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Antiviral Drug Resistance of Human Cytomegalovirus

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INTRODUCTION

Characterization of human cytomegalovirus (HCMV) antiviral drug resistance mutations has contributed to the advancement of HCMV therapy and to knowledge of the basic biological functions of the virus-encoded products that serve as antiviral drug targets. The current anti-HCMV drugs, i.e., ganciclovir (GCV), its prodrug valganciclovir (val-GCV), foscarnet (FOS), and cidofovir (CDV), all target the viral DNA polymerase. GCV-resistant HCMV was isolated after *in vitro* propagation under the drug (14) before its initial licensing and was detected *in vivo* shortly after the first use of GCV in immunosuppressed patients who received prolonged therapy (73). Over the following 2 decades, there have been many reports of HCMV isolates resistant to one or more of the approved drugs, which has created a need for antiviral resistance testing. Currently this is usually based on the detection of mutations in the relevant viral *UL97* kinase and *UL54* DNA polymerase genes. Accurate interpretation of these genotypic assays depends on an understanding of the levels of resistance and cross-resistance conferred by various mutations and recognition of sequence variants unrelated to drug resistance. Experimental compounds with different viral targets are under investigation and may become therapeutic options for drug-resistant HCMV disease (50, 72, 114). This review will summarize the virological and clinical data pertaining to HCMV antiviral drug resistance, including the target genes and their functions, diagnostic mutations, and treatment options for patients requiring antiviral therapy.

HCMV GENOME STRUCTURE AND REPLICATION

HCMV shares the structural and genomic characteristics of other members of the herpesvirus family (153). It contains an icosahedral nucleocapsid surrounded by a lipid envelope. Between the nucleocapsid and envelope is the tegument, which contains viral phosphoproteins, including the abundant pp65 antigen. The structure of the large 230-kbp, linear, double-stranded DNA genome is shown in Fig. 1. There are two regions containing unique sequences bounded by inverted repeats. The open reading frames (ORFs) are named by the region and the numerical order in which they occur. Thus, the two target genes involved in GCV anabolism, *UL54* (*pol*) and *UL97*, occur in the unique long region as the 54th and 97th ORFs, respectively, counting from left to right. Genomes of clinical strains contain a block of ORFs that has been partially or completely deleted from the genomes of the well-characterized HCMV laboratory strains AD169 and Towne during extensive passage over many years of culture (30, 33) (Fig. 1). None of the deleted ORFs has been linked to antiviral drug anabolism.

Fresh clinical HCMV isolates have a cell-associated phenotype and produce very little extracellular virus. After multiple passages in cell culture, measurable titers of extracellular virus are gradually produced. By comparison, well-characterized, high-passage HCMV laboratory strains such as AD169 and Towne produce relatively high extracellular viral titers (10^6 to 10^7 PFU/ml). Differences in growth characteristics between these laboratory strains and clinical isolates affect the design and readout of phenotypic antiviral drug resistance assays (see "Phenotypic Methods" below).

Similar to the case for other herpesviruses, HCMV replication involves temporally ordered gene expression (34, 153). A set of immediate-early (IE) genes, which are mainly transcriptional regulators, is expressed on entry of the virus into the nucleus. Expression of these genes is required for transcription of the second temporal class, the early genes, which are involved mainly in DNA replication. The *pol* gene belongs to this temporal class. Once DNA replication begins, late genes encoding viral structural and assembly proteins are expressed from the replicated genomes. Some genes can be assigned to more than one temporal class. For example, the UL97 kinase (pUL97) is expressed with both early and late kinetics. The protein can be detected early in infection; however, the level of expression is reduced when DNA replication is inhibited, which is characteristic of late genes (151).

Three basic virological factors that appear to play a role in the development of drug resistance are as follows: (i) HCMV establishes lifelong latency following a primary infection, which allows for later reactivation, particularly under the conditions of immunosuppression; (ii) the lytic replication cycle of HCMV is slow *in vitro*, taking up to 72 h for release of infectious virus, although there is evidence that this may be more rapid *in vivo* (71, 153); and (iii) the viral DNA polymerase (Pol), which has proofreading function, replicates the genome with high fidelity (159).

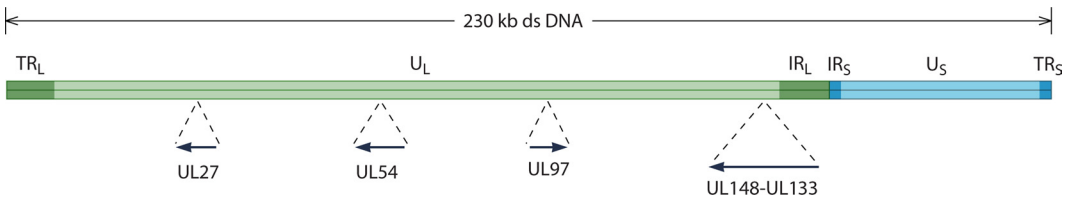

FIG. 1. Map of the structural components of the human CMV genome. There are two sets of unique sequences: unique long (U_L) and unique short (U_S). Each is flanked by inverted repeats (TR and IR). Positions of genes of interest are indicated by dotted lines. Arrows show the direction of the open reading frame (ORF) for each gene (65). ORFs UL148 to -133 (reverse numerical order from direction of ORFs) are found in low-passage clinical isolates but are deleted from the prototype laboratory strain AD169. Horizontal arrows designate directions of open reading frames of interest for drug resistance (UL27, UL54, and UL97).

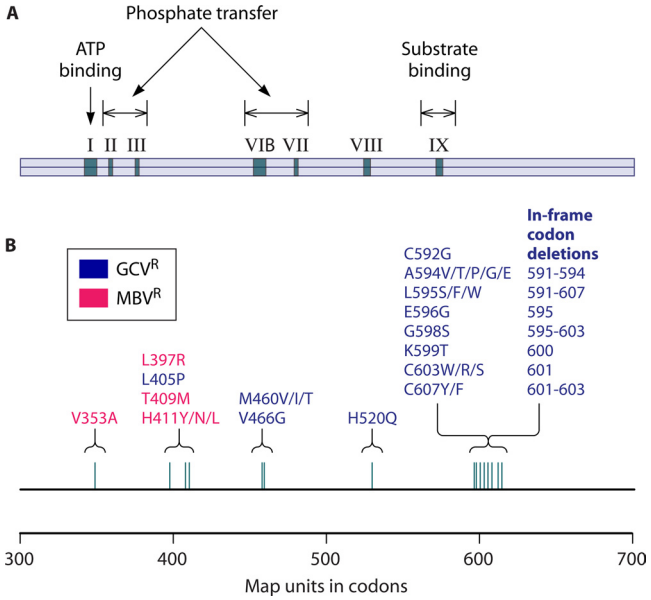

FIG. 2. Map of the kinase subdomain of the *UL97* gene. (A) Conserved functional regions of the UL97 kinase (pUL97). Codons defined for each region are as follows: region I, 338 to 345; region II, catalytic lysine 355; region III, glutamate 380; region VIB, 453 to 462; region VII, 481 to 483; region VIII, 520 to 527; and region IX, 574 to 579 (34, 89). (B) Confirmed mutations are color coded by resistance profile. Multiple mutations at a single codon are listed in order of frequency. Map units correspond to codons of *UL97*.

GENE TARGETS OF ANTIVIRAL DRUGS

UL97 Kinase

Early sequence analysis of the HCMV AD169 genome suggested that pUL97, encoded by the *UL97* ORF, was a phosphotransferase related to protein kinases. The pUL97 amino acid sequence contains conserved serine/threonine kinase functional motifs (Fig. 2A) based on homology with a large number of characterized protein kinases from different host species, including yeast, bovine, rat, and human (34, 89). The critical lysine residue in pUL97 that is conserved in all kinases is K355; mutants such as those carrying K355Q (92) or K355M (140) are devoid of any phosphorylating activity and are important controls for biochemical studies of pUL97.

There was a delay in identifying pUL97 as the GCV kinase, because GCV and the structurally similar compound acyclovir (ACV) (Fig. 3) are nucleoside analogs that are phosphorylated in cells infected with herpes simplex virus type 1 (HSV-1) and HSV-2 by a virus-encoded thymidine kinase (TK). However, HCMV does not encode a TK. It was later determined that HCMV pUL97 phosphorylates GCV and ACV (128, 187, 188) but not natural nucleosides such as guanosine (151). ACV is very active against HSV but has only weak activity against HCMV (76).

The role of pUL97 in GCV anabolism is shown in Fig. 4. GCV requires three phosphorylation steps for its antiviral activity; pUL97 performs the initial phosphorylation, and two additional phosphate groups are then added by cellular enzymes. The triphosphate has a free hydroxyl group, which can continue chain elongation; however, incorporation of phosphorylated GCV by Pol alters the DNA conformation, which slows and eventually terminates replication (61).

The normal functions of pUL97 in HCMV replication have been studied for some time but are not yet completely defined. The enzyme is a serine/threonine kinase capable of autophosphorylation (6, 92, 150) and is a component of the mature virion (164). The virus yield of a *UL97* deletion mutant was greatly reduced, accompanied by a severe defect in virion morphogenesis and maturation. Electron microscopy studies suggested that there were defects in encapsidation and/or nuclear egress (114, 208). There is an abnormal intranuclear aggregation of the viral pp65 and other tegument proteins in the absence of pUL97 (106, 164), which results in an atypical cytopathic effect (CPE) in cell culture. Exposure of HCMV to the experimental UL97 kinase inhibitor maribavir (MBV) produces effects similar to those of deletion of *UL97* (114, 164). Neither the deletion of *UL97* nor treatment with MBV completely prevents HCMV replication, which may continue at variably attenuated levels depending on cell culture conditions (163).

More recent studies have focused on viral and cellular substrates of pUL97. The viral UL44 protein, which is the DNA polymerase processivity factor, is phosphorylated by pUL97 (115, 138). Therefore, pUL97 may have a role in regulation of viral DNA synthesis, which appears to be variably decreased in the absence of the enzyme (208). The HCMV transcriptional regulator and mRNA export factor pUL69 is another pUL97 substrate. These two proteins have been shown to physically interact, resulting in upregulation of the pUL69 mRNA export function (191).

The retinoblastoma tumor suppressor protein (Rb) is a cellular substrate of pUL97 (99, 166). Rb is inactivated by direct phosphorylation, and the result is release of control of cell cycle progression, creating a favorable environment for viral replication. A second cellular protein that is phosphorylated by pUL97 is the nuclear lamina component lamin A/C, which produces breaks in the nuclear lamina that are predicted to aid in viral nuclear egress (88, 139).

It is evident from these studies that pUL97 has a wide spectrum of both cellular and viral substrates, which may explain its importance for normal viral replication. At the same time, the enzyme is fortuitously active in the anabolism of antiviral nucleoside analogs.

