After triphosphorylation, GCV, a guanosine analog, becomes a substrate competitor for UL54-encoded DNA polymerase. The initial GCV phosphorylation is carried out by a viral phosphotransferase encoded by the UL97 gene (18, 27). PFA, on the other hand, does not require any previous activation, and it blocks the viral DNA polymerase by a noncompetitive mechanism (4).

GCV is the anti-HCMV drug most widely used in clinical practice, and detection of GCV-resistant HCMV strains is becoming more and more frequent in patients with AIDS (6–8, 11, 12, 14). Mutations in the HCMV DNA polymerase (20, 25) or in the UL97 gene (1, 3, 19, 27) may lead to GCV resistance, but the latter mechanism seems to occur in vivo most frequently (3, 24). PFA was introduced in clinical practice more recently and was used for rescue therapy in patients with no response to GCV treatment (7, 14). HCMV PFA-resistant strains exhibiting cross-resistance to other DNA polymerase inhibitors were identified in both the laboratory and the clinical settings (5, 12, 15, 23, 26, 28). However, despite the common assumption that a single mutation in the DNA polymerase gene could be responsible for cross-resistance, there were no experimental data elucidating the molecular basis of PFA resistance, nor were the confirmatory genetic experiments performed.

In this report, we describe the molecular basis of the double resistance to both GCV and PFA of three HCMV strains recovered from three patients with AIDS who underwent multiple sequential courses of GCV and PFA treatment (12, 23). These HCMV strains were found to carry two amino acid changes, one in UL97 and the other in domain II of the DNA

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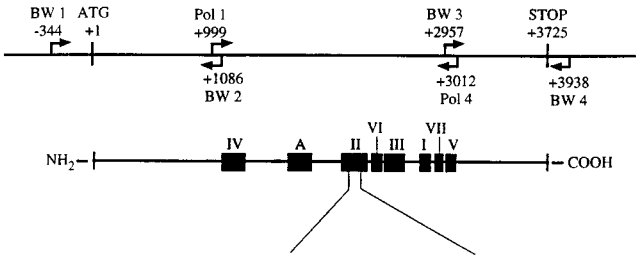


| Virus | amino acid sequence | | |
|---|---|---|---|
| HCMV | 695- | SYQGATVFEPEVGYYNDPVAVFDFASLYPSI | -725 |
| EBV | 562- | GYQGATVIQPLSGFYNSPVLVVDFASLYPSI | -592 |
| HSV1 | 695- | GYQGARVLDPTSGFHVNPVVVFDFASLYPSI | -725 |
| HSV2 | 700- | GYQGARVLDPTSGFHVDPVVVFDFASLYPSI | -730 |
| VZV | 660- | GYKGARVFDPDTGFYIDPVVVLDFASLYPSI | -690 |
| HHV6 | 550- | GYKGATVLEPKTGYYAVPTVVFDFQSLYPSI | -580 |
| VR4760 | 695- | SYQGATVFEPEVGYYNDPVA**M**FDFASLYPSI | -725 |
| VR5120 | 695- | SYQGATVFEPEVGYYNDPVA**M**FDFASLYPSI | -725 |
| VR4955 | 695- | SYQGA**A**VFEPEVGYYNDPVAVFDFASLYPSI | -725 |

FIG. 1. Structure of the HCMV DNA polymerase gene and primer positions. Solid boxes on the corresponding schematic of the protein represent the conserved domains of the enzyme. The amino acid sequences of fragments of VR4760, VR5120, and VR4955 domain II (residues 695 to 725) containing the mutations conferring PFA resistance (in boldface) are shown; for comparison, the amino acid sequences of the same domain II fragments from HCMV, Epstein-Barr virus (EBV), HSV-1, HSV-2, varicella-zoster virus (VZV), and human herpesvirus 6 (HHV6) DNA polymerases are given.

polymerase gene, which conferred GCV and PFA resistance, respectively. The recombinant strains carrying the polymerase mutations were sensitive to GCV, which suggested that the double resistance was due to independent mechanisms. In addition, we demonstrate that the polymerase mutations conferring PFA resistance caused slower viral growth in cell cultures of the recombinant strains than of the reference strain AD169, from which they were derived.

