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# A human cytomegalovirus mutant resistant to the nucleoside analog 9-{[2-hydroxy-1-(hydroxymethyl)ethoxy]methyl}guanine (BW B759U) induces reduced levels of BW B759U triphosphate

(antiviral chemotherapy/drug mechanisms/nucleoside kinases/viral genetics)

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ABSTRACT **We have isolated a human cytomegalovirus mutant that is resistant to the antiviral drug 9-{[2-hydroxy-1-(hydroxymethyl)ethoxy]methyl}guanine (BW B759U), yet exhibits wild-type sensitivity to inhibitors of herpesvirus DNA polymerases such as phosphonoformic acid and aphidicolin. Cells infected with the mutant accumulate ≈1/10th the amount of drug triphosphate as do those infected with the wild-type parent. This reduction in drug triphosphate could not be attributed to altered drug uptake or to reduced stability of the triphosphate, once formed. The induction of normal nucleoside and deoxynucleoside triphosphates and certain cellular nucleoside kinases was comparable in mutant and wild-type virus infections. These results provide strong evidence for the importance of phosphorylation in the selectivity of this antiviral compound and raise the possibility that human cytomegalovirus encodes a nucleoside kinase. The mutant may identify the existence of a cytomegalovirus function whose properties could facilitate genetic analysis of this important pathogen.**

Human cytomegalovirus (HCMV) infections can produce serious consequences, including birth defects in newborns and life-threatening and sight-threatening disease in immunosuppressed individuals, such as transplant and cancer chemotherapy recipients as well as patients with acquired immunodeficiency syndrome (1).

Currently, there are no licensed treatments for HCMV infections in the U.S. One promising anti-HCMV agent is the deoxyguanosine analog, 9-{[2-hydroxy-1-(hydroxymethyl)-ethoxy]methyl}guanine (BW B759U; also known as 2'NDG, DHPG, and BIOLF-62) (2–4). BW B759U is a congener of the antiherpetic drug acyclovir (ACV), which is licensed for the treatment of herpes simplex virus (HSV). However, BW B759U is much more potent against HCMV than is ACV (2–6). Recent clinical studies suggest that BW B759U has consistent antiviral activity against HCMV *in vivo* and may be useful in treatment of HCMV disease (7–9).

BW B759U is converted to its triphosphate form in HCMV-infected cells 10–100 times more efficiently than in uninfected cells, and much more efficiently than is ACV (5, 6). BW B759U triphosphate can then inhibit HCMV-induced DNA polymerase with $K_i$ values below $1.5 \times 10^{-6}$ M (4, 6, 10). As yet, an enzyme(s) specifically induced by HCMV infection that phosphorylates BW B759U has not been identified. Furthermore, it has not been possible to detect a HCMV-encoded enzyme with properties similar to the HSV-encoded thymidine kinase (TK) (refs. 11 and 12; J.A.F. and K.K.B., unpublished data), an enzyme that is principally responsible for the monophosphorylation of BW B759U and ACV in HSV-infected cells (13–15). On the other hand, it has been shown that HCMV induces increases in host cell nucleoside kinases (5, 6, 11, 12, 16, 17), but none of these has been identified as the enzyme responsible for BW B759U phosphorylation. Thus, it is still an open question whether BW B759U anabolism in the HCMV-infected cell results from the activity of an unidentified virus-encoded enzyme or from a virus-induced increase or alteration of a host enzyme.

Understanding of HCMV has been hampered by a lack of genetic markers. In contrast, studies of HSV have been aided greatly by temperature-sensitive mutants (18), plaque morphology (19), and drug-resistant mutants (15, 20–23). The latter have been invaluable in establishing the selectivity and mechanisms of action of antiviral drugs (14, 15, 21–23), in identifying and dissecting viral genes whose products are drug targets (15, 20–24), and in studies of HSV mutation (25), packaging, recombination, and gene expression (26–29). The few reports of HCMV temperature-sensitive mutants have thus far yielded little information regarding specific HCMV functions (30–32), and we know of no reports of drug-resistant HCMV mutants. To aid in the analysis of HCMV and its interaction with BW B759U, we have isolated and partially characterized a mutant resistant to BW B759U. The resistance of this mutant is associated with reduced phosphorylation of BW B759U in the infected cell.