UL54 DNA Polymerase

The large coding capacity of herpesvirus genomes includes a virus-specific DNA polymerase (Pol), which has conserved functional regions shared not only among the DNA polymerases of all of the herpesviruses (190) but also with DNA polymerases encoded by a wide range of organisms (31). These homologies allow the functional mapping of mutations and structural modeling. For HCMV, the closest available crystal structure is the HSV DNA polymerase (129). Conserved regions of homology are designated Exo I through III (3′-5′ exonuclease) and I through VII (polymerization) (Fig. 5A). There is an additional region of homology in the herpesvirus polymerases that is shared with some mammalian and yeast delta DNA polymerases (214), and this is designated δ-region C. Within all of these homologous regions there are amino acid

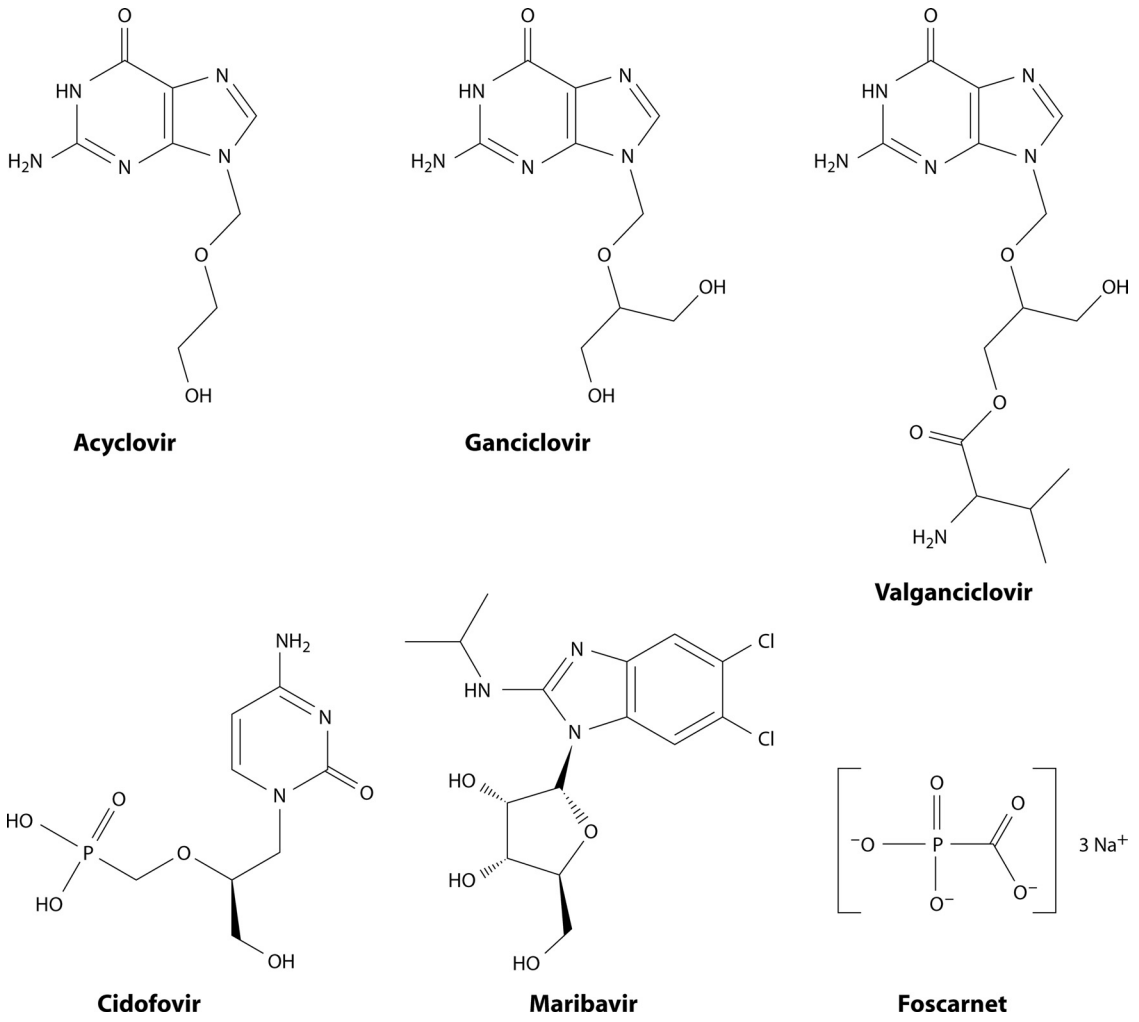

FIG. 3. Structures of antiviral drugs with anti-HCMV activity.

residues that are either invariant or highly conserved among DNA polymerases from many groups of organisms.

Pol is expressed in the early phase of the HCMV life cycle and is the enzyme responsible for viral DNA replication, acting in conjunction with several other essential components of the viral replication complex, including the polymerase accessory protein pUL44, the single-stranded DNA binding protein (UL57), and the primase-helicase complex (UL70, UL102, and UL105) (3, 212). The amino acid sequences of all of these components of the replication complex are highly conserved among HCMV strains. The accessory protein pUL44 forms homodimers that interact through N-terminal residues with the C terminus of Pol (Fig. 5A), and the specific amino acids involved in the interaction are completely conserved (212).

The main functions associated with Pol are (i) nucleotide polymerization, including binding of the incoming nucleotide triphosphate and release of pyrophosphate, and (ii) 3′-5′-exonuclease activity (proofreading). The latter function is responsible for the high fidelity of replication, which results in a low mutation rate. As seen in Fig. 5A, the regions of homology among DNA polymerases are also linked to the two main functions of the enzyme, and there is overlap of Exo II with polymerization region IV and of Exo III with δ-region C. Drug resistance may result from amino acid sequence changes that either prevent binding of the active drug to the enzyme or alter the balance of exonuclease and polymerase activities to favor the removal of the incorporated drug. (16, 60, 87, 190).

No drug resistance mutations map to the Pol region that interacts with pUL44 (Fig. 5B) (27); however, the accessory protein homodimer binding is essential for long-chain DNA synthesis. For this reason, there are two potential interactions in the Pol-pUL44 complex that are attractive targets for future antivirals. The first is inhibition of the homodimerization of the pUL44 subunits (179), and the second is inhibition of binding of the pUL44 homodimers to Pol. The latter interaction can be disrupted by a single amino acid substitution in pUL44 (131).

Anabolism of HCMV Antiviral Drugs

The current drug of choice for treatment of systemic HCMV infections is GCV, a guanosine analog (Fig. 3). GCV is inactive as administered and requires pUL97-mediated phosphorylation for antiviral activity (Fig. 4). The drug was initially FDA approved in 1989 for intravenous use. An oral capsule form

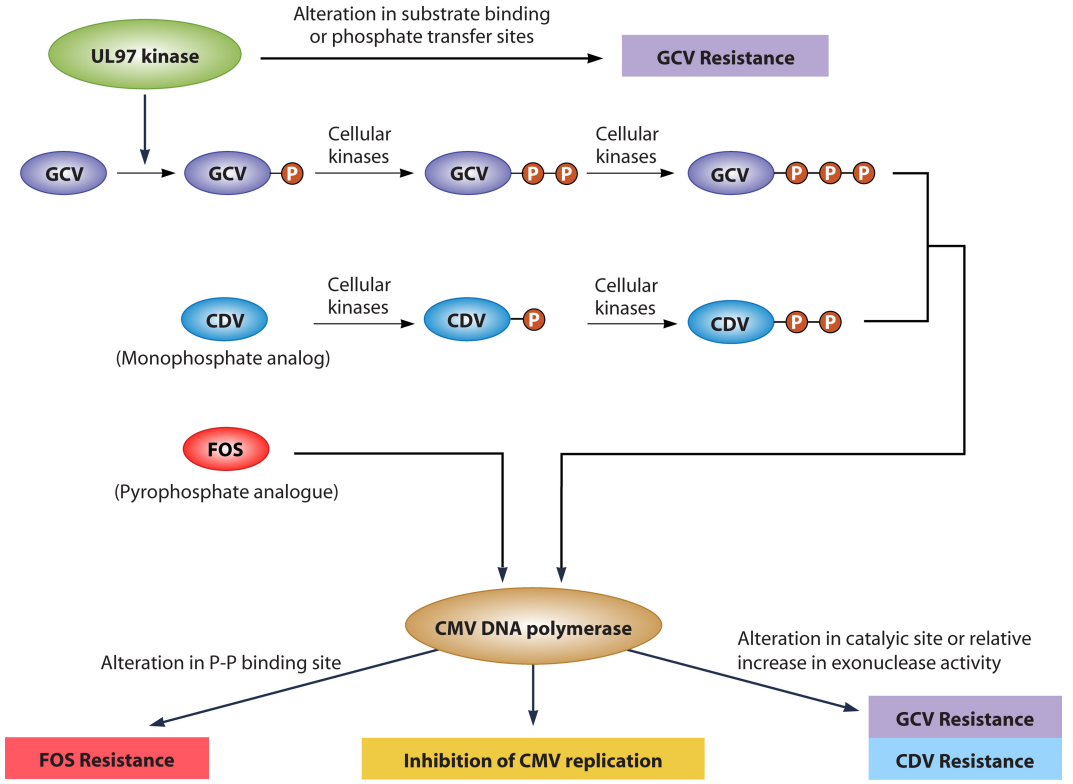

FIG. 4. Anabolism of approved HCMV antiviral drugs. UL97 kinase adds the initial phosphate to GCV. Cellular kinases add two additional phosphates. GCV triphosphate is the active form of the drug incorporated into viral DNA by the viral DNA polymerase. Resistance results from UL97 or DNA polymerase mutations. CDV is a monophosphate analog and does not require initial viral kinase activity. Cellular kinases add additional phosphates to produce CDV diphosphate, the triphosphorylated active form of the drug. Resistance is conferred only by DNA polymerase mutations. FOS is a pyrophosphate analog, which does not require activation. Resistance is also conferred only by DNA polymerase mutations. The DNA polymerase is the ultimate target of all three drugs.

was released in 1994, but it has poor bioavailability (5.6%) and has been largely superseded by the valyl ester prodrug, val-GCV, which has much better bioavailability (60%) and is a suitable replacement for intravenous GCV in many clinical applications (23, 96, 101, 142).

FOS (phosphonoformate sodium) is a pyrophosphate analog (Fig. 3), which does not require intracellular activation. It blocks the release of pyrophosphate by Pol, which results in chain termination (58) (Fig. 4). It is administered as large-volume intravenous solutions and was FDA approved in 1991.

CDV is a CMP analog (Fig. 3), which does not require initial phosphorylation by a viral kinase but is dependent on diphosphorylation by cellular kinases for activation (Fig. 4) (63). Because the requirement for a viral kinase for monophosphorylation is bypassed, the only HCMV target is Pol. CDV is also an inhibitor of DNA polymerases of a number of other DNA viruses (63). It was FDA approved in 1996 for intravenous use. An experimental lipid-conjugated oral prodrug formulation is in clinical trials (95).

HCMV DISEASE AND ANTIVIRAL THERAPY

Indications for Anti-HCMV Therapy

The incidence of HCMV infection in the general population ranges from 36 to 90% (12, 182). Seropositivity increases with age and tends to be highest among lower socioeconomic groups. In the majority of HCMV-infected persons the immune system maintains the virus in a state of latency or low-level reactivation that is clinically undetectable. Symptomatic infections are rare in immunocompetent persons, who do not typically require antiviral therapy. However, HCMV disease is consistently associated with three groups of patients: congenitally infected infants, patients with AIDS, and transplant recipients.

HCMV is the most common cause of congenital viral infection, with an incidence in newborns of approximately 1%. Only about 10% of these infections are symptomatic with classic findings such as hepatosplenomegaly, thrombocytopenia, and central nervous system (CNS) disease (microcephaly, chorioretinitis, psychomotor retardation, or hearing loss) (158, 213). Asymptomatically infected newborns may also be at risk of hearing loss.

GCV has been used to treat congenital infections (2, 35, 147, 204). A randomized controlled trial of a 6-week course of intravenous GCV therapy in symptomatic neonates showed significantly improved hearing at follow-up (110). Other small studies have found the use of GCV therapy for congenital infections to be effective in reducing hearing loss (2, 147, 158), although there remain concerns regarding GCV toxicity, mainly myelosuppression, and optimal treatment regimens have not been established.