(These results were presented in part at the 5th International Cytomegalovirus Conference, 20 to 24 May 1995, Stockholm, Sweden.)

## MATERIALS AND METHODS

**Virus isolation, plaque purification, genome restriction analysis, and chemosensitivity testing.** The HCMV isolates VR4760, VR4955, and VR5120 were recovered from the blood of three patients with AIDS during a course of PFA treatment at a full dosage with no clinical and virological response. All three patients had previously received GCV treatment courses with initial clinical and virological response. Patients were then shifted to PFA treatment because of clinical failure of GCV therapy. Initial effectiveness of PFA therapy in controlling HCMV infections was followed by lack of clinical and virological response to treatment (23). The viral isolates were plaque purified and submitted to drug susceptibility testing by an immediate-early antigen (IEA) plaque reduction assay (11) as previously reported (12, 23). In addition, comparative restriction analyses of multiple genome regions amplified from clinical isolates and the relevant plaque-purified strains were performed (23).

Antiviral drug susceptibility of recombinant plaque-purified viral progeny obtained from marker transfer experiments was determined by using the conventional plaque reduction assay (26).

**UL97 and UL54 PCR amplification and sequencing.** A 1,629-nucleotide (nt) fragment of the UL97 gene, spanning the conserved domains of the enzyme (2) and the region containing all of the mutations conferring GCV resistance so far identified (1, 3, 19, 27), was amplified by using the primer pair UL97 1-UL97 6 and the PCR protocol previously reported (1).

The entire UL54 gene coding for the HCMV DNA polymerase was amplified either in a single step using the primer pair BW1-BW4 (nt −344 to 3938) or in three overlapping fragments using primer pairs BW1-BW2 (nt −344 to 1086), POL 1-POL 4 (nt 999 to 3012), and BW3-BW4 (nt 2957 to 3938) (Fig. 1). A detailed sequence analysis of the conserved domains of the enzyme was achieved with the primer pairs POL 1-POL 2 and POL 3-POL 4. POL 1, POL 2, POL 3, and POL 4 primer sequences as well as the PCR protocol for POL 1-POL 2 and POL 3-POL 4 fragment amplification were previously reported (1). The remaining primer sequences were as follows: BW1, 5′-CCAACGAGCAGGCTTAC C-3′ (forward); BW2, 5′-AGACCCGAGTGCCCAAAG-3′ (reverse); BW3, 5′-AGTTCGTCAAGGGCGTCA-3′ (forward); and BW4, 5′-GTCGTCCTACGC GATACG-3′ (reverse). Amplification protocols were as follows: 1 min at 94°C, 45 s at 65°C, and 2.5 min at 72°C for 10 cycles followed by 30 s at 94°C, 30 s at 58.4°C, and 2.5 min at 72°C for 30 cycles for the BW1-BW2 fragment (1,430 bp); 1 min at 94°C, 1 min at 58°C, and 2 min at 72°C for 40 cycles for the BW3-BW4 fragment (973 bp); 1 min at 94°C, 1 min at 58°C, and 3 min at 72°C for 45 cycles for the POL 1-POL 4 fragment (2,013 bp); and 45 s at 94°C, 45 s at 65°C, and 4 min 15 s at 72°C for 10 cycles followed by 30 s at 94°C, 30 s at 58.4°C, and 4 min 15 s at 72°C for 40 cycles for the BW1-BW4 fragment (4,282 bp). Sample lysis and PCRs were performed as reported previously (30) except that UL97 1-UL97 6, BW1-BW4, BW1-BW2, and POL 1-POL 4 amplification reactions were carried out in the presence of 10% dimethyl sulfoxide. In addition, for BW1-BW4 and POL 1-POL 4 PCRs, the Taq Extender PCR additive (Stratagene, La Jolla, Calif.) was used as instructed by the manufacturer.