## MATERIALS AND METHODS

**Cells and Viruses.** Human foreskin fibroblast (HFF) cells (kindly provided by J. Nelson) and human diploid lung fibroblast cells (MRC-5) were maintained as described (5). Wild-type HCMV strain AD169 (33) and mutant BW B759$^r$D100 (this report) were prepared as described (5).

**Drug-Sensitivity Assays.** Plaque-reduction assays were performed as described (5). Measurements of drug sensitivity in terms of HCMV DNA synthesis were performed by the method of Gadler (34) using HFF cells and a multiplicity of infection (moi) of 0.5 plaque-forming unit per cell. The infected cell DNAs were hybridized with $^{32}$P-labeled cosmid DNA containing a fragment of HCMV DNA that does not hybridize to human DNA sequences (P. Medveczky and C. Mulder, personal communication). 2'-Fluoro-5-iodoarabinosylcytosine was generously supplied by C. McLaren (Bristol–Myers, Syracuse, NY) and was dissolved in distilled water and filter-sterilized to prepare a concentrated stock solution. Phosphonoacetic acid, phosphonoformic acid, and aphidicolin were kindly supplied by E. Woroch (Abbott), B.

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Abbreviations: HCMV, human cytomegalovirus; HSV, herpes simplex virus; ACV, acyclovir; HFF, human foreskin fibroblast; moi, multiplicity of infection.

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Erikkson (Astra, Sodertalje, Sweden), and A. H. Todd (Imperial Chemical Industries, Macclesfield, England), respectively. Arabinosyladenine was purchased from Calbiochem–Behring. Drug solutions were prepared as described (5, 22–24, 35).

**Measurements of Drug and Nucleoside Anabolites.** BW B759U and normal nucleotide pools were measured by using a rapid-wash technique, followed by perchloric acid extraction and HPLC analysis as described (5); deoxyribonucleotide pools were measured following sodium periodate treatment to remove ribonucleotides (36). BW B759U anabolites in perchloric acid extracts were also measured by adapting a described method (37) that utilizes styrene cation exchanger columns. BW B759U remained adsorbed to the exchanger, while di- and triphosphates eluted with 0.1 M HCl, and monophosphate eluted with 30% methanol.

**Enzyme Assays.** Cell-free extracts prepared as described (14) were assayed for deoxycytidine, deoxyguanosine, and thymidine phosphorylating activities essentially as described for deoxycytidine and thymidine kinases (5), except that reactions contained 50–100 $\mu$M of the appropriate $^{14}$C-labeled nucleoside (50–100 cpm/pmol), 5 mM ATP, 5 mM $MgCl_2$, 100 mM Tris·HCl (pH 7.5), and 0.5 mM dithiothreitol. Phosphorylated thymidine was measured as described (5). Aliquots of reactions with deoxy[$^{14}$C]cytidine were analyzed by spotting onto PEI thin-layer plates with appropriate standards and developing with water (14). Phosphorylated deoxyguanosine was separated from radioactive guanine and deoxyguanosine on cellulose thin-layer plates developed with water. It was important to effect good separation of dGMP from guanine, since HCMV infection increased the amount of deoxyguanosine cleaving activity (J.A.F. and K.K.B., unpublished data).

## RESULTS

**Isolation of a Mutant Resistant to BW B759U.** Preliminary experiments indicated that if mutants resistant to 10 $\mu$M or greater BW B759U were present in our stocks of HCMV laboratory strain AD169, they were present at a frequency of <1 in 5000. This suggested that single-step selection methods that have been successful in isolation of HSV drug-resistant mutants (22, 23) would not be suitable. Instead, we adopted a strategy of serial passage of virus in increasing concentrations of drug. We first prepared a virus stock from a plaque isolated from HCMV strain AD169 and then serially passaged virus in HFF cells at a moi of 0.02–0.2 plaque-forming unit per cell in BW B759U concentrations of 2.5, 5, 10, 20, 50, and 100 $\mu$M. After the final passage, virus was cloned by limiting dilution in 100 $\mu$M BW B759U, and then cloned twice more in the absence of drug. The mutant was designated BW B759$^r$D100.