Sight-threatening retinitis is most characteristically diagnosed

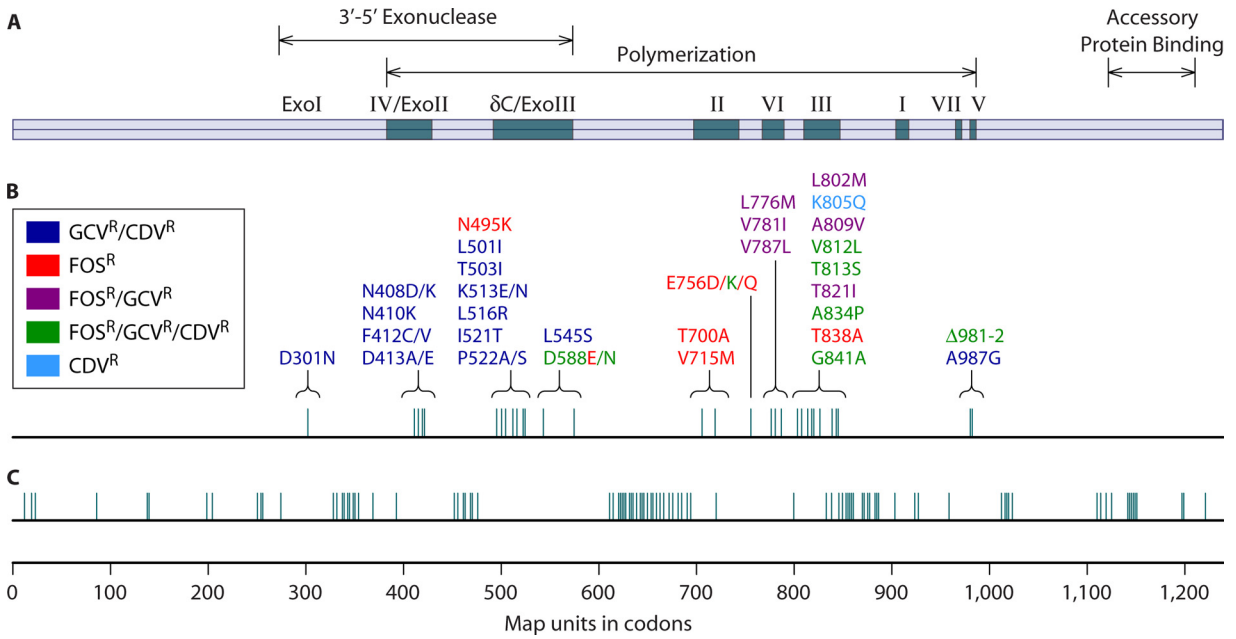

FIG. 5. Map of the DNA polymerase gene. (A) Functional regions of HCMV DNA polymerase. Codon ranges for each region are as follows: region IV/ExoII, 379 to 421; region delta-C/ExoIII, 492 to 588; region II, 696 to 742; region VI, 771 to 790; region III, 805 to 845; region I, 905 to 919; region VII, 962 to 970; and region V, 978 to 988 (190, 214). (B) Confirmed mutations color coded by resistance profile. (C) Polymorphisms observed in drug-sensitive isolates. Map units correspond to codons of the DNA polymerase gene (UL54, *pol*).

in immunocompromised patients with AIDS but also occurs in other patients with prolonged immunosuppression and viremia. HCMV retinitis is always treated upon diagnosis, and the use of several weeks of higher-dose induction therapy followed by maintenance therapy for the duration of severe immunosuppression became an early standard for the antiviral therapy of invasive HCMV disease (107). The advent of effective combination antiretroviral regimens has markedly decreased the incidence of HCMV retinitis and drug resistance related to its treatment (141).

Tissue-invasive HCMV disease, including pneumonia, hepatitis, and gastrointestinal ulcerations, is a serious complication in immunosuppressed transplant recipients (78, 194). HCMV pneumonia remains a deadly disease despite antiviral therapy. In recent years, morbidity and mortality have been reduced by the widespread, proactive use of anti-HCMV therapy before the onset of invasive disease, using either prophylaxis or preemptive therapy (98, 113, 193). For prophylaxis, antiviral therapy is begun early posttransplant and continued for a designated period of time, commonly 100 days. This approach is simple to implement, at the cost of treating more individuals than may be necessary, with attendant risks of adverse medication effects. Late-onset HCMV disease often follows discontinuation of prophylaxis, and this has prompted clinical trials of prolonged prophylaxis to 200 days (97). For preemptive therapy, HCMV viral loads are monitored to determine when a short course of antiviral therapy should be initiated. Problems associated with this approach include the need for frequent quantitative viral monitoring and the lack of a universally accepted standardized assay for early detection of rising viral loads. Some individuals may develop invasive disease with no history of detectable viral loads. No prospective clinical trials have established the superiority of either management approach. The extensive use of antiviral therapy with both strategies has made the transplant population the most likely to develop problems with HCMV drug resistance today (98, 125, 132, 136, 193).

Risk Factors and Incidence of HCMV Drug Resistance

General factors in the emergence of HCMV drug resistance are the duration of drug exposure and ongoing viral replication resulting from insufficient antiviral drug activity or impaired host defenses. Antiviral potency, bioavailability, and effectiveness of drug delivery are important, but the category and degree of host immunosuppression appear to have the greatest influence on the risk of drug resistance. In AIDS patients, ongoing profound immunodeficiency with decreased CD4 cell counts (often less than 50/μl) and a requirement for indefinite therapy led to a greater than 20% incidence of resistance to all available anti-HCMV therapy at 9 to 12 months (102). More recently, with effective combination antiretroviral therapy, the incidence has decreased to about 5%, and HCMV drug resistance in the HIV-infected population is no longer a prominent management problem (141).

Currently, the risk of resistance is highest among transplant recipients. Incidence varies greatly within subsets of these patients, with HCMV-seronegative recipients of seropositive solid organs (D+/R− status) accounting for most of the cases. Depending on the transplanted organ and immunosuppressive regimen, the incidence of drug resistance among D+/R− recipients being treated for HCMV disease is usually in the 5% to 10% range, and it is higher in lung transplant recipients (24, 122, 125, 126, 132). During preemptive or prophylactic use of GCV or val-GCV, the incidence of drug resistance is expected to be lower, because fewer patients have active

disease or high viral loads. In stem cell recipients no resistance was reported after preemptive use of GCV (84), and during clinical trials of GCV or val-GCV prophylaxis, there was a relatively low (0% to 4%) incidence of drug resistance after approximately 3 months of drug exposure and ensuing follow-up (21, 23, 24).

Clinical Consequences of Drug Resistance

Drug resistance may be suspected if persistent or increasing plasma viral loads or overt HCMV disease occur after several weeks of therapy of appropriate dose and delivery. It is unusual for resistance to develop during the first 6 weeks of therapy, although this has been reported to occur in immunodeficient pediatric populations (69, 211). Drug resistance results from the evolution of single or multiple mutations that confer various levels of resistance, with the overall level rising over time as mutations accumulate (180). Ultimately, this may make antiviral therapy completely ineffective. Depending on the patient's immune status, outcomes can vary from asymptomatic infection to life-threatening or fatal disease. Most clinical reports of HCMV drug resistance have emphasized the more severe outcomes (122–125), because the same host factors that predispose to drug resistance are also associated with more severe HCMV disease. In clinical trials where monitoring of viral loads and genotypes is done without regard to symptoms, drug resistance mutations have sometimes been observed in relatively asymptomatic subjects who appeared to respond to therapy (22, 144).

VIRAL MUTATIONS LINKED TO DRUG RESISTANCE

Tables 1 to 4 list UL97 and UL54 *pol* mutations with a confirmed drug susceptibility phenotype. These are listed by gene as either drug resistant (Tables 1 and 3) or drug sensitive (Tables 2 and 4). Some phenotypes are borderline, and their clinical significance is open to interpretation.

Table 5 contains a list of mutations that are changes from the original baseline sequences of HCMV strains, which arose after exposure of the strains to antiviral drugs either *in vivo* or *in vitro*. However, these mutations have not been subjected to recombinant phenotyping, and therefore their significance for drug resistance is undetermined. Not listed in these tables are a large number of sequence variants or mutations that have been observed in connection with antiviral drug exposure but for which pretherapy baseline sequences or adequate phenotypic characterization is unavailable. The status of many of these variants should be resolved as confirmatory data become available in the future.

Mutations Selected after *In Vitro* Propagation under Drug Exposure

***UL97* mutations.** GCV resistance was first demonstrated by serially propagating the standard laboratory strain AD169 in cell culture under GCV exposure, resulting in selection of a strain, 759rD100, that showed a 10-fold decrease in GCV susceptibility compared to the parental wild-type strain AD169 (14). Although the mechanism of resistance was unknown at that time, the observed reduction in GCV phosphorylation pointed to the role of a nucleoside kinase in drug anabolism. Cosmid clones of the GCV-resistant strain were used to map the genetic locus of drug resistance, which was an in-frame *UL97* deletion (codons 590 to 593). The deletion resulted in decreased phosphorylation of GCV (187), probably by altering substrate recognition, because the putative ATP binding and phosphotransfer domains of the kinase were not affected. A later propagation of HCMV *in vitro* resulted in the selection of *UL97* mutation M460I (133), which maps to the catalytic domain of the kinase. Subsequent clinical data have established *UL97* as the preferred locus of GCV resistance mutations (Table 1). The mutations commonly found in GCV-resistant clinical isolates do not substantially impair virus replication in cell culture but result in reduced phosphorylation of GCV while maintaining the normal protein kinase functions of pUL97 (152). Although not perceptibly attenuated in routine cell cultures, drug-resistant UL97 mutants may have some subtle loss of growth fitness, as supported by *in vivo* viral population dynamics studies (71).

The essential role of pUL97 in phosphorylating GCV implies that any mutation that impairs its phosphotransfer function will confer GCV resistance and cross-resistance to any drug that depends on pUL97 for phosphorylation or uses pUL97 as a viral target. This includes, for example, any mutation at the critical lysine residue 355 (140) or mutations that truncate the expressed pUL97 (57, 149). A site-directed mutagenesis study of *UL97* functional domains using vaccinia virus recombinants showed that mutations G340V, A442V, L446R, and F523C impair biological UL97 kinase function as judged by loss of autophosphorylation (149) and GCV phosphorylation. Such mutations, although technically GCV resistance mutations, are not expected to occur in clinical isolates, because a severe growth deficiency accompanies loss of normal pUL97 function (165).

Since the UL97 kinase also phosphorylates ACV (188), a concern is that exposure of HCMV to ACV may select for cross-resistance to GCV. An *in vitro* study suggests that this could be the case. Three HCMV isolates obtained from subjects who had not received GCV, when propagated under ACV, displayed decreased GCV susceptibility. M460V, a well-established and common GCV resistance marker, was among the *UL97* mutations that emerged in cultures examined after propagation (56, 148) (Table 1).

***pol* mutations.** The first GCV-resistant strain obtained after propagation under drug exposure, 759rD100, was found to contain not only the *UL97* mutation discussed above but also the mutation A987G in region V of *pol*, which confers dual GCV-CDV resistance (186) (Fig. 5B). Although not among the first *pol* mutations to be encountered in clinical isolates, it has since been reported with some frequency (118, 132, 175). Later studies of mutations arising after *in vitro* propagation included the finding of *pol* mutations F412V, L501I, L545S, and V812L under GCV exposure (59, 82, 134), which were later confirmed as resistance mutations (Table 3). A number of other mutations observed after *in vitro* drug exposure have not been confirmed by marker transfer (Table 5), and they illustrate the potential diversity and distribution of *pol* mutations, which may not be the same in laboratory strains as in clinical isolates.