Sequencing was performed directly on PCR products by the dideoxy-chain termination method (22), using either a $^{35}$S-labeled dATP incorporation procedure or an automatic sequencer (373 DNA sequencing system; Applied Biosystems, Foster City, Calif.). Primers for sequencing were synthesized from published HCMV sequences at 200- to 300-bp intervals. Sequencher 2.1 (Gene Code Co., Ann Arbor, Mich.) software was used for analysis and alignment of the electropherograms obtained by the automatic sequencer.

UL97 and UL54 sequences from VR4760, VR4955, and VR5120 were compared with reported AD169 sequences (2, 16, 29) as well as 50 UL54 sequences from 50 HCMV field isolates.

**Marker transfer experiments.** Marker transfer experiments were carried out with BW1-BW2, POL 1-POL 4, and BW3-BW4 PCR products from plaque-purified VR4760 and VR4955. Full-length infectious AD169 DNA was extracted from virion pellet as reported previously (1, 3). MRC-5 cells were plated at a density of $10^6$ cells per 60-mm-diameter dish 16 to 20 h before transfection. Two micrograms of AD169 full-length infectious DNA and 0.2 μg of each DNA polymerase PCR product were cotransfected into 75% confluent MRC-5 cells by the calcium phosphate transfection method (21). Viral plaques formed 7 to 21 days after transfection, and the infection was allowed to progress until 100% cytopathic effect was observed. Supernatant virus was collected and plated in the presence of 400 μM PFA. The resulting possible recombinants were picked, propagated, and directly assayed for sensitivity to PFA, GCV, and (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine (HPMPC), using the cytopathic effect plaque reduction method. To verify the successful transfer of VR4760 and VR4955 UL54 mutations to the AD169 UL54 gene and the absence of additional changes potentially occurring during the recombination process, viruses that showed a greater than threefold elevation in PFA 50% infectious doses ($ID_{50}$s) over values for the reference strain AD169 were sequenced across the entire transfected fragment.

**GCV anabolism.** GCV phosphorylation assays were performed on the clinical isolates VR4760, VR4955, and VR5120 as well as on recombinant strains VR4760rec and VR4955rec, using $^3$H-labeled GCV and the technique previously described by Fyfe et al. (10). Controls included in the experiment were MRC-5 mock-infected cells and cells infected with AD169 and GCV-resistant UL97 mutant HCMV strain Xba F4-3-1 (27).

**Slow-growth phenotype analysis.** AD169, VR4760rec, and VR4955rec cell-free viruses were used to infect confluent MRC-5 cultures grown in 60-mm-diameter dishes. Cultures were fixed 2, 4, and 7 days postinfection (p.i.), using 100% ethanol. Dishes were then stained with the monoclonal antibody 5D2 to the major IEA (11) for detection of single infected cells and IEA plaques.

To perform a comparative evaluation of AD169, VR4760rec, and VR4955rec virus yields, MRC-5 cultures grown in 12-well plates were infected in parallel with AD169 and the recombinant strain cell-free virus at a multiplicity of infection of 2. Infections were performed in duplicate, and supernatant virus was collected 72, 96, and 120 h p.i. The virus titer of each supernatant collection was then determined.

## RESULTS

**Chemosensitivity testing of clinical isolates and viral population analysis.** As previously reported, three HCMV strains showing a high level of resistance to both GCV and PFA were recovered from three patients with AIDS (12, 23). All plaque-purified strains from VR4760, VR4955, and VR5120 showed a level of double resistance comparable to that of the relevant clinical isolate (23), with >5-fold $ID_{50}$ elevation over the mean GCV and PFA $ID_{50}$ values of sensitive HCMV clinical isolates (23). In addition, comparative DNA restriction analysis of multiple genome regions performed on the clinical isolates as well as on the relevant plaque-purified strains showed that (i) the

