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Isolation and Characterization of Dna Polymerase Mutants of Human Cytomegalovirus and Herpes Simplex Virus

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ISOLATION AND CHARACTERIZATION OF DNA POLYMERASE MUTANTS OF HUMAN CYTOMEGALOVIRUS AND HERPES SIMPLEX VIRUS

by

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ACV	acyclovir
Aph	aphidicolin
AraA	adenine 9-ß-D-arabinofuranoside
AraT	thymine-1-D-arabinofuranoside
CPE	cytopathic effect
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EBV	Epstein-Barr virus
ELISA	enzyme-linked immunosorbent assay
GCV	ganciclovir
HCMV	human cytomegalovirus
HFF	human foreskin fibroblasts
HPLC	high pressure liquid chromatography
HPMPA	(S)-9-(3-hydroxy-2-phosphonylmethoxypropyl)-adenine
HPMPC	(S)-1-(3-hydroxy-2-phosphonylmethoxypropyl)-cytosine
HSV-1	herpes simplex virus type 1
HSV-2	herpes simplex virus type 2
IC_{50}	inhibitory concentration 50
kb	kilobase
µl	microliter

μM	micromolar
ml	milliliter
mM	millimolar
MOI	multiplicity of infection
OD	optical density
PAA	phosphonoacetic acid
PFA	phosphonoformic acid
PFU	plaque forming units
PPi	pyrophosphate
PRA	plaque reduction assay
TK	thymidine kinase
VZV	varicella zoster virus

INTRODUCTION

The focus of this project involved the study of antiviral drug resistance in human cytomegalovirus (HCMV). The project was divided into three sections: 1) development of a rapid antiviral susceptibility assay for HCMV, 2) isolation of a ganciclovir (GCV)-resistant clinical strain of HCMV, and 3) construction of a GCV-resistant mutant of HCMV.

In order to more rapidly screen for HCMV antiviral susceptibility, an in situ ELISA was developed. Evaluation of the ELISA showed that it 1) was more rapid than the standard plaque reduction assay (PRA), 2) objectively measured viral replication, and 3) more accurately revealed drug resistance.

Clinical isolates of HCMV from transplant patients at Loyola University Medical Center were collected and screened for GCV-susceptibility using the ELISA assay. One isolate, H51856, was shown to be GCV-resistant. Further experiments revealed that <1% of the isolate was actually GCV-resistant. Clones of the GCV-resistant subpopulation were purified. The ELISA assay revealed that the clones were also cross-resistant to DNA polymerase inhibitors phosphonoformic acid and phosphonoacetic acid. GCV anabolism studies showed that the clones had wild-type GCV-kinase activity which is necessary for activation of the drug. Therefore, a mutation in the viral DNA polymerase was probably responsible for drug

resistance. Sequence analysis of the DNA polymerase gene of the GCV-resistant clones was performed to locate the possible mutation.

Construction of a drug-resistant mutant of HCMV was carried out by first introducing a single base substitution in the wild-type HCMV AD169 DNA polymerase gene. Evidence exists suggesting that this substitution may result in drug resistance in herpesviruses. Recombination experiments were performed using the mutagenized plasmid and wild-type HCMV AD169 DNA. Although numerous experiments were unsuccessful, several drug-resistant plaques were eventually isolated and are currently being purified.

Since significant amino acid homology exists between the HCMV and HSV DNA polymerase proteins, recombination experiments were performed using the mutagenized plasmid and wild-type HSV-1 KOS DNA. Sequence analysis of the DNA polymerase gene of a drug-resistant plaque suggested that the clone was not a recombinant, but a spontaneous mutant.

Finally, a GCV-resistant clinical isolate of HSV-2 was characterized. In contrast to the HCMV results, experiments with the HSV-2 mutant suggested that a mutation in the gene responsible for drug phosphorylation was the most probable cause of drug resistance.

LITERATURE REVIEW

Structure and molecular characteristics of HCMV and HSV. Human cytomegalovirus (HCMV) and herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) are members of the herpesvirus family. Herpesvirus particles characteristically consist of an icosahedral capsid containing a linear, double-stranded DNA genome (3,24,84). The nucleocapsid is surrounded by the tegument, an amorphous layer of viral proteins, which in turn is enclosed by a lipid envelope carrying surface glycoproteins.

The genome sizes of HCMV and HSV are approximately 230 and 152 kilobase pairs (kb), respectively (14,84). The HSV and HCMV genomes are composed of two covalently joined segments, the long (L) and short (S) regions (3,84). The L region consists of a unique sequence (U_L) flanked by a pair of oppositely orientated repeat elements. Similarly, the S region consists of the U_S sequence flanked by its own repeat elements. Since the L and S components can invert during replication, virions contain any one of four isomers of the viral genome.