Fig. 1*A* shows plaque-reduction curves comparing mutant BW B759$^r$D100 with AD169. The mutant typically exhibited an $ED_{50}$ (dose that reduces virus by 50%) that was ≈10-fold that of AD169. Similar differences were seen in yield-reduction measurements (not shown). BW B759$^r$D100 formed typical HCMV plaques, similar in size to those of AD169. The number of plaques formed by the mutant in drug was proportional to the concentration of the virus; thus, a single mutant virion was sufficient to infect a cell in the presence of drug. Mutant-infected cells synthesized DNA complementary to a cloned HCMV probe in the presence of concentrations of BW B759U that abolished synthesis of HCMV DNA directed by AD169 (Fig. 1*B*). No such synthesis was seen when cosmid vector sequences were used as probe or in uninfected HFF cells. By this procedure (34), the mutant again exhibited an $ED_{50}$ ≈10-fold greater than that of AD169 (Table 1).

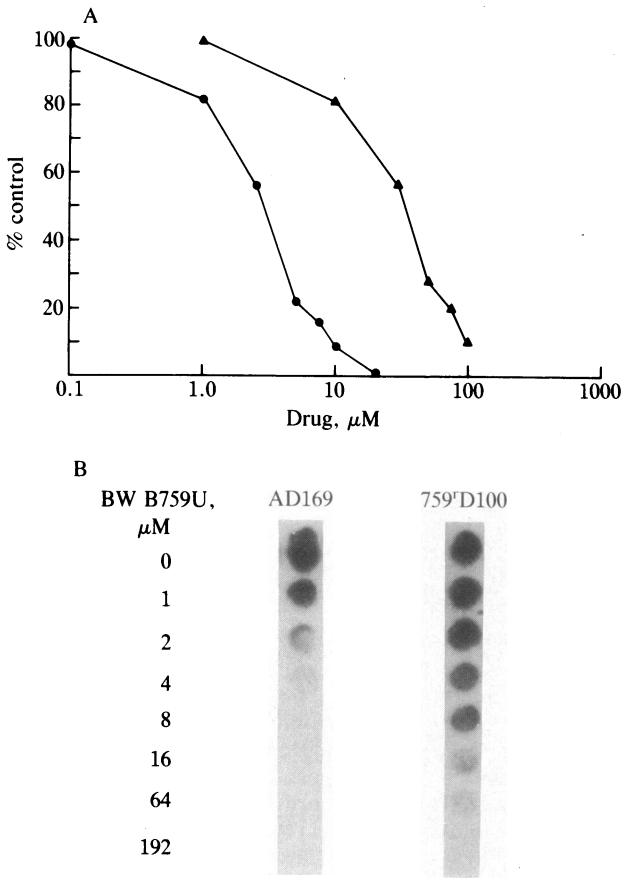


FIG. 1. Effect of BW B759U on wild-type and mutant HCMV viruses. (*A*) Inhibition of plaque formation of wild-type strain AD169 (●) and mutant BW B759$^r$D100 (▲) on MRC-5 cells. (*B*) Inhibition of synthesis of HCMV DNA of wild-type strain AD169 and mutant BW B759$^r$D100. The relative levels of HCMV DNA synthesis in HFF cells infected with either wild-type or mutant virus incubated in the indicated concentrations of drug were determined by the method of Gadler (34).