TABLE 1. Amino acid changes present in UL97 and UL54 of clinical isolates VR4760, VR4955, and VR5120 and of DNA polymerase recombinant strains VR4760rec and VR4955rec with respect to AD169 sequence

| HCMV strain | Enzyme (gene) | Mutation(s) |
|---|---|---|
| VR4760 | Phosphotransferase (UL97) | Met-460→Ile |
| | DNA polymerase (UL54) | Val-715→Met, Ala-885→Thr,[a] Asn-898→Asp[a] |
| VR4955 | Phosphotransferase (UL97) | Ala-594→Val |
| | DNA polymerase (UL54) | Pro-628→Leu,[a] Ser-655→Leu,[a] Asn-685→Ser,[a] Thr-700→Ala, Ser-885 insertion,[a] Ala-886→Thr[a] |
| VR5120 | Phosphotransferase (UL97) | Leu-595→Ser |
| | DNA polymerase (UL54) | Val-715→Met, Ala-885→Thr,[a] Ser-897→Leu[a] |
| VR4760rec | Phosphotransferase (UL97) | None |
| | DNA polymerase (UL54) | Val-715→Met |
| VR4955rec | Phosphotransferase (UL97) | None |
| | DNA polymerase (UL54) | Pro-628→Leu,[a] Ser-655→Leu,[a] Asn-685→Ser,[a] Thr-700→Ala, Ser-885 insertion,[a] Ala-886→Thr[a] |

[a] Mutation outside of conserved domains and present in at least one drug-sensitive HCMV clinical isolate.

three strains were genetically unrelated and (ii) each clinical isolate consisted of a single viral population (23).

**UL97 and UL54 sequencing.** The UL97 1-UL97 6 fragment (1) containing the conserved domains of the enzyme and the sites of mutations shown to confer GCV resistance (1, 3, 19, 27) was amplified by PCR from plaque-purified VR4760, VR4955, and VR5120 and sequenced. Comparison of sequencing data with the published AD169 sequence showed a Met-to-Ile change in position 460 of VR4760, an Ala-to-Val change in position 594 of VR4955, and a Leu-to-Ser change in position 595 of VR5120. These changes were previously described as responsible for GCV resistance in clinical HCMV isolates with impaired GCV phosphorylation (3, 19). Similarly, plaque-purified VR4760, VR4955, and VR5120 UL54 genes were amplified by PCR using primer pairs BW1-BW4, POL 1-POL 2, POL 3-POL 4, and POL 1-POL 4 and directly sequenced. Sequencing showed, among several amino acid mutations in nonconserved regions of the enzyme, a Val-to-Met change at position 715 in the VR4760 and VR5120 UL54 genes and a Thr-to-Ala change at position 700 in the VR4955 UL54 gene (Fig. 1; Table 1). Both mutations are within the conserved domain II of the viral DNA polymerase, which is one of the most conserved regions among all herpesvirus (17, 29). Single amino acid changes in domain II of herpes simplex virus (HSV) DNA polymerase were shown to lead to phosphonoacetic (PAA) resistance (9, 13, 17), suggesting a role of domain II in pyrophosphate binding. It is interesting that Gibbs et al. showed that a particular Arg-to-Gly change in position 700 of the HSV type 1 (HSV-1) DNA polymerase gene, corresponding to position 700 of the HCMV amino acid sequence (Fig. 1), was responsible for PAA resistance (13). In addition, by comparing VR4760, VR4955, and VR5120 sequencing data with the UL54 sequences from 50 HCMV field isolates, we observed that neither the Val-715→Met nor the Thr-700→Ala change was present in sensitive pretherapy HCMV strains (data not shown). All the other mutations found outside the known conserved domains (Table 1) were also detected in at least one drug-sensitive HCMV clinical isolate, and some of them were found to be very common among field isolates, which suggested that they represent normal strain variations (Table 1).