Growth of HCMV and HSV. HSV has a broad host range and can infect most tissue culture cell lines that have been derived from vertebrate species (24). The replication cycle is fairly short (approximately 24 h), resulting in release of

virions into the culture medium. In contrast, HCMV is species-specific and efficiently replicates very slowly (up to 72 h) only in certain permissive human primary cell cultures (116). The virus is generally cell-associated with little release of virus into the culture medium. Repeated passage of primary HCMV isolates may result in increased titers of virus in the culture medium.

Both HSV and HCMV bind to as of yet unidentified receptors on the permissive cell membrane. After attachment, fusion between the viral envelope and the cell membrane allows entry of the nucleocapsid into the cytoplasm followed by release of the viral DNA.

Once the viral DNA genome reaches the cell nucleus, expression of viral genes occurs in a highly regulated temporal manner. Three classes of genes have been defined based on the timing and requirements of their expression. Alpha genes are expressed earliest in infection (4-6 h for HSV, 12-24 h for HCMV) without any requirement for prior viral protein synthesis. Beta gene expression requires prior alpha protein synthesis but not replication of viral DNA. The beta proteins include regulatory proteins and enzymes required for replication of viral DNA (e.g. viral DNA polymerase). Expression of the third class of genes, gamma genes, is dependent on replication of viral DNA. Most viral structural proteins are gamma proteins.

After the viral genome has been replicated and structural

proteins have been synthesized, nucleocapsids are assembled in the nucleus and bud through the inner nuclear membrane into the perinuclear space. Virions are then transported through the endoplasmic reticulum and Golgi apparatus to the cell surface where they are released.

Epidemiology of HSV and HCMV. Primary HSV-1 infection typically occurs in childhood following contact with a virus positive individual. Initial HSV-2 infection occurs either at delivery through contact with the birth canal of an infected mother, or later in life as a sexually transmitted disease. Primary infections with both serotypes can be asymptomatic. When apparent, the infection is self-limiting. However, in immunocompromised individuals and newborns, HSV infection can spread from the primary site of infection to other parts of the body and is frequently fatal. The clinical manifestations and course of HSV disease depend on the anatomical site of infection, the age and immune status of the host, and the antigenic type of the virus.

After primary infection, HSV travels to the regional sensory ganglia that innervates the site of inoculation. Although the mechanism is not fully understood, the most rational hypothesis is retrograde axonal spread or transport of HSV along microtubules by movement of membrane bound organelles. The virus permanently maintains a latent state within the ganglia although infectious virus cannot be detected. Several latency-associated transcripts (LATs) have

been detected by in situ hybridization (112). Experiments performed using mutants containing deletions within the LAT gene suggest that LATs are not essential for establishment or maintenance of latency (67,115), but may play a role in reactivation of the virus (9). Once reactivated, the virus migrates back to the site of the primary infection and begins the replication process. The clinical manifestations of these periodic reactivations can be life-threatening for individuals who are immunosuppressed (40,42,75,102).

Acquisition of HCMV can occur through natural routes or through transfusion and transplantation (1,44). In the first case, acquisition appears to require close or intimate contact with persons who are excreting HCMV in their urine, saliva, semen, tears, or other secretions (113). Development of HCMV disease after transfusion and transplantation can be the result of a natural infection, a donor-induced infection, or reactivation of a latent infection. HCMV infection in healthy individuals is usually asymptomatic or results in a self-limited infectious mononucleosis-like syndrome. However, severe and life-threatening HCMV disease may develop in immunocompromised patients such as transplant recipients or AIDS patients (1,3,21,44,122). In these patients, the clinical manifestations include pneumonia, hepatitis, GI involvement, and chorioretinitis (1).

HCMV is the most frequent viral agent causing congenital malformations (1,3,44,127). Neonatal infection occurs as the

result of either primary infection in the mother or reactivation or reinfection followed by passage of the virus across the placenta (113). Clinically apparent HCMV infection can be demonstrated in about 1% of newborns, and about 10-15% of the infected children suffer either from immediate symptoms or late sequelae such as mental retardation, chorioretinitis, microencephaly, and hearing loss.

The site of latency of HCMV has not yet been determined. HCMV latency may be associated with a nonproductive cell that permits only limited viral gene expression (116). Polymorphonuclear and mononuclear leukocytes can be infected in vitro resulting, however, in an abortive infection (100). Limited viral expression occurs in these cells since HCMV DNA and RNA can be demonstrated in these cell types by in situ hybridization (27,100,104). Data also suggest that latent HCMV is transferred from donor to recipient during blood transfusion (44). Although factors controlling HCMV latency and reactivation are not understood, immunosuppression appears to promote reactivation (44).