**Sensitivity of Mutant BW B759$^r$D100 to Antiviral Compounds.** Since the HCMV DNA polymerase is sensitive to BW B759U triphosphate (4, 6, 10), we hypothesized that our mutant might be resistant because of an altered DNA polymerase. In the HSV system, all well-characterized mutants that are drug resistant because of an altered polymerase exhibit altered sensitivities to two or more of the following compounds: Phosphonoacetic acid, phosphonoformic acid,

Table 1. Sensitivities of mutant BW B759$^r$D100 and wild-type strain AD169 to various drugs

| Drug | $ED_{50}$ (μM) for AD169 | $ED_{50}$ (μM) for BW B759$^r$D100 |
|---|---|---|
| BW B759 | 1 | 12 |
| Phosphonoacetic acid | 150 | 150 |
| Phosphonoformic acid | 120 | 190 |
| ACV | 190 | 380 |
| FIaC | 2 | 2 |
| araA | 50 | 50 |
| Aphidicolin | 6 | 6 |

$ED_{50}$ values were determined by the method of Gadler (34). Values presented are either the average of two or more determinations or are from single representative determinations. $ED_{50}$ differences of 2-fold were not regarded as meaningful; in these cases, there was also little difference between wild-type and mutant viruses at higher drug concentrations. FIaC, 2′-fluoro-5-iodoarabinosylcytosine; araA, arabinosyladenine.

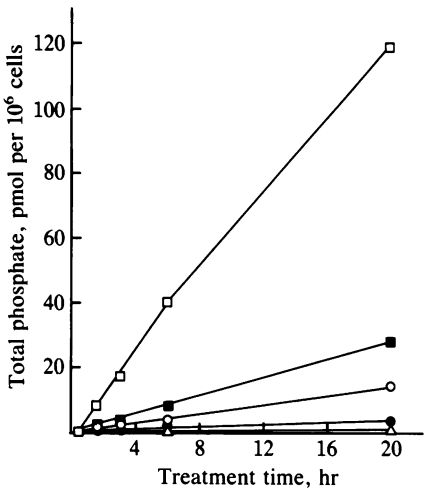


FIG. 2. Accumulation of $^{14}$C-labeled BW B759U anabolites in MRC-5 cells infected with AD169 or with BW B759$^r$D100. On day 3 post-infection (moi = 0.5, verified by back titration), radiolabeled compound at the indicated concentration was added to the medium. At the indicated times, cultures were washed with phosphate-buffered saline (0.0072 M $Na_2HPO_4$/0.0028 M $KH_2PO_4$/0.15 M NaCl) and extracted with 0.5 M perchloric acid. Anabolism of BW B759U was measured by using columns of styrene cation exchanger as outlined in *Materials and Methods*. Total phosphates of BW B759U (pmol per $10^6$ cells) are indicated for AD169 (□, 25 μM; ■, 5 μM) and for BW B759$^r$D100 (○, 25 μM; ●, 5 μM). Mock-infected MRC-5 cells were incubated with 25 μM $^{14}$C-labeled BW B759U (△).

ACV, arabinosyladenine, and aphidicolin (15, 21–25, 35). We therefore compared the BW B759U-resistant mutant with wild-type AD169 in terms of its sensitivity to these compounds and to 2′-fluoro-5-iodoarabinosylcytosine. As summarized in Table 1, although the mutant exhibited substantial resistance to BW B759U, it was indistinguishable from wild type in terms of its sensitivity to all the other compounds, except ACV, to which it was, at most, marginally more resistant. Similar results were obtained in plaque-reduction assays. In contrast, a HCMV mutant selected for resistance to phosphonoformic acid, an inhibitor of herpesvirus DNA polymerases, exhibited substantial resistance to phosphonoacetic acid, phosphonoformic acid, and ACV, and substantial hypersensitivity to aphidicolin (L.K.L. and D.M.C., unpublished results). These data are consistent with the hypothesis that mutation in some locus other than the HCMV DNA polymerase gene is responsible for the resistance to BW B759U.