**Marker transfer and analysis of recombinant VR4760rec and VR4955rec strains.** Since the change in domain II of the UL54 gene was the same for VR4760 and VR5120, and mutations present in the UL97 gene of the double-resistant strains are well known to be related to GCV resistance, we focused our marker transfer experiments on the VR4760 and VR4955 DNA polymerases. The overlapping fragments BW1-BW2, BW3-BW4, and POL 1-POL 4, spanning the entire UL54 open reading frame (Fig. 1), were amplified by PCR from plaque-purified VR4760 as well as VR4955 and used individually in marker transfer experiments. Progeny viruses from cotransfection in MRC-5 cells of full-length infectious AD169 DNA and each of the UL54 fragments from VR4760 and VR4955 were plated in the presence of 400 μM PFA. Viral plaques were obtained in the presence of PFA only from progeny of the cotransfection of AD169 DNA with the POL 1-POL 4 fragment of the two clinical isolates. Plaques were picked, propagated, and assayed for PFA and GCV by the conventional plaque reduction method. Mean PFA $ID_{50}$ values from three separate experiments were 210 and 180 μM for VR4760rec and VR4955rec, respectively, compared with the wild-type AD169 mean value of 38 μM (Table 2). Thus, the recombinant strains showed a ~5-fold elevation in PFA $ID_{50}$ compared with the value for AD169. Similarly, the parent strains VR4760 and VR4955 showed a ≥5-fold elevation in PFA $ID_{50}$ compared with mean $ID_{50}$ levels of PFA-sensitive clinical isolates (23). In contrast, the recombinant strains VR4760rec and VR4955rec were GCV sensitive (Table 2). In addition, we tested the recombinant strains for HPMPC susceptibility, since the previously described UL54 laboratory-induced mutant resistant to GCV was also cross-resistant to HPMPC (25). HPMPC is a nucleotide analog DNA polymerase inhibitor that does not require virus-specific enzymes for its activation. Both VR4760rec and VR4955rec strains were shown to be HPMPC sensitive (Table 2).

The entire VR4760rec and VR4955rec DNA polymerase genes (BW1-BW4) were amplified by PCR, and products were used for the complete sequencing of the transfected POL 1-POL 4 fragment (codons 333 to 1004). Silent nucleotide changes present in the VR4760 and VR4955 UL54 genes with respect to the AD169 sequence allowed us to map the region of the VR4760 and VR4955 POL 1-POL 4 fragment which was inserted into the AD169 DNA polymerase gene during the recombination process. In detail, nucleotide changes present in

TABLE 2. Susceptibility levels of recombinant and parental HCMV strains[a]

| HCMV strain | PFA | | GCV | | HPMPC | |
|---|---|---|---|---|---|---|
| | $ID_{50}$ (μM) | $SI_{50}$[a] | $ID_{50}$ (μM) | $SI_{50}$ | $ID_{50}$ (μM) | $SI_{50}$ |
| AD169 | 38.0 | | 7.80 | | 0.49 | |
| VR4760rec | 210.0 | 5.5 | 7.00 | 0.9 | 0.55 | 1.1 |
| VR4955rec | 180.0 | 4.7 | 7.80 | 1.0 | 0.75 | 1.5 |

[a] Mean values from three separate experiments. $SI_{50}$ (50% sensitivity index) = recombinant strain $ID_{50}$/AD169 $ID_{50}$.