TABLE 1. *UL97* resistance mutations confirmed by marker transfer or recombinant phenotyping[a]

Codon no.	Amino acid(s)/mutation		No. reported[b]	GCV ratio[c]	Reference(s)	Comment
	Wild type	Mutant				
405	L	P		2.5	41	
460	**M**	**I**	**20**	**5**	1, 28, 56, 132, 173, 180, 209	
460	M	T		9.3	41	
460	**M**	**V**	**41**	**8.3**	20, 54, 56, 74, 104, 125, 132, 136, 173, 180, 209	
466	V	G		3.5	144	
520	**H**	**Q**	**22**	**10**	20, 56, 74, 90, 104, 126, 132, 173	
591–594	ACRA	Del 4[d]	2	3–10	20, 56, 187	
591–607	ACRALENGKLTHCSDAC	Del 17		6.2	56	Laboratory mutant
592	**C**	**G**	**23**	**2.9**	28, 54, 56, 74, 104, 125, 173, 180	
594	A	E		3.0	41	
594	A	G	1	13.5	26, 173	
594	A	P	3	NA[e]	28, 100, 132, 173	Vaccinia virus recombinant[f]
594	A	T	7	2.7	1, 20, 28, 56, 74, 132, 173, 180	
594	**A**	**V**	**81**	**8.3**	1, 20, 28, 54, 56, 74, 104, 125, 126, 132, 180, 209	
595	L	F	9	15.7	1, 56, 125, 173, 180, 209	
595	**L**	**S**	**65**	**9.2**	20, 28, 54, 56, 74, 104, 125, 132, 180	
595	L	W	5	5.1	56, 104, 132	
595	L	Del		13.3	9	
595–603	LENGKLTHC	Del 9		8.4	52	
596	E	G	2	2.3	28, 56	
598	G	S		NA	7	Vaccinia virus recombinant
599	K	T		5.3	75	
600	L	Del	1	1.9	56	
601	T	Del		NA	91	Vaccinia virus recombinant
601–603	THC	Del 3		11	136	
603	C	R	1	3.6–8.3	41 132, 144	
603	C	S	1	1.9	41, 132	
603	**C**	**W**	**31**	**8**	20, 28, 41, 46, 56, 126, 132, 173, 180	
607	C	F	3	1.9	20, 56, 173	
607	C	Y	4	12.5	11, 20, 56, 132, 173	

[a] Boldface indicates the seven most common ("canonical") UL97 mutations conferring GCV resistance.
[b] Number of distinct isolates in a total of 322 cases reported in several series (20, 28, 56, 74, 104, 125, 132, 173, 180). No number is shown for the unusual mutations reported outside these series.
[c] IC_{50} of mutant/IC_{50} of wild type. These ratios may fluctuate by 20 to 30% in replicate assays of the same mutation, and a range is shown where reported results vary by more than this amount.
[d] Deletion starting at the designated codon and continuing through the number of codons shown. Deletion of codons 591 to 594 results in the same mutant virus as deletion of codons 590 to 593.
[e] NA, not available.
[f] Vaccinia virus recombinants do not have GCV IC_{50} ratios.

Mutations Observed *In Vivo* after Antiviral Therapy

***UL97* mutations.** GCV resistance was reported in isolates from three patients shortly after the first therapeutic use of the drug (73) and before any information about relevant viral mutations was published. When the function of pUL97 in GCV anabolism became known, it was discovered that the resistant isolates from these cases contained *UL97* mutations A594V and L595S (42), which are among the most frequently encountered in GCV-resistant isolates. M460V was also reported among GCV-resistant isolates at around the same time (42). Marker transfer studies (see "Recombinant Phenotyping" below) confirmed that the three mutations conferred phenotypic GCV resistance and decreased GCV phosphorylation (42). Subsequent compilations of UL97 mutations found in clinical isolates have shown that GCV resistance mutations are strongly clustered at codons 460, 520, and 590 to 607 (Fig. 2B; Table 1), with one of the "canonical" mutations M460V/I, H520Q, C592G, A594V, L595S, and C603W appearing in >80% of resistant isolates from patients who received GCV as initial therapy (56, 173). The codon range from 591 to 607

TABLE 2. *UL97* drug-sensitive mutations

Mutation category	Codon no.	Amino acid		Reference(s)
		Wild type	Mutant	
Confirmed drug sensitive by transfer to control strain	68	N	D	41, 135
	126	L	Q	41, 135
	244	I	V	41, 135
	329	D	H	56
	427	A	V	143
	449	Q	K	57, 135
	466	V	M	41
	469	H	Y	41, 135
	478	A	V	41
	510	N	S	41
	550	M	I	143
	582	A	V	143
	587	H	Y	54
	588	A	V	41
	591	A	V	57
	597	N	D	101
	599	K	R	41
	600	L	I	41
	605	D	E	41, 135
	615	M	V	144
	617	Y	H	143
	623	G	S	41
	634	L	Q	56
	659	T	I	41
	665	V	I	41
	674	A	T	143
Observed in baseline sequences or drug-sensitive isolates	19	Q	E	135
	95	T	S	135
	108	S	N	135
	112	R	C	135
	137	R	C	135
	227	E	D	135
	582	A	T	135

appears to be completely dispensable (56). This allows for the selection of a large variety of uncommon mutations and in-frame codon deletions that confer various degrees of GCV resistance ranging from none (D605E) to approximately 15-fold over baseline (Tables 1 and 2). This supports the hypothesis that mutations in this region impair the recognition of GCV as a substrate while preserving the normal biological functions of pUL97. Mutations at codons 460 and 520 are located at conserved kinase domains more likely to be critical to overall kinase function, which probably explains the more limited range of mutations observed at these sites. Two recent publications report the rare occurrence of noncanonical GCV resistance mutations in clinical specimens, outside the previously defined range (41, 144). Mutations L405P and V466G each conferred low-grade GCV resistance. While the L405P mutant was reported to have relatively normal growth, further study is required to determine if the V466G mutation causes a growth defect associated with mutations that impair UL97 autophosphorylation (149).

The frequency with which prior treatment with ACV elicits GCV resistance *in vivo* remains unclear. One study based only on phenotypic testing of available HCMV isolates from HIV-infected subjects receiving prolonged high-dose ACV did not detect GCV resistance (66). On the other hand, one of the first three reported cases of GCV resistance had evidence of preexisting resistance before receiving any GCV but after receiving ACV (73).

***pol* mutations.** A wide variety and distribution of *pol* mutations have been found in drug-resistant HCMV clinical isolates. It was noted early on, and confirmed by later observations, that *pol* mutations *in vivo* were more likely to emerge after prolonged GCV exposure (180) and add to the level of resistance conferred by preexisting *UL97* mutations (54). The appearance of a *pol* resistance mutation in the absence of a *UL97* mutation, after initial therapy with GCV, is uncommon but has been reported (21, 74). Several dozen *pol* mutations from clinical isolates have now been found to confer various patterns of cross-resistance, as shown in Fig. 5B and detailed in Table 3. As a broad generalization, mutations conferring dual GCV-CDV resistance are located in the exonuclease domains and region V, while those conferring FOS resistance range widely but are concentrated at and between regions II and III. Some mutations in and near region III confer FOS resistance with low-grade GCV cross-resistance, and a few mutations (e.g., A834P and Δ981-2) confer moderate resistance to GCV, FOS, and CDV. Unlike the situation with *UL97*, there is not a short list of mutations that covers the vast majority of resistant isolates. Therefore, *pol* genotypic resistance testing requires more extensive sequencing, typically covering the codon range of 300 to 1000. Interstrain sequence polymorphisms are relatively common in this gene, which makes it more complicated to distinguish them from true resistance mutations (45, 77). However, the majority of resistance mutations occur within the conserved regions of homology, and many of these mutations are at amino acid residues that are very highly conserved among enzymes from herpesviruses (190) as well as enzymes from a wide spectrum of unrelated organisms such as yeasts, bacteria, and humans (31). Conversely, the majority of amino acid polymorphisms that are not associated with drug resistance occur at residues outside the conserved regions (Fig. 5C and Table 4). No drug resistance mutations have so far been detected in regions I and VII. In contrast to the case for *UL97*, perceptible attenuation or retardation of viral growth in cell culture has been documented with many *pol* mutations that confer drug resistance (10, 60) (51). This may be a factor in *pol* mutations not being a preferred genetic pathway of drug resistance.

Recombinant Phenotyping

For continued antiviral drug development and for accurate interpretation of genotypic resistance assays, the roles of specific mutations in HCMV drug resistance are assessed by transferring them to control laboratory strains of HCMV and testing for altered drug susceptibility in the resulting mutant, using a standardized phenotypic assay. This process is termed "marker transfer" or "recombinant phenotyping" and is analogous to current methods for HIV resistance phenotyping (94). Because of the large size of the HCMV genome, recombinant phenotyping has historically been technically difficult, but its efficiency has been considerably improved in recent years.

Homologous recombination by cotransfection. Originally, putative drug resistance mutations were transferred to control

TABLE 3. UL54 *pol* resistance mutations characterized by marker transfer/recombinant phenotyping

Region	Codon no.	Amino acid(s)/mutation		Ratio[a]			Reference(s)
		Wild type	Mutant	GCV	FOS	CDV	
ExoI	301	D	N	**2.6**	0.5	**3**	44
ExoII	408	N	D	**4.9**	1.3	**5.6**	59
	408	N	K	**4.2**	0.7	**21**	176
	410	N	K	**2.9**	0.8	**3**	44
	412	F	C	**4.2**	1.2	**18**	46
	412	F	V	**4.3**	1.1	**15.5**	59
	413	D	A	**6.5**	0.8	**11**	136
	413	D	E	**4.8**	0.8	**4.3**	44
IV	495	N	K	1.1	**3.4**	1.1	68
ExoIII	501	L	I	**6**	1.4	**9.1**	59
	503	T	I	**2.9**	0.5	**6.1**	44
	513	K	E	**5**	1.4	**9.1**	59
	513	K	N	**6**	1.1	**12.5**	60
	516	L	R	**2.1**	0.8	**5.1**	44
	521	I	T	**3.1**	0.9	**3.9**	47
	522	P	A	**3**	1	**4.1**	47
	522	P	S	**3.1**	1.1	**3.6**	59
	545	L	S	**3.5**	1.2	**9.1**	59
	588	D	E	1.3	**2.3**	1.1	59
	588	D	N	**3.8**	**3.2–9**	**2.7**	154, 181
II	700	T	A	0.9	**4.7**	1.5	10
	715	V	M	1	**5.5**	1.1	10
	756	E	D	1.2	**3.4**	0.7	44
	756	E	K	**3.5**	**>8**	**2.2**	44
	756	E	Q	1.7	**4.3**	1	202
VI	776	L	M	**2.5**	**3.5**	1	177
	781[b]	V	I	**1–4**	**4–5.2**	1.2	59, 154
	787	V	L	**2.4**	**4.1**	1	202
III	802[b]	L	M	**1.1–3.5**	**3.2–10.8**	0.9–1.8	46, 59
	805	K	Q	1	0.18	**2.2**	59
	809	A	V	**2.6**	**6.3**	1.7	48
	812	V	L	**2.5**	**2.9**	**3.2**	60
	813	T	S	**2.5**	**4.9**	**2.7**	51
	821	T	I	**4.5**	**21**	1.9	59
	834	A	P	**5.4**	**6.4**	**3**	176
	838	T	A	1.8	**2.4**	0.8	181
	841	G	A	**3.2**	**4.3**	**2.6**	51
V	981–982	DL	Del 2[c]	**8.3**	**3.6**	**2.8**	53
	987	A	G	**5.3**	1.2	**11.3**	186

[a] IC_{50} of mutant/IC_{50} of wild type. These ratios may fluctuate by 20 to 30% in replicate assays of the same mutation, and a range is shown where reported results vary by more than this amount. Boldface indicates reduced susceptibility according to the references cited.
[b] Resistance patterns for mutations differ between the references cited.
[c] Deletion starting at the designated codon and continuing through the number of codons shown.