**Decreased Phosphorylation of BW B759U in Mutant-Infected Cells.** Since BW B759U is preferentially activated to its triphosphate in HCMV-infected cells (5, 6), we determined whether the mutant would exhibit altered drug activation. Three days after infection with either AD169 or the mutant, cultures of MRC-5 human lung fibroblasts were pulse-labeled with $^{14}$C-labeled BW B759U at either 5 or 25 μM. After various times, the cells were analyzed for phosphorylated anabolites of $^{14}$C-labeled BW B759U (Fig. 2). The mutant induced the formation of ≈1/10th the levels of BW B759U diphosphate plus triphosphate as did wild-type AD169 at both concentrations of drug tested. The levels of BW B759U anabolites formed in mock-infected MRC-5 cells remained low regardless of the concentration of BW B759U. In a similar experiment, infected or mock-infected cells were treated with 50 μM $^{14}$C-labeled BW B759U from 48 to 72 hr postinfection and analyzed by HPLC to quantitate the intracellular drug and normal nucleoside and deoxynucleoside triphosphate (NTP and dNTP) pools. The relative amounts of BW B759U anabolites in mutant and wild-type infected cells are shown in Fig. 3; in this experiment, the mutant induced 1/6th the BW B759U triphosphate as did AD169 (Table 2).

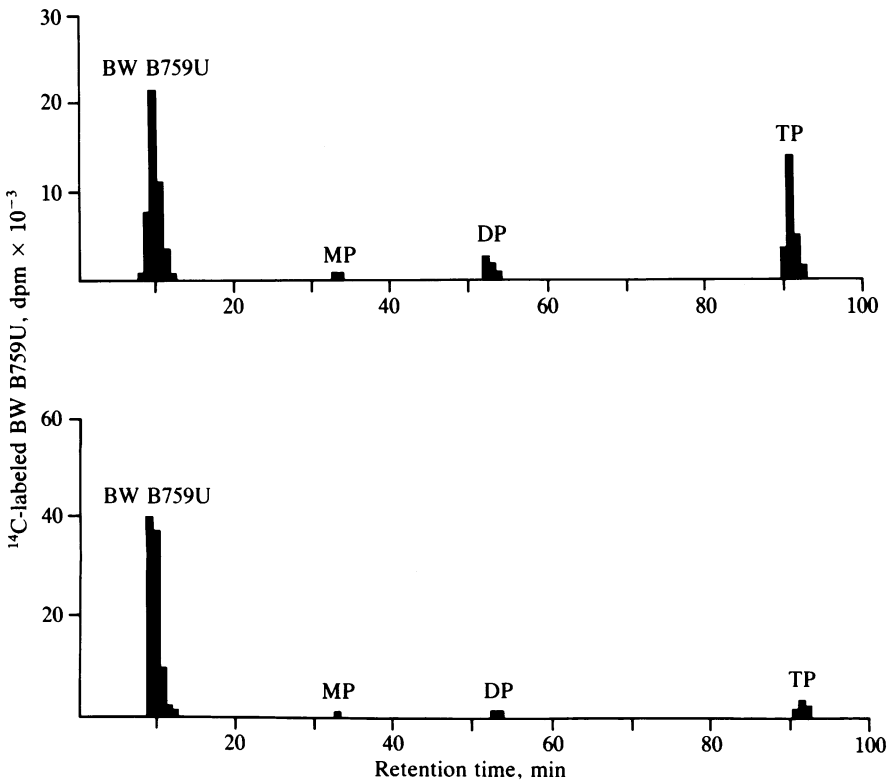


FIG. 3. HPLC profiles of $^{14}$C-labeled BW B759U anabolites in MRC-5 cells infected with AD169 (*Upper*) or with BW B759$^r$D100 (*Lower*). Cells were infected as described in the legend to Fig. 2, labeled with 50 μM $^{14}$C-labeled BW B759U from 48 to 72 hr post-infection, and analyzed by HPLC as described (5). The upper profile is derived from AD169-infected cells and the lower profile is from mutant-infected cells. MP, DP, and TP, BW B759U monophosphates, diphosphates, and triphosphates.