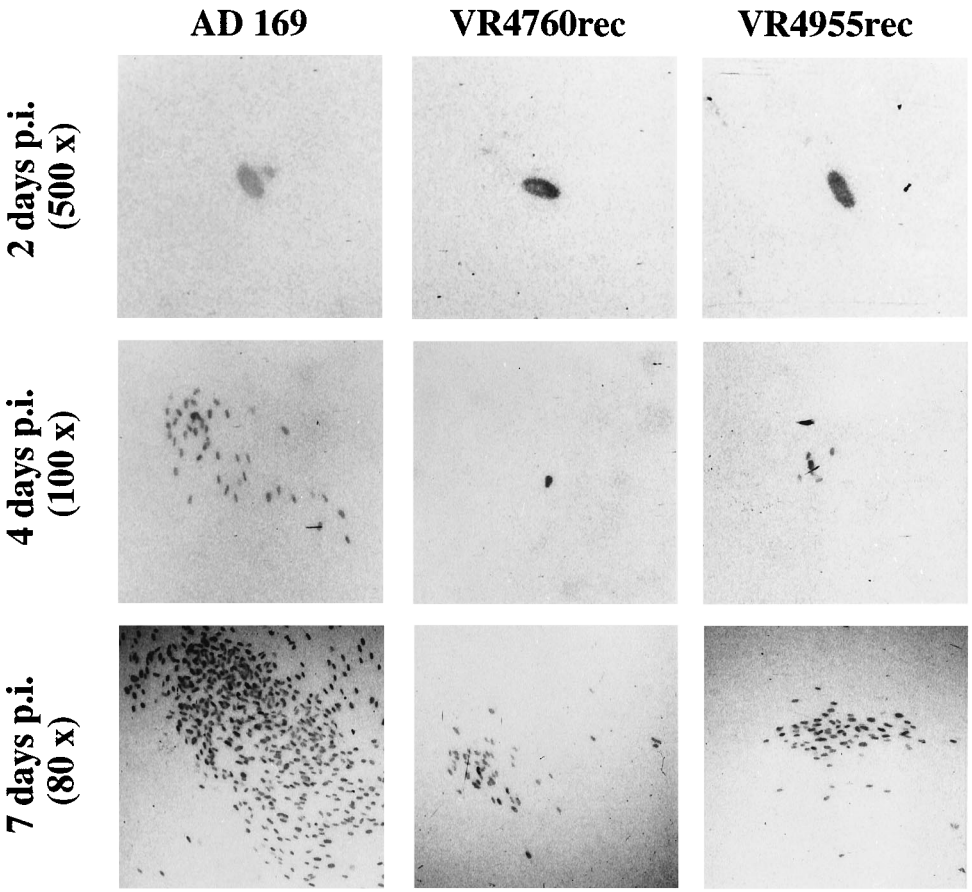


FIG. 2. AD169-, VR4760rec-, and VR4955rec-infected MRC-5 monolayers at 2, 4, and 7 days p.i., stained with a monoclonal antibody to the major IEA by the immunoperoxidase technique. For each row, the magnification is given in parentheses.

VR4760 between codons 440 and 822 were also present in VR4760rec; similarly, nucleotide changes present in VR4955 between codons 489 and 886 were detected in VR4955rec. Thus, we demonstrated that the amino acid changes present in the recombinant DNA polymerase did not arise ex novo during PFA selection of progeny virus.

**Slow-growth phenotype analysis.** We previously observed that HCMV strains with double resistance to GCV and PFA showed a delay in plaque formation in cell cultures compared with HCMV-sensitive or GCV-resistant strains (12, 23). To verify the hypothesis that the PFA resistance could be associated with a slower replication of HCMV strains, we studied the time course of the IEA plaque formation of AD169, VR4760rec, and VR4955rec cell-free viruses on confluent MRC-5 cultures. Results are shown in Fig. 2. At 4 days p.i. in VR4760rec-infected cultures, only single infected cells were present, while the VR4955rec-infected cultures showed small IEA plaques (<10 infected cells); on the other hand, AD169-infected dishes showed large IEA plaques (>30 infected cells). Moreover, at 7 days p.i., VR4760rec and VR4955rec plaques were smaller than those of AD169, suggesting a slower replication of the recombinant strains with respect to the reference strain from which they were derived.

In addition, virus yield analysis showed that supernatant virus titers at 72, 96, and 120 h p.i. were lower in VR4760rec- and VR4955rec-infected than in AD169-infected cultures (Fig. 3). Thus, the slower replication of the recombinant strains was further confirmed, strengthening the hypothesis of a linkage between PFA resistance and slower viral replication, possibly due to impaired DNA replication.

**Phosphorylation assay.** Previous studies of Lurain et al. (19) and Chou et al. (3) reported that the Met-460→Ile, Ala-594→Val, and Leu-595→Ser substitutions in the UL97 gene product (each present in one of the three double-resistant isolates) impair the GCV phosphorylation and confer resistance to the drug. Results from GCV anabolism assays of VR4760, VR4955, and VR5120 confirmed that these strains were unable to phosphorylate the drug, whereas their polymerase recombinants VR4760rec and VR4955rec retained this ability and showed GCV sensitivity (Fig. 4).