HCMV strains by cotransfecting into fibroblast cultures a segment of viral DNA containing the mutation of interest together with genomic viral DNA of the control strain (42, 187). The resulting rare recombinant mutant virus was then isolated and plaque purified after limited propagation under drug exposure, and its drug resistance phenotype was determined by a standardized assay such as plaque reduction. This method is tedious, risks introducing new mutations after propagation under drug exposure, and is unlikely to succeed if the desired mutation confers a low level of drug resistance (less than 3-fold) or significant growth impairment. Despite these deficiencies, common *UL97* and *pol* resistance mutations were validated in this manner (9, 10, 80, 90, 186), and the reported GCV resistance phenotypes are generally consistent with later findings using newer techniques.

Recombination of cosmid clones. By cotransfecting about seven overlapping cosmids missing the wild-type *pol* sequence, along with *pol* DNA containing the mutation of interest to fill the gap, live virus that should contain nothing but the mutant recombinant can be reconstituted. Although this technique enabled the phenotyping of 17 *pol* mutations in one study (59), the tendency of cosmid clones to become defective and the multiple recombination events required to create an infectious viral genome frequently result in no recovery of live virus.

Genetically modified control strains. Unique restriction sites, such as PmeI or SwaI sites, were introduced into the

TABLE 4. UL54 *pol* drug-sensitive mutations

Mutation category	Codon no.	Amino acid(s)/mutation		Reference(s)
		Wild type	Mutant	
Confirmed drug sensitive by transfer to control strain	515	D	G	45
	522	P	L	47
	692	A	S	44, 77
	971	G	D	44
Observed in baseline sequences or drug-sensitive isolates	15	A	T	45
	24	S	L	45
	32	S	P	45
	95	V	E	45
	142	H	Y	45
	143	G	S	45
	341	I	T	21
	345	N	S	21
	347	G	D	45
	355	V	A	45
	464	S	F	45
	626	A	V	45
	628	P	L	45
	629	G	S	21
	631	A	G	45
	633	S	F	77
	640	M	R	45
	655	S	L	45
	663	S	N	45
	669	F	L	45
	676	S	G	45
	678	G	S	45
	681	VSNDNHGA	Del 8	77
	685	N	S	45
	688	A	V	45
	691	T	A	77
	693	A	T	45
	695	S	T	175
	697	Q	H	45
	737	L	M	176
	759	V	M	29
	868	Q	R	45
	870	D	H	45
	873	V	L	45
	874	G	R	45
	884	S	Insert T	45
	885	A	S	45
	885	A	T	45
	887	P	S	45
	890	L	F	45
	892	T	I	21
	897	S	L	45
	898	N	D	45
	899	E	K	45
	927	V	M	36
	953	V	A	45
	1012	A	V	156
	1020	L	I	45
	1108	T	A	65
	1116	N	H	45
	1122	A	T	45
	1133	G	S	45
	1146	S	N	45
	1147	N	S	45
	1149	R	T	45
	1151	G	Del	45
	1153	P	S	45
	1154	A	P	180
	1156	K	Del	45
	1162	P	L	45
	1235	S	T	45

TABLE 5. *pol* and *UL97* changes from baseline after drug exposure *in vivo* or *in vitro* with undetermined significance[a]

Gene	Codon no.	Amino acid(s)/mutation		Reference
		Wild type	Mutant	
UL97	590	AACRALENGKL	Del 11[b]	210
	590	AACRALENGKLTHC	Del 14	29
	592	C	F	210
	594–595	AL	Del 2	171
	596	E	Del	24
	599	K	E	24
	599	K	M	210
	600	LT	Del 2	104
	601	T	M	8
pol	412	F	S	132
	419	T	M	155
	500	K	N	82
	501	L	F	74
	552	T	N	82
	578	Q	H	155
	585	S	A	82
	757	N	K	82
	773	L	V	155
	802	L	R	82
	802	L	V	82
	829	P	S	82
	841	G	S	24
	879	D	G	82
	926	L	V	82
	957	L	F	82

[a] This table contains examples of sequence changes from baseline observed after exposure to antiviral drugs but not confirmed by marker transfer.
[b] Deletion starting at the designated codon and continuing through the number of codons shown.

standard laboratory strain AD169 at the desired sites of recombination (*UL97* or *pol*). Genomic viral DNA could then be digested with the enzymes (PmeI or SwaI), causing a targeted break which could be repaired by recombination with a cotransfected overlapping DNA segment containing the mutation of interest (44, 56). This enabled the routine recovery of recombinant virus without the requirement for any propagation under drug exposure, provided that the mutation did not severely impair viral viability. This technical approach was used to phenotype various *UL97* mutations, including ones that confer low-grade GCV resistance (56), and various *pol* mutations as well (44).

Phenotyping of recombinant viruses using the standard plaque reduction assay is labor-intensive and subjective (as discussed below), making it difficult to perform an adequate number of replicates to ensure reliability of phenotypic data. To increase operational efficiency, reporter gene cassettes have been inserted into the control laboratory strains used for recombinant phenotyping. For example, a secreted alkaline phosphatase (SEAP) gene driven by the HCMV major immediate-early promoter is used to quantitate growth over multiple cycles of viral replication at days 4 to 8 postinfection by assaying culture supernatant for enzyme activity using a chemiluminescent substrate (54). Drug resistance phenotypes associated with particular mutations were determined by measuring the drug concentrations required to decrease SEAP activity by 50% at 6 days. This method appears to correlate well with data obtained by more traditional techniques.

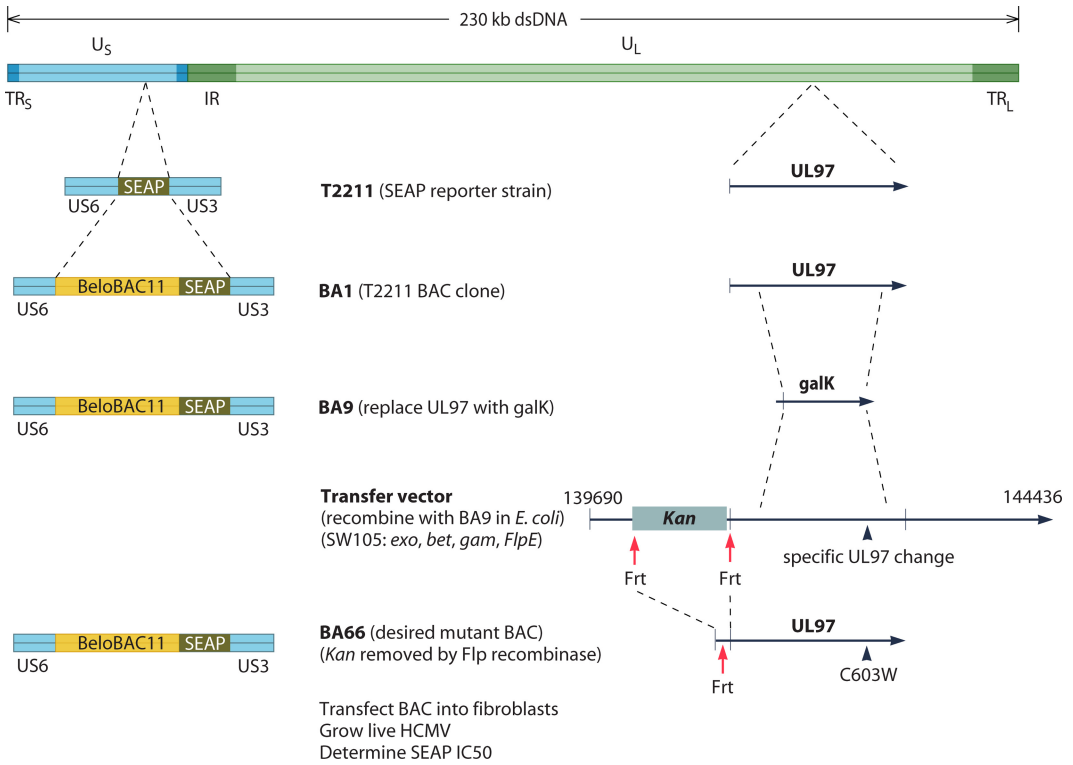

FIG. 6. Recombinant phenotyping. Sequential genetic constructs from top to bottom illustrate a contemporary method for determining the drug resistance phenotype of a viral mutation. Standard laboratory strain AD169 is modified with a secreted alkaline phosphatase (SEAP) reporter gene between genes *US3* and *US6* (strain T2211) (54). A bacterial artificial chromosome (BAC) vector is then ligated into strain T2211 DNA at this site and a full-length clone (BA1) is isolated in *E. coli.* The gene region to be mutagenized (*UL97*) is replaced with a selectable *galK* gene (BA9) by conditional recombination ("recombineering") in *E. coli* strain SW105 (200). The desired mutant *UL97* sequence is then introduced by additional recombineering, using a transfer vector containing a selectable kanamycin marker (Kan) flanked by 34-nucleotide Frt motifs. The Kan marker is subsequently removed by an inducible Flp recombinase, leaving a BAC clone (BA66) containing a specific *UL97* mutation (in this case C603W) and one upstream Frt motif (41). The mutant BAC is transfected into human fibroblast cell cultures, resulting in live virus that is phenotyped for drug susceptibility using culture supernatant SEAP activity as a measure of viral growth (41, 54). (Adapted from reference 41.)

BAC clones. The most recent trend in HCMV recombinant phenotyping is to mutagenize bacterial artificial chromosome (BAC) clones of the virus. Although the initial cloning and characterization of a given HCMV strain as a BAC takes some time and effort, its subsequent mutagenesis is greatly facilitated by a variety of elegant methods that have been developed for BACs in general, including "recombineering" (200) in special strains of *Escherichia coli* (e.g., SW102 or SW105). These strains have temperature-inducible expression of genetic elements *exo*, *bet*, and *gam*, which enable the transient high-frequency recombination of introduced DNA segments with as little as 50 nucleotides of homology at their ends. Recombineering can introduce various selectable markers, such as a galactokinase (*galK*) cassette that is capable of both positive and negative selection (200) or a Frt-Kan-Frt cassette (Flp recombinase recognition target sites flanking a kanamycin resistance gene) (143), to facilitate site-specific mutagenesis. An early BAC clone (25) of HCMV strain AD169 (pHB5) was mutagenized in one study to show that several *UL97* sequence variants from a clinical trial had no effect on GCV susceptibility (143) and in another study to show that *pol* region III mutation L845P resulted in a nonviable virus (51) with a "knockout" mutation that could not have been validated using earlier technology. More recently, a SEAP-expressing derivative of the laboratory strain AD169 has been cloned as a BAC and used for the recombinant phenotyping of many UL97 mutants (Fig. 6) (41). Although BAC clones are sufficiently stable to produce infectious HCMV DNA routinely, it remains unclear how frequently nucleotide sequence errors and deletions occur during BAC propagation compared with the replication of infectious virus in cell culture, where defective genomes have a survival disadvantage. Attention must be given to the quality and purity of BACs at each stage of mutagenesis, using such diagnostic tools as restriction digests, PCR to detect the presence or absence of nonviral genetic markers, and DNA sequencing.

Biochemical Assays of Expressed Mutant Enzymes

Indirect evidence of drug resistance may be obtained by expressing a mutant enzyme in a heterologous system outside the context of the HCMV genome and measuring the effect of the mutation on enzyme activities such as GCV phosphorylation or DNA polymerization under antiviral drug exposure. For example, various *UL97* sequences encoding mutations previously detected in drug-resistant clinical isolates were expressed in a recombinant vaccinia virus system. The level of

drug resistance was measured by a quantitative decrease in GCV phosphorylation activity, which correlated with levels of GCV resistance conferred by the mutations in recombinant phenotyping assays (7). In addition, a newly observed mutation, G598S, was predicted to confer GCV resistance based on this system (Table 1). *UL97* has also been expressed by transient transfection of expression constructs into cell cultures (170). Mutant and wild-type *pol* genes were expressed by *in vitro* translation in a reticulocyte lysate system (67). The enzymatic activity of Pol was assayed using a radioactive or colorimetric readout to test for inhibition by FOS and appeared to identify several mutations known to confer phenotypic FOS resistance.