Table 2. Ribonucleoside and deoxyribonucleoside triphosphate pools and nucleoside kinase activities in extracts of uninfected and HCMV-infected MRC-5 cells

| Sample | $^{14}$C-labeled BW B759U | NTP, pmol per $10^6$ cells | | | | dNTP, pmol per $10^6$ cells | | | | | Kinase, pmol per min per mg of protein | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | CTP | UTP | ATP | GTP | dCTP | dTTP | dATP | dGTP | BW B759TP | dCyd | dGuo | dThd |
| MRC-5 | − | — | — | — | — | — | — | — | — | — | 10 | 18 | 29 |
| MRC-5 | + | 868 | 2429 | 8,570 | 1950 | 2 | 4 | 2 | 2 | <1 | — | — | — |
| AD169 | − | 615 | 3232 | 6,714 | 1553 | 24 | 83 | 14 | 13 | — | 140 | 101 | 255 |
| AD169 | + | 727 | 3228 | 7,945 | 1685 | 36 | 86 | 31 | 17 | 18 | — | — | — |
| BW B759$^r$D100 | − | 1018 | 5095 | 10,533 | 2299 | 26 | 101 | 12 | 14 | — | 210 | 115 | 314 |
| BW B759$^r$D100 | + | 1036 | 4365 | 10,828 | 2239 | 56 | 202 | 30 | 24 | 3 | — | — | — |

MRC-5 cells were mock-infected or infected with AD169 or BW B759$^r$D100 (moi = 0.1). At 2 days post-infection, cells were pulsed with 50 μM $^{14}$C-labeled BW B759U for 24 hr or were mock-pulsed. NTP, dNTP, and BW B759U triphosphate pools and nucleoside kinase activities were analyzed as outlined in *Materials and Methods*.

The normal NTP and dNTP pools were similar in mutant and wild-type infected cells; thus, the reduction in drug phosphorylation in mutant-infected cultures was not the result of a generalized reduction in NTP and dNTP levels. Indeed, both mutant and wild-type viruses induced a substantial increase in dNTP pools above mock-infected levels as reported for dTTP (38), indicating that the mutant is not impaired in the functions that induce this increase.

The lower levels of BW B759U anabolites in mutant-infected cells could result from decreased uptake, decreased activation, or decreased stability of the phosphorylated analog. Comparable intracellular levels of unmodified BW B759U were detected in mutant- and wild-type-infected cells in several different experiments. In addition, the BW B759U anabolites formed in mutant-infected cells were as stable as those formed in wild-type-infected cells (Fig. 4). Thus, the mutant appears to be specifically defective in the activation of BW B759U.

**Normal Induction of Cellular Nucleoside Kinases in Mutant-Infected Cells.** If cellular kinases are responsible for the phosphorylation of BW B759U, one might postulate that mutant BW B759$^r$D100 would be deficient in the ability to stimulate host cellular kinases. However, mutant and wild-type HCMV induced comparable increases in the activities of deoxycytidine, deoxyguanosine, and thymidine kinases (Table 2), and the inductions followed similar time courses (not shown). Thus, decreased induction of these enzymes cannot explain the mutant phenotype.

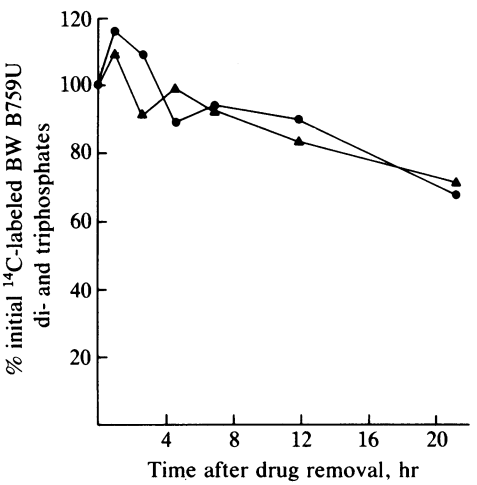


FIG. 4. Stability of BW B759U diphosphate plus triphosphate in extracts of MRC-5 cells infected with AD169 (●) or BW B759$^r$D100 (▲). Cells were incubated from 48 to 72 hr post-infection with concentrations of $^{14}$C-labeled BW B759U that allowed accumulation of similar levels of diphosphate plus triphosphate in cells infected with each virus. Cultures were then washed, refed with drug-free medium, and incubated until the indicated times. Levels of BW B759U di- and triphosphates were determined by using styrene cation exchange columns.