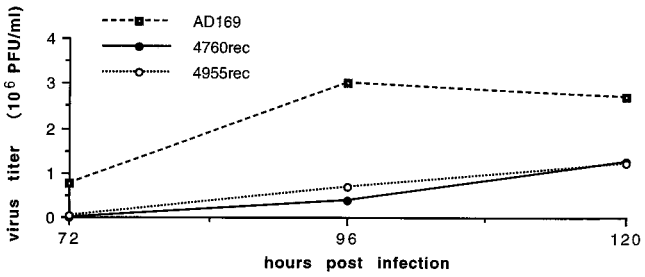


FIG. 3. Virus yields for AD169, VR4760rec, and VR4955rec at 72 to 120 h p.i.

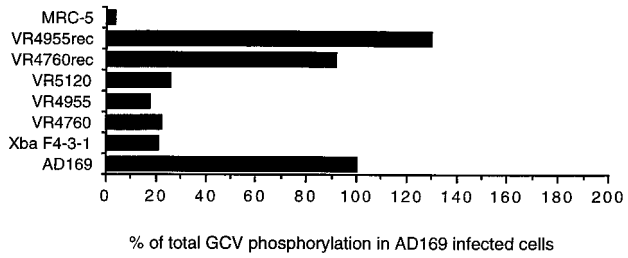


FIG. 4. Anabolism of GCV by HCMV isolates. MRC-5 cells were mock infected or infected with clinical isolates VR4760, VR4955, and VR5120 and DNA polymerase recombinants VR4760rec and VR4955rec. As controls, cells were infected with reference HCMV strain AD169 and recombinant HCMV GCV-resistant strain Xba F4-3-1 (27). $^{3}$H-labeled GCV was pulsed for 20 h; then cells were harvested, and total phosphorylated GCV (mono-, di-, and triphosphates of GCV) was measured for each isolate (10). Histograms represent total GCV phosphorylation in mock-infected and virus-infected cells expressed as percentages of total GCV phosphorylation in AD169-infected cells.

## DISCUSSION

We previously documented the emergence of three HCMV strains which are highly resistant to both GCV and PFA (12, 23). Mutations in both the UL97 gene (1, 3, 19, 27) and the UL54 gene (20, 26) could be responsible for GCV resistance. On the basis of the PFA mechanism of action and on data already available for HSV PAA- and PFA-resistant laboratory strains (9, 13, 17), HCMV PFA-resistant strains were predicted to have DNA polymerase mutations. However, reports of HCMV PFA-resistant isolates are few (5, 12, 15, 23, 26, 28), and no data are available on the molecular basis of resistance. When one is analyzing the simultaneous resistance to GCV and PFA of a single HCMV strain, multiple causative scenarios should be considered: (i) simultaneous mutations in different target genes (UL97 and UL54); (ii) different mutations in the same gene (UL54); and (iii) a cross-resistance due to a single mutation in the same target gene (UL54). We used sequencing and marker rescue experiments to document that in VR4760, VR4955, and VR5120, the GCV resistance was due to a single amino acid change in the UL97-encoded phosphotransferase and that a single change in the DNA polymerase was responsible for the PFA resistance. Both polymerase recombinant strains, carrying either the Val-715→Met or the Thr-700→Ala change in domain II of the enzyme, were PFA resistant but GCV and HPMPC sensitive. Since a UL54 mutation conferring GCV resistance was also reported to be responsible for a cross-resistance to HPMPC (25), this finding confirms that the mechanisms of PFA resistance described here are unrelated to GCV resistance. Thus, in the cases reported here, the double resistance was not a cross-resistance to PFA and GCV but was likely due to sequential addition of the two different mutations providing resistance first to GCV (UL97 change) and then to PFA (UL54 change). In clinical practice, GCV is a first-choice drug for management of HCMV infections in patients with AIDS. Patients are shifted to PFA therapy because of GCV toxicity or after clinical and virological failure of GCV therapy suggesting the emergence of a GCV-resistant HCMV strain (7, 14). It was previously reported that in patients with AIDS, GCV-resistant strains are selected after 3 to 6 months of GCV therapy (6). In this situation, it is easy to hypothesize that a GCV-resistant strain could account for the majority of viral burden in blood (11, 23) and represent the viral background for the selection of a new PFA-resistant mutant, resulting in an HCMV strain dually resistant to GCV and PFA. The clinical history of the patients with AIDS from whom the three double-resistant isolates were recovered supports this conclusion, but more data are needed to evaluate the average PFA therapy period needed for selection of resistant strains.