Biochemical assay systems are not likely to replace recombinant phenotyping methods that involve live HCMV grown under standard cell culture conditions for a number of reasons. First, *in vitro* translation and expressed-enzyme assays are technically complex and experimentally variable. Second, there has not been sufficient quantitative correlation of biochemical assay data with the measured levels of drug resistance conferred by specific HCMV mutations. Finally, GCV triphosphate and CDV diphosphate, the active forms of the nucleoside drugs, are required in cell-free Pol assay systems but are not readily available.

LABORATORY DIAGNOSIS OF DRUG RESISTANCE

There are two types of assays used to diagnose antiviral drug resistance: phenotypic and genotypic. Phenotypic assays were the first to be developed, and they have been of great importance in identifying and characterizing mutations that arise in the target genes in response to antiviral therapy. For clinical diagnostic purposes, phenotypic methods are too time-consuming, although they remain essential for validation of genotypic assays, which are now routinely used for diagnosis of drug resistance.

Phenotypic Methods

Phenotypic methods to detect drug resistance are based on determination of the drug concentration required to reduce viral growth by a specified amount in cell culture. This requires preparation of a standardized inoculum and quantitative viral replication in the presence and absence of the drug. The steps involved in cell culture and detection of viral replication vary among the assays. Cutoff levels for each drug have been determined using virus strains known to be sensitive to each of the approved drugs. These include laboratory strains such as AD169 and Towne and clinical strains isolated from patients who have never been exposed to these drugs. It is important to note that the laboratory strains are high-passage isolates, which produce cell-free virus; therefore, the inoculum is obtained from cell culture supernatants. Low-passage clinical strains are highly cell associated, which means that the inoculum consists of infected cells that are trypsinized from monolayer cultures.

Plaque reduction assay. The plaque reduction assay (PRA) has been considered the "gold standard" for phenotypic drug resistance testing, because the CPE of the virus is observed and quantitated by plaque counting, a traditional measure of viral infectivity. The virus sample must first be cultured and standardized to determine either the cell-free titer or the percentage of infected cells in permissive cell monolayers, usually human foreskin fibroblasts (HFF). It normally requires 3 to 4 weeks to obtain optimal infectivity (at least 60% of the monolayer) from a clinical sample, which produces only cell-associated virus. The inoculum is obtained by trypsinizing the infected monolayer and adjusting the concentration of infected cells to produce a readable number of plaques in monolayers in a multiwell plate. This is commonly approximately 60 to 80 plaques per monolayer in a 24-well plate in the absence of drug (120). The newly infected monolayers are exposed to a range of drug concentrations, which has been determined for each drug to cover the established cutoff for drug susceptibility. The number of plaques produced at each drug concentration is compared to the number of plaques produced in the absence of drug. The concentration at which there is a 50% reduction in plaque number is calculated as the 50% inhibitory concentration (IC_{50}). Proposed cutoffs for susceptibility are 6 μM GCV, 2 μM CDV, and 400 μM FOS (38, 132), but these need to be calibrated in each laboratory based on known susceptible and resistant control strains. To improve the correlation of results from different laboratories, cutoffs may be defined as the ratio of the IC_{50} to that of a susceptible control. This is typically a 2-fold or greater increase, given the standard deviations usually observed in replicate phenotyping assays. The time required for this quantitative part of the assay is an additional 7 to 10 days, which makes the total time to obtain results approximately 4 to 6 weeks.

A consensus PRA protocol has been published (120); however, there are a number of problems in standardizing the assay. The extracellular high-passage, drug-sensitive laboratory strains have replication kinetics different from those of cell-associated clinical strains, which makes it difficult to use the laboratory strains as drug-susceptible controls. Quantitation of the inoculum of cell-associated clinical isolates is problematic, because it is not possible to determine the viral load per cell. For technical personnel the assay is labor-intensive, and there is subjectivity in determining the optimal time to read the assay as well as in the actual counting of plaques. For example, plaques produced by drug-resistant isolates in the presence of drug develop more slowly and are often smaller than those in control wells without drug. These problems together with the lengthy time required to perform the assay preclude the use of the standard PRA for therapeutic monitoring. However, the assay is still useful in the research and developmental setting to characterize HCMV strains carrying potential drug resistance mutations (see below). For this purpose, the ability to monitor and quantitate viral CPE in the presence of antiviral agents is not restricted by the length of time required to perform the assay.

Other phenotypic methods. Because the value of culture-based methods for confirming drug resistance mutations is well recognized, a number of modifications to reduce the extended time frame and subjectivity in quantitating infectivity have been reported. All assays published so far attempt to address these problems by shortening the time required for culture of a clinical sample and/or by enhancing detection techniques. None of the alternative methods discussed below has been widely adopted or considered a reference standard similar to the PRA.

Yield reduction assays are based on the concept that drug-

sensitive strains "yield" lower measurable titers of infectious virus when propagated under increasing drug concentrations. Although yield reduction protocols that reduce the time required for the growth of virus in the presence of drug have been published (167), a calibrated initial viral inoculum is still necessary.

An *in situ* enzyme-linked immunosorbent assay (ELISA) protocol was reported to be somewhat faster than the PRA (189). The inoculum preparation is similar to that for the PRA, but the assay is performed using HFF monolayers in 96-well plates. Production of a late HCMV antigen is detected colorimetrically by ELISA using a standard 96-well plate reader.

Phenotypic assays based on flow cytometry have been published (121, 146). They also require time to prepare the inoculum, consisting of virus-infected cells, which can be added to monolayers in 25-cm^2 flasks and incubated for 3 to 4 days in the presence of a range of drug concentrations. An alternative method of inoculation that can further shorten the assay is to accelerate transfer of virus infection by mixing infected with uninfected cells and adding them to six-well plates (121). In each case the cells are harvested, permeabilized, and treated with a fluorescein isothiocyanate (FITC)-labeled monoclonal antibody to an HCMV immediate-early (IE) antigen. The IC_{50} is determined by flow cytometric analysis of expression of the IE antigen in the presence of drug, which is less labor-intensive and more objective than the PRA. A problem with flow cytometry is the relatively high background resulting from input virus-infected cells.

A simplified PRA protocol was published by Prix et al. (168), in which the inoculum was prepared from primary isolates that were passaged on monolayers in 25-cm^2 flasks. Similar to the procedure for the flow cytometry assay described above, these infected cells were mixed with uninfected cells in 96-well plates to accelerate the transfer of HCMV infection. The cocultures were exposed to increasing concentrations of drug for 5 days. Immunoperoxidase staining of an IE antigen was used to detect and quantitate plaques. Effective standardization of this type of assay could be difficult.

Other methods have also addressed the lengthy time required to expand the amount of infectious virus for preparation of the inoculum. One approach is to use primary cultures of clinical specimens. Leukocytes from patients with HCMV viremia have been used as a direct inoculum in a modified plaque assay (79). Growth in shell vials in the presence and absence of drug was detected by immunoperoxidase-tagged monoclonal antibodies to the HCMV major IE protein. The assay could be read at 4 to 6 days postinoculation; however, it still required microscopic counting of plaques. Others reported a similar assay for urine, bronchoalveolar lavage fluid, and amniotic fluid specimens (161). An antibody to a late HCMV antigen was used, which, unlike the IE antigen, is highly dependent on viral replication for its expression. The main problem with these assays is that they require specimens that contain a sufficient amount of infectious virus to give quantitative results.

A DNA-DNA hybridization assay (62) was formerly available as a commercial assay (103). The preparation of the inoculum and assay format were similar to those for the standard PRA, but instead of reading plaques, the monolayers were lysed to extract the viral DNA, which was hybridized to HCMV-specific ^{125}I-labeled probes. The hybridization was quantitated in a gamma counter, and the IC_{50} was determined by a 50% reduction in radioactivity compared to controls unexposed to drug. This method of quantitation is more efficient than plaque counting, but acceptance of the assay was limited by the radioactivity hazard and associated regulations.

A recently reported assay uses real-time PCR to quantitate viral DNA extracted from monolayers exposed to a range of drug concentrations. HCMV isolates are grown for 4 days in the presence or absence of drug. The IC_{50} is determined by the concentration of drug that produces a 50% reduction in viral DNA copy numbers (172). The advantage of this molecular phenotypic assay is the sensitivity and objectivity in measuring the endpoint by an automated system.

All of these methods address the problem of subjectivity of the plaque reduction assay by direct detection of virus-specific antigens or viral DNA, which tends to make the quantitative portion of the assays more objective, rapid, and reproducible. However, in most cases prior culture of the original clinical sample is still required to amplify the inoculum before performing the assays. The variability in the quantity of virus in the initial sample remains a problem for standardization (79, 161), and even with shorter culture and detection modifications, the total time is still too long for the assays to be of value in making therapeutic decisions. In addition, many clinical laboratories no longer use cell culture for diagnostic purposes; therefore, virus isolates are not available for phenotypic resistance testing. However, some form of phenotypic assay is still required for confirmation of potential new drug resistance mutations identified by genotypic methods described below. Recombinant phenotyping approaches using genetically modified control strains or BAC clones (see above) are now preferred for this purpose (Fig. 6).

Genotypic Methods

Rapid laboratory confirmation of HCMV drug resistance is important because increasing viral loads or disease during prolonged antiviral therapy may be due to host factors rather than to drug resistance. Empirical changes in treatment regimens are complicated by the toxicity and logistical complexities associated with the alternative drug therapies. Thus, except in pressing clinical situations (life- or sight-threatening disease), it is desirable to confirm a clinical suspicion of drug resistance by laboratory testing, which should also include the degree of drug resistance and the extent of cross-resistance with other drugs.

This need for rapid diagnosis of drug resistance has led to the development of genotypic methods for identification of resistance mutations. The length of time to perform genotypic assays varies from a few hours to up to 3 days, which is significantly less time than for any of the phenotypic assays, and the results are completely objective. The main problem with genotypic assays is that resistance mutations cannot be distinguished from sequence polymorphisms without prior confirmation by phenotypic analysis.

Restriction endonuclease analysis. One of the earliest published genotypic methods is based on detection of mutations by changes in restriction endonuclease recognition sequences in *UL97* PCR products amplified from suspected drug-resistant virus strains. These changes result in either gain or loss of

restriction sites, which are detected by running a set of restriction digests of the PCR products on an agarose gel (19, 20, 42, 43, 74, 83). The pattern of fragments produced by different enzymes is indicative of the presence of specific drug resistance mutations.

Screening of *UL97* by restriction endonuclease analysis is effective, because PCR products can be directly obtained from low copy numbers of HCMV DNA in clinical samples, and no culture or DNA sequencing of the viral genome is required. The assay is relatively simple and inexpensive to run. The main problems are that not every mutation will produce a change in a restriction site and that changes in restriction patterns could be the result of polymorphisms close to previously mapped mutations, which yield fragments of similar size. In addition, the assay was developed only for mutations in the *UL97* coding sequence, which occur in a limited number of codons in a short region of the gene. The assay has not been expanded to *pol*, because the mutations and polymorphisms in that gene are more numerous and scattered across a wider range of nucleotides (38, 45, 81).

Real-time PCR. A real-time PCR assay with melting curve analysis to identify confirmed drug resistance mutations in *UL97* has recently been published (85, 86). Hybridization probes specific for each mutation site are labeled with different fluorescent dyes. Changes in the melting curve introduced by mutations are analyzed following PCR amplification. The advantages of the assay are that (i) very low DNA copy numbers can be amplified without cell culture, (ii) mixed virus populations can be analyzed semiquantitatively, and (iii) multiple mutations can be detected simultaneously using different dye labels. The disadvantages are that polymorphisms near known mutations may affect the melting curve and individual probes are required to identify each mutated codon. In addition, the assay cannot distinguish different point mutations that occur in the same codon, such as M460I (ATT/ATA) and M460V (GTG).