## DISCUSSION

Studies comparing the antiviral effect and cytotoxicity of BW B759U have indicated that this drug may be one of the first antiviral agents that is selective against HCMV (2–4). The isolation of a mutant resistant to BW B759U confirms that this drug is indeed a selective anti-HCMV agent. Biochemical studies have suggested that this selectivity could result from its preferential activation in HCMV-infected cells and perhaps from its subsequent inhibition of HCMV-induced polymerase (4, 6, 10). The sensitivity of the BW B759U-resistant mutant to DNA polymerase inhibitors lends no support to the hypothesis that its resistance is due to an altered viral DNA polymerase. Since the degree of reduction in drug phosphorylation induced by the mutant virus corresponds closely to the degree of resistance, it seems likely that reduced phosphorylation accounts for the resistance of the mutant. Thus, the mutant's properties lend strength to the suggestion (5, 6) that specific activation of BW B759U in HCMV-infected cells contributes to its anti-HCMV selectivity. Nevertheless, we cannot rule out the possibility that some other defect also contributes to resistance.

The simplest interpretation of our results is that the mutation in BW B759$^r$D100 results in a reduction in the activity of a HCMV-encoded enzyme that contributes to the phosphorylation of BW B759U. The mutant's marginal increase in insensitivity to ACV, a similar acyclic guanosine analog, is compatible with the idea that such an HCMV-encoded enzyme could contribute to the small increase in ACV phosphorylation observed after HCMV infection (5, 6).

A second interpretation is that BW B759$^r$D100 is defective in a function that specifically increases a preexisting host-cell enzyme activity that contributes to drug phosphorylation. Evidence for this second interpretation has not been forthcoming. Although phosphorylated BW B759U can be detected in the HCMV-infected intact cell, no consistent significant increase in the amount of BW B759U phosphorylating activity has been observed in extracts of infected versus uninfected cells (5). Cytosolic deoxycytidine, deoxyguanosine, and thymidine kinase activities were induced with HCMV infection (5, 6, 11, 12, 16); however, mutant BW B759$^r$D100 induced wild-type levels of these enzyme activities. Moreover, negligible phosphorylation of BW B759U has been observed after purification of these cytosolic enzymes and mitochondrial deoxyguanosine kinase (refs. 6, 17, and 39; J.A.F. and K.K.B., unpublished results). If the BW B759U phosphorylating enzyme is indeed a host-cell enzyme induced by HCMV, this activity would seem to be under different regulation than other host nucleoside kinases, since the temporal occurrences of these enzymes do not coincide

with the optimal rate of drug triphosphate formation during the virus infectious cycle (5). Additional data to be presented elsewhere (K.K.B. and J.A.F.) also demonstrate that the ability of HCMV to activate BW B759U can be separated from its ability to induce host-cell nucleoside kinases and DNA synthesis.

A less likely explanation for our results is that BW B759$^r$D100 is defective in a HCMV function that induces the host cell to elaborate a previously unexpressed enzyme activity that contributes to drug phosphorylation.

Although we cannot exclude these latter possible explanations for our results, we favor the much simpler first interpretation offered above. The HCMV mutant BW B759$^r$D100 should facilitate the identification and characterization of a promising anti-HCMV drug target, the enzyme that phosphorylates BW B759U.

The mutant described here invites comparisons with HSV mutants that are TK defective and drug resistant. Because HSV TK is not essential for HSV replication in cell culture, it has been possible using selection for resistance to antiviral drugs to construct recombinant viruses containing other portions of the HSV genome or engineered mutations within the *TK* gene (26–29, 40). Studies of such recombinants have contributed to understanding fundamental processes of HSV infection, such as recombination, packaging, and gene expression (26–29, 40). Regardless of whether the BW B759$^r$D100 lesion alters a kinase-encoding gene or some other gene, it suggests the existence of a HCMV function that, like HSV TK, is nonessential in cell culture and whose inactivation can be selected by drug resistance. Just as the *TK* gene has been instrumental in furthering the understanding of HSV, perhaps this gene will facilitate studies of HCMV.

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