PAA-resistant HSV strains contain mutations in conserved regions I, II, III, VII, and A of the DNA polymerase (9, 13, 17). Domain II is one of the most conserved regions of the herpesvirus DNA polymerases (17, 29) and was suggested as a strong candidate for the pyrophosphate binding site (17) or as part of such a site along with other conserved regions (13) on the basis of genetic analysis of HSV PAA-resistant strains. It is therefore interesting that the three HCMV strains with PFA resistance have a change in domain II of the DNA polymerase. In addition, the same single mutation in VR4760 and VR5120 is at a residue that is conserved among six herpesviruses (29). VR4955 had a change only 15 residues away from that present in the other two isolates and in the same position as a reported change in a HSV-1 PAA-resistant strain (13). However, in HSV, single changes in several conserved domains of DNA polymerase can impair PAA susceptibility, showing that the pyrophosphate binding site is not restricted to domain II (13). Thus, a possibility that should be taken into consideration when one is analyzing HCMV PFA resistance is that multiple changes simultaneously present in the DNA polymerase (even outside the conserved domains) can interfere with pyrophosphate binding and contribute to the final level of the PFA resistance. Indeed, it must be noted that our isolates showed multiple changes in the enzyme. Although all of the mutations except Val-715→Met and Thr-700→Ala were found to be present in PFA-sensitive isolates (as single changes or as different combinations of mutations), we cannot exclude that these changes, in association with Val-715→Met or Thr-700→Ala, can modify the PFA resistance level. However, it must also be noted that Met-715 and Ala-700 were never found in PFA-sensitive isolates. In addition, two genetically unrelated PFA-resistant HCMV strains (VR4760 and VR5120), recovered from different patients from different geographical areas in Italy (12), showed the same Val-715→Met change. Moreover, Met-715 was the only amino acid change transferred to AD169 DNA polymerase in the recombinant strain VR4760rec. Thus, it seems very likely that Val-715→Met and Thr-700→Ala changes alone are the major cause of PFA resistance and the other changes represent only common strain variations.

We first observed that a slower growth of HCMV strains in cell cultures could be associated with PFA resistance (12, 23). This finding is confirmed by the slower replication rate associated with the Val-715→Met and Thr-700→Ala mutations in domain II of UL54, as demonstrated by the delay in plaque formation of VR4760rec and VR4955rec with respect to AD169, from which they were derived. The findings that the Val-715 residue is conserved among all herpesviruses and that all three PFA-resistant strains carry a mutation in domain II of the enzyme (two of them showing the same change) may suggest that a very restricted region of HCMV polymerase is involved in PFA recognition and binding and that this domain can play an important role in the DNA polymerase function. The biological disadvantage of a defective DNA replication of PFA-resistant strains could be balanced in vivo by a selective advantage in the presence of the drug. Importantly, this slower replication in cell culture could introduce a bias in the evaluation of the drug susceptibility of a mixed viral population, since sensitive strains could likely overgrow the resistant ones in cell cultures. Thus, after some passages in vitro, the resistant viral component could be dominated by the sensitive one. Since patients with AIDS frequently carry mixed viral populations with different resistance levels (11), this possibility should be taken into account when one is analyzing a clinical isolate for PFA resistance.

We conclude that single amino acid changes in certain regions of the HCMV UL97 and UL54 gene products can impair drug susceptibility in HCMV strains and confer GCV and PFA resistance in patients with AIDS. From a biological point of view, a more extended series of resistant strains should be analyzed to better understand the functions and structures of HCMV UL97 and DNA polymerase genes; from a clinical perspective, more data are needed to establish the time period required in vivo for the emergence of mutations conferring PFA resistance and their practical significance in HCMV disease management as well as to determine guidelines for a correct alternative treatment.

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