DNA sequencing. The standard genotypic method for detection of drug resistance mutations has been DNA sequencing of PCR products amplified from the antiviral target genes, because all nucleotide and amino acid substitutions within the amplified region can be identified. Sequencing of both strands resolves most ambiguities. Fluorescent dideoxy sequencing of PCR products is able to detect a subpopulation of mutant sequences within a wild-type background when the mutant sequence is greater than 20 to 30% of the total, based on experience with HIV-1 diagnostic sequencing (174). Newer, "deep" sequencing technologies enable the detection of far smaller subpopulations of mutants, even <1%, and have been initially explored in connection with HIV-1 and hepatitis B virus resistance mutations (137, 178, 199). Preliminary data indicate that ultradeep sequencing enables the earlier detection of emerging drug resistance mutations. Although the technology is currently expensive and impractical for diagnostic purposes, it may be useful for studying how drug exposure histories influence the early evolution of mutations that result in various degrees of viral growth fitness and drug resistance. From this information potent antiviral regimens that decrease the risk of drug resistance may be designed, and sentinel mutations may be targeted for sensitive detection of impending resistance.

Since GCV is the current drug of choice for prophylaxis and treatment of HCMV disease and *UL97* is usually the initial site of drug resistance mutations, most current diagnostic sequencing protocols examine *UL97* first. A recommended codon range for sequencing is 400 to 670 after GCV exposure. In the future, screening for resistance to UL97 inhibitors such as MBV may require expansion of the range to codons 300 to 670 to include the ATP binding domain.

Under current protocols, if no mutations are found in *UL97* or if the patient is being treated with FOS or CDV, *pol* can be sequenced. Because *pol* is larger than *UL97* and confirmed drug resistance mutations are widely dispersed across the gene, it is necessary to sequence from codon 300 to 1000. As additional mutations are analyzed in the future, this region may need to be expanded.

Interpretation of Genotypic Assays

The genotypic assays described above that do not involve direct DNA sequencing are designed to detect only a select group of confirmed resistance mutations. Therefore, interpretation of results is relatively straightforward. DNA sequencing assays, on the other hand, detect all nucleotide and amino acid codon changes in the PCR products amplified from the regions of the target genes that contain confirmed resistance mutations. This has generated a long list of variant codons, some of which have been phenotypically tested. However, the phenotypes of a large number of these variants remain undetermined, which complicates the interpretation of sequencing assay results. To address this problem, evolving databases of reported variant codons have been maintained by various groups but are not yet standardized. A web-based search tool was recently published to interpret user-supplied sequences and provide links to published mutations with the supporting phenotypic data (37). The variant codons that have been reported for both *UL97* and *pol* fall into the following general categories: (i) no drug resistance conferred after transfer to drug-sensitive control strain, (ii) observed in baseline or drug-sensitive isolates, (iii) observed in isolates from treated individuals with no baseline sequence available, (iv) change from known baseline sequence after drug exposure *in vivo* or *in vitro*, and (v) drug resistance conferred when transferred to drug-sensitive control strain.

The variants in the first category have been proven by phenotypic methods not to confer resistance and probably represent the naturally occurring polymorphisms within each gene (Tables 2 and 4). Those variants in the next three categories represent a spectrum of potential resistance mutations ranging from very unlikely to highly likely to confer resistance, depending on drug exposure history and position within the gene coding sequence. The last category represents the mutations that have been proven by a recombinant phenotyping method to confer resistance to one or more of the approved anti-HCMV drugs. These are the only ones that should be reported to the clinician as validated resistance mutations (Tables 1 and 3). The relative level of resistance is expressed as a ratio of the IC_{50} of the mutant to that of the drug-sensitive wild-type control where available.

Confirmed mutations. (i) *UL97* mutations. The baseline sequence variability of *UL97* is <2% among drug-sensitive clinical HCMV strains (135). The only licensed drug that selects

for resistance mutations in this gene is GCV. As stated above, the sites of these mutations have remained quite consistent over many years of study, namely, codons 460, 520, and 590 to 607 (39, 42, 46, 52, 56, 90, 101, 133), although codons 405 and 466 are two recent additions (41, 144). The codon range from 590 to 607 includes both point mutations and codon deletions. The frequency at which these mutations have been reported and the level of resistance conferred are listed in Table 1. It should be noted that not all sequence changes within the codon range from 590 to 607 are associated with resistance (for example, N597D, K599R, L600I, and D605E) (54, 101) (Table 2). Thus, a drug resistance phenotype cannot be assumed to be conferred by a given mutation based solely on proximity to other genotypically identified mutations.

(ii) *pol* mutations. The coding sequence of *pol* is almost twice as long as that of *UL97.* The baseline sequence is more variable and the observed number of resistance mutations is greater in *pol* than in *UL97*. However, resistance mutations in *pol* are detected much less frequently in clinical isolates than mutations in *UL97*. Some individual mutations such as P522S, V781I, L802M, A809V, A834P, and A987G, have been reported in multiple isolates (Table 3), but unlike in *UL97*, there are no well-defined codons or codon ranges where *pol* mutations consistently occur. What is striking is that almost all confirmed resistance mutations occur in regions of the *pol* sequence that are highly conserved among all types of organisms (Fig. 5). However, within these conserved regions, mutations in the same or adjacent codons can have different phenotypes. For example, L516R and P522S/A confer resistance (59, 60) (Table 3), while D515G and P522L (45) do not (Table 4). N495K confers only FOS resistance (68), but nearby L501I confers only GCV and CDV resistance (59, 134) (Fig. 5). Thus, as with *UL97*, it cannot be assumed that variant codons close to proven resistance mutations also confer resistance or have the same antiviral phenotype. Only those mutations that have been confirmed by a phenotypic method should be reported for diagnostic purposes (Table 3).

Polymorphisms and uncharacterized sequence changes. Available publications document some sequence changes in *UL97* (135) and many more in *pol* (45, 77) that have occurred in baseline HCMV isolates from patients who have never received antiviral therapy or isolates that have tested phenotypically sensitive to anti-HCMV drugs. These can be considered to be instances of baseline interstrain sequence polymorphisms (Tables 2 and 4), unrelated to drug resistance, although cases of misunderstanding arising from speculation in the published literature have required the use of recombinant phenotyping for definitive confirmation of the drug-susceptible phenotypes of such baseline changes as *UL97* H469Y, N510S, and D605E (41, 54, 135).

There is a category of mutations in both *UL97* and *pol* which have been observed in isolates showing a change from a known baseline sequence after drug exposure *in vitro* or *in vivo* which may plausibly be linked to drug resistance. Recombinant phenotyping will be required for confirmation of this association. Examples of these mutations are listed in Table 5.

Despite the dozens of phenotypically characterized HCMV drug resistance mutations, many other sequence changes noted in the *UL97* and *pol* genes are of unknown significance for resistance, based on their presence in clinical specimens from individuals who have received antiviral therapy. Uncharacterized sequence variants are a continuing source of difficulty in the interpretation of clinical genotyping data. We currently estimate that over 20 *UL97* (135) and over 150 *pol* sequence changes fall into this category, based on the published literature (24) and inquiries from laboratories providing HCMV genotyping services.

In *UL97* these variants include uncommon changes at codons 460, 520, or 590 to 607. In-frame codon deletions in the range from codon 590 to 607 may reasonably be suspected to confer some degree of GCV resistance, especially with the larger deletions involving several codons. Mutations outside the typical codon range for GCV resistance are sometimes investigated and usually found to confer no GCV resistance (41, 143), with rare exceptions (41, 144).

Similar to the case for confirmed polymorphisms, the majority of the uncharacterized *pol* changes lie outside the defined functional domains and conserved residues where resistance-related mutations have been validated (Fig. 5C). These changes are not expected to be resistance related, but there are others that involve conserved residues or domains. Depending on treatment history and sequence location, many of the uncharacterized changes are expected to be resolved by recombinant phenotyping in the near future.

So far, recombinant phenotyping has focused on the transfer of single nucleotide changes into well-characterized laboratory control HCMV strains, but the underlying strain-specific baseline sequence polymorphisms may have a significant impact on the degree of resistance conferred by a given mutation, and this has not been sufficiently examined. Interstrain variation may affect the function of the particular gene under study (*UL97* or *pol*) or of one or more unrelated genes that influence the metabolic state of the host cell, which in turn affects its permissiveness for viral replication. To address these uncertainties, future recombinant phenotyping experiments will need to involve more diverse control strains rather than a few long-established laboratory strains that have been extensively propagated in fibroblast cultures.

TREATMENT OPTIONS FOR HCMV DRUG RESISTANCE

No controlled clinical trials exist to support the use of specific treatment options where HCMV drug resistance is suspected or proven. A rigorously standardized approach is difficult to envision with the diversity of individual host factors that affect clinical outcome as much as or more so than antiviral drug resistance. Decisions on switching therapy must also consider the potency, toxicity, and logistical complexity of the available alternatives, which are currently limited. Clinical management guidelines have been proposed based largely on expert opinion assessing published case reports and anecdotal experience (17, 113, 162, 173). Although the guidelines do not agree on every detail, a consensus is as outlined below.

Antiviral drug resistance is generally suspected only when there is a rising or persistently high viral load or overt disease after several weeks of drug exposure, including a full 2 weeks of induction-dose therapy, usually with GCV. Initial attention must be given to stratifying the risk of serious life- or sight-threatening invasive disease in the treated subject. In cases

where exogenous immunosuppression is a major contributor to disease risk, the first step is to reduce such treatment to the minimum possible. Risk factors such as solid organ D+/R− HCMV serostatus, lung or pancreas transplantation, depressed markers of immune competence, recent posttransplant status, and treatment with antithymocyte antibodies are assessed. The higher the risk, the more reasonable it may be to consider a change or intensification of treatment. Empirical alterations in the face of immediately threatening disease might include the addition of FOS to a GCV regimen (157, 184) or switching from GCV to FOS.

Time permitting, genotypic testing data provide an indication of the level of resistance to one or more available drugs. The detection of no resistance mutations, as often happens, suggests staying the course with GCV at induction doses and working toward improving host factors.

If a typical *UL97* mutation that confers a 5- to 10-fold increase in GCV resistance is detected, a switch to FOS is indicated, especially in the presence of ongoing disease or high and increasing viral loads. For a mutation such as *UL97* C592G that confers low-grade resistance, it may be feasible to increase the GCV dosage up to double the standard induction dose and avoid the therapeutic complexity of an immediate switch to FOS (203) if the clinical situation is not severe as discussed above. In cases where it is elected to continue GCV in the face of *UL97* mutations, it is advisable to check for coexisting *pol* mutations that combine to increase the level of GCV resistance beyond the reach of GCV monotherapy. Depending on the specific *pol* mutation, a switch to FOS is most likely indicated, assuming that GCV was initially used. Almost all *pol* mutations that confer GCV resistance confer CDV cross-resistance, while there is more limited and usually lower-grade FOS-GCV cross-resistance. Eventually, multiple *UL97* and *pol* mutations that confer high-level resistance to all licensed drugs may accumulate, which may lead to empirical therapy with both FOS and GCV (157, 184) or to consideration of experimental treatments. CDV may be considered where GCV resistance is based solely on *UL97* mutation, but experience with this treatment option in the transplant population is limited (130), and there is concern that a *pol* mutation conferring GCV-CDV resistance may already exist as an undetected subpopulation and be rapidly selected.

EXPERIMENTAL DRUGS AND NEW ANTIVIRAL TARGETS

Drugs with Defined Viral Targets

Maribavir: a UL97 kinase inhibitor. The same UL97 viral kinase (pUL97) that is responsible for the initial phosphorylation of GCV also plays an important role in viral replication. In cell culture, HCMV growth is severely inhibited after genetic deletion of *UL97*, making it a logical antiviral target distinct from the *pol* target of current drugs (163). After screening a series of compounds for anti-HCMV activity, the benzimidazole L-riboside MBV (Fig. 3) was discovered to be a potent and highly specific pUL97 inhibitor with low host cell toxicity (15). Favorable preclinical and phase I and II clinical trials of MBV (119, 207) supported the lack of limiting toxicity (notable primarily for taste disturbance) and suggested clinical efficacy in decreasing viral shedding and prevention of HCMV reactivation after stem cell transplantation (207). Unfortunately, phase III trials of low-dose MBV as HCMV prophylaxis in stem cell and liver recipients were unsuccessful, with details yet to be published and no explanation so far for the discordance with earlier results. Several subjects given a higher dose of MBV as salvage therapy for drug-resistant HCMV infection appeared to respond (5), but more clinical data are needed to establish an appropriate therapeutic role for MBV.

In vitro propagation of HCMV under MBV exposure selects for several *UL97* mutations (V353A, L397R, T409M, and H411L/N/Y) (Fig. 2B), which confer 9-fold to >200-fold increases in MBV resistance without cross-resistance to GCV (15, 49, 57). Mutations T409M and H411Y were subsequently observed in plasma HCMV sequences and in a clinical isolate from the first treated patient known to have developed MBV resistance. This occurred during an attempt to treat an extremely high viral load resulting from an infection with GCV- and FOS-resistant virus in a D+R− heart transplant recipient (183).

Structure modeling indicates that *UL97* mutations conferring MBV resistance are in the vicinity of the ATP binding site and support the concept of MBV as a small-molecule ATP-competitive kinase inhibitor (49). Diverse *UL27* gene mutations that also confer low-grade (2- to 5-fold) MBV resistance are observed (40, 50, 111). Although the function of the *UL27* gene is presently unknown, it appears that the *UL27* mutations are adaptations to loss of pUL97 kinase activity, because *UL27* mutations are also observed after serial propagation of *UL97*-deleted HCMV strains without exposure to MBV (40). Further study of *UL27* MBV resistance mutations will expand our knowledge of the function of this viral gene.

Terminase inhibitors. Some benzimidazole D-ribosides (e.g., GW275175X) (195, 206) and other unrelated compounds such as tomeglovir (Bay 38-4766) (169) act to inhibit viral DNA cleavage and processing into unit-length genomes, which are necessary steps in replication (18). This mechanism of action is supported by biochemical data and by resistance mutations that have been mapped to viral genes *UL89*, *UL56*, and *UL104* (32, 112, 116, 169). After limited phase I studies some years ago, clinical development of the two compounds cited as examples was halted despite the appeal of a distinct antiviral target. Because the terminase inhibitors target an essential set of viral genes, identification and assessment of new candidate compounds in this drug class are worthwhile (127).

Nucleoside and nucleotide analog DNA polymerase inhibitors. The *pol* target of current HCMV antivirals remains attractive for the development of more potent and specific inhibitors with less bone marrow, renal, and metabolic toxicity than current treatments. An orally bioavailable lipid conjugate of CDV, hexadecyloxypropyl-CDV (CMX001), is reported to be more potent and less nephrotoxic than the current intravenous CDV formulation (95) and is in phase II clinical trials for the prevention of posttransplant HCMV infection. A potential advantage is the weekly dosing interval. Relevant *pol* drug resistance mutations are expected to be the same as those for CDV (Fig. 5B and Table 3). Another nucleoside analog, cyclopropavir (108), has undergone only preclinical testing but appears to have greater *in vitro* potency than GCV and lower host cell toxicity. Cyclopropavir is phosphorylated by pUL97,

similar to the case for GCV. While some GCV resistance mutations were reported to confer little or no resistance to cyclopropavir (109), recent data indicate that *UL97* mutations M460I and H520Q confer significant GCV and cyclopropavir cross-resistance (S. Chou, unpublished data). The nature of *pol* mutations conferring cyclopropavir resistance and cross-resistance with GCV remains undetermined. Nonnucleoside 4-oxo-dihydroquinoline DNA polymerase inhibitors also underwent preclinical testing (192) and appeared to lack cross-resistance to GCV, but data are limited as clinical development of this drug class was not pursued.

Drugs Approved for Other Diseases or Conditions

Several drugs that are approved for treatment of other diseases have been reported to have anti-HCMV activity. However, no controlled clinical studies confirm the antiviral efficacy of these drugs, which have been used largely for patients whose HCMV strain is multidrug resistant or who have underlying conditions that preclude use of one or more of the approved anti-HCMV drugs.

Leflunomide. Leflunomide, a drug approved for the treatment of rheumatoid arthritis, has been reported to be immunosuppressive and to have anti-HCMV activity (197, 205). The drug inhibits protein kinase activity and pyrimidine synthesis, and there is evidence that it may interfere with virion assembly (198). *In vitro* phenotypic assays indicated that leflunomide has activity against both wild-type and GCV-resistant HCMV strains (197), and leflunomide has been used to treat multidrug-resistant HCMV strains in a few patients, with variable results (4, 13, 185). The role of this drug in treatment of HCMV strains that are refractory to treatment with the approved drugs remains undetermined.

Artesunate. Artesunate is an antimalarial drug that has antiviral activity *in vitro* against both wild-type and GCV-resistant strains of HCMV (173). *In vitro* antiviral activity was first reported against laboratory strains of HCMV, and the mechanism of action was suggested to be interference with steps in host cell kinase signaling and NF-κB and Sp1 activation pathways (70). Since the drug targets appear to be cellular pathways, drug resistance may be less likely to develop. A stem cell transplant recipient was treated with artesunate on a compassionate basis, because the patient's HCMV viral load did not respond to GCV and FOS (177). The response to artesunate was an approximately 2-log-unit decrease in peripheral blood viral load. HCMV retinitis that subsequently developed responded to combined artesunate and GCV therapy. This is the only reported case so far where artesunate was used to treat HCMV that had developed multidrug resistance. Clinical studies are needed to confirm the limited observational data that suggest that artesunate may be an effective inhibitor of HCMV replication.

Sirolimus and everolimus. Several recent studies have reported that the immunosuppressive agents sirolimus (rapamycin) and its derivative everolimus are associated with a significantly reduced incidence of HCMV infections in both solid organ transplant recipients (64, 93, 196) and allogeneic hematopoietic stem cell recipients (145). A switch to sirolimus has also been reported to be associated with reduced viral loads in patients infected with GCV-resistant HCMV strains (160). These two drugs are mammalian target of rapamycin (mTOR) inhibitors (93, 201); however, the mechanism behind the observed anti-HCMV activity is not fully understood. *In vitro* exposure of infected cells to rapamycin results in transient or incomplete inhibition of virus infection (55, 105, 117), but no specific antiviral target has been identified. It is assumed that there is an indirect effect through inhibition of host cell proliferation and signaling pathways (105, 117, 196). Drugs that have both immunosuppressive and anti-HCMV activity could be therapeutically advantageous in transplant recipients.

SUMMARY

Antiviral drug resistance in clinical HCMV isolates was initially observed mostly in patients with HIV/AIDS, but the incidence in this population has greatly declined following the introduction of combination antiretroviral therapy. Currently, resistance is reported most frequently among transplant recipients, especially solid organ recipients with a D+/R− serostatus. Drug resistance generally appears after weeks to months of antiviral drug exposure during periods of immunosuppression and may manifest clinically as rising viral loads or progressive disease despite therapy. Because these clinical findings are not specific for drug resistance, laboratory testing is needed for confirmation. However, the ability to detect resistance is low if antiviral treatment has been in place for less than 6 weeks.

The three systemic antiviral drugs currently approved for treatment of HCMV (GCV, FOS, and CDV) all target the DNA polymerase, and GCV anabolism requires an additional viral product, the UL97 kinase. Resistance mutations can arise in the gene sequences encoding one or both enzymes, depending on the drug exposure. Because GCV is the drug of choice for prophylactic and preemptive therapy, *UL97* resistance mutations are detected most frequently and are consistently found in a limited number of codons. There are a much larger number of *pol* mutations, which tend to be selected only after extended periods of treatment with GCV or FOS, and all known GCV resistance mutations in *pol* confer cross-resistance to CDV and/or FOS.

The original identification of drug resistance in clinical HCMV isolates depended on phenotypic methods, which are too time-consuming to provide therapeutically relevant results. They have been replaced by rapid genotypic assays for resistance mutations in viral sequences directly amplified from blood or other clinical specimens without use of cell cultures. HCMV drug resistance genotyping is increasingly available through reference and clinical laboratories. However, accurate interpretation of these assays is dependent on validation of resistance mutations by a phenotypic or recombinant phenotypic method. The complexity of recombinant phenotyping requires the expertise of research laboratories, which have played a key role in the development of HCMV genotypic assays, in particular the differentiation of true resistance mutations from baseline sequence variation. Diagnostic laboratories should be careful to identify confirmed resistance mutations and not confuse them with uncharacterized sequence variants.

Timely results of resistance testing are useful for making clinical decisions. If no drug resistance is identified, clinical management may focus on improving host defenses rather

than switching antivirals. If there is confirmed genotypic evidence of resistance, the specific mutation(s), host immune status, and disease severity should all factor into these decisions, such as to continue or intensify current treatment, to switch to a non-cross-resistant drug, to use drug combinations, or to try experimental drugs. Management algorithms have been proposed by several groups (17, 113, 162, 193).

The study of HCMV drug resistance mutations is an important component of antiviral drug development as well as basic virological research. Early on, GCV resistance led to the discovery of the important role of *UL97* in GCV anabolism, followed by mapping of pUL97 functional domains as additional mutations were identified. Mutations conferring resistance to each of the approved drugs have served to map the functional domains of *pol*. More recent work with MBV has identified resistance mutations in *UL27* and new mutations in *UL97* in functional domains distinct from those associated with GCV resistance. As new antiviral drug targets are explored, mutations identified in the targeted genes may offer new insights into virological functions and interactions of HCMV with host cells.

FUTURE DIRECTIONS AND NEEDS

With the continued use of antiviral therapy and the potential introduction of new antiviral drugs, it will be increasingly necessary to develop a standardized resistance genotyping database of mutations confirmed by recombinant phenotyping methods. To be of value for therapeutic purposes, this database will need to be continuously updated and accessible, preferably online.

Larger studies to characterize drug resistance in clinical specimens from patients on antiviral therapy would contribute to our understanding of the development of resistance mutations and support the predictive value of genotypic testing. Rapidly improving sequencing technologies should help to define the early evolution and genetic pathways of resistance after various drug exposures, thus identifying treatments that are less likely to cause drug resistance. Analysis of the pattern of polymorphisms in baseline sequences of drug-resistant mutants may reveal modulating effects on the phenotypes of confirmed resistance mutations.

Current clinical management guidelines for drug-resistant HCMV are mostly empirical and not evidence based. Controlled trials are needed, but it is difficult to recruit and stratify adequate numbers of study subjects based on host factors that prominently affect clinical outcomes. Evolving treatment options that include different dosing regimens of currently approved or experimental drugs either alone or in combination must also be considered.

There is a pressing need for new antiviral treatment options with greater potency, less toxicity, and lack of cross-resistance with current therapies. Pol remains a target for development of new compounds with these improved pharmacologic properties. The multiple functional interactions of pUL97 with products of other HCMV genes, such as *UL27* and *UL44*, and host proteins, such as Rb and nuclear lamin proteins, are also potential antiviral targets. Other HCMV targets, including those involved in the viral DNA replication complex and viral DNA cleavage, processing, and packaging, are all candidates for drug development and are unlikely to show cross-resistance with current drugs.

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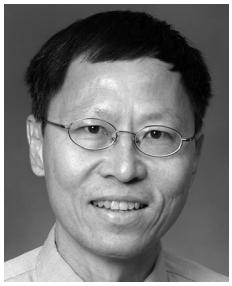

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