Both HSV and HCMV gene products have been shown to transactivate the HIV-1 long terminal repeat (LTR) in vitro, suggesting that they may play a role as cofactors in the development of AIDS (4,98). In addition, in vitro studies suggest that there may be bidirectional potentiation between HCMV and HIV (60,107). In vivo evidence exists for coinfection of HCMV and HIV-1 in cells from the white matter

of AIDS patients (89). Potentially, herpesvirus gene products may act through the LTR to increase initiation of HIV-1 transcripts.

Recently, Lerner-Tung et al. (74) demonstrated formation of guinea pig cytomegalovirus (GPCMV) and guinea pig retrovirus (GPRTV) pseudotypes in cultured guinea pig cells. Formation of GPRTV pseudotypes which now express GPCMV antigens on their envelopes would extend the host cell range of the retrovirus. These interactions may have serious implications for human patients with retroviral infection and a supervening HCMV infection.

Normal HSV- and HCMV-specific cytotoxic T lymphocyte response, normal levels of natural killer cells, and normal levels of antibody-dependent cellular cytotoxicity appear to play an important role in prevention of HSV and HCMV diseases (24,101). In contrast, HSV and HCMV infections in patients with primary B-cell disorders such as agammaglobulinemia are usually not severe.

Oncogenicity. Both HSV and HCMV can transform cells in vitro and induce malignant disease in animals. Small segments of the HSV and HCMV genomes were able to transform NIH 3T3 cells (48,88). Epidemiologic evidence suggests that HSV infection may play a role in cervical (87) and vulvar (69) carcinomas. The presence of HCMV nucleic acid in human tumors is difficult to interpret because HCMV can infect these organs latently in a high percentage of individuals.

Studies have shown that HCMV infection induces cell DNA synthesis and cell proliferation (52). Colberg-Poley and Santomenna (19) showed that permissive infection of HFF with HCMV increases the abundance of transcripts from selected growth-related HFF genes. HCMV infection also increases the level of c-fos, c-jun, and c-myc RNA within infected cells (10). These proto-oncogenes play a pivotal role in regulation of cell proliferation. Increased cell proliferation mediated by HCMV infection through increased RNA levels of genes which regulate cell growth may be the first step in HCMV oncogenesis.

Laboratory diagnosis. The primary method of detection of HSV and HCMV in clinical samples is by conventional cell culture. However, demonstration of HCMV in cell culture can take several weeks due to the increased replication time and cell-associated nature of the virus. Paya et al. (95) developed a rapid and reliable shell vial assay which is routinely used in conjunction with conventional tube culture. Patient samples are inoculated into shell vials containing HFF seeded glass coverslips. The vials are centrifuged to promote adherence and absorption of the virus. After 24 to 48 h incubation, the monolayers are stained with an indirect fluorescent antibody technique using a HCMV-specific monoclonal antibody. Preliminary positive results can be reported as early as 24 h post inoculation.

Serological and histological tests can aid in the

detection of HCMV disease. Demonstration of a four-fold rise in titer of HCMV-specific antibodies is indicative of active disease. However, acute and convalescent sera are required and results may not be reliable in immunosuppressed patients. Histology performed on biopsy samples may also reveal the presence of characteristic herpesvirus inclusion bodies in infected cells.

Several additional techniques have been described for rapid detection of HCMV infection. Radiolabeled and non-radiolabeled HCMV probes have been developed for in situ hybridization or Southern blot hybridization of patients samples (16,77,85,111). However, these techniques are labor-intensive and are not as sensitive as the combined shell vial and immunofluorescence method. Demmler et al. (30) and Olive et al. (93) reported polymerase chain reaction (PCR) amplification of HCMV DNA in urine specimens. Primers were used to amplify the major immediate-early and late antigen genes of HCMV. Flow cytometry has been used to detect HCMV early nuclear antigen in infected fibroblasts (38). Positive results were obtained earlier than with the shell vial assay or conventional culture.

Strategy of anti-herpesvirus therapy. The first class of anti-herpesvirus agents are the viral DNA polymerase inhibitors. HSV encodes a DNA polymerase enzyme characterized by 1) salt-stimulated polymerase activity, 2) 3'-5' exonuclease activity, 3) a deoxynucleotide (dNTP) binding

site, 4) a pyrophosphate (PPi) binding site, 5) a DNA binding site, and 6) aphidicolin and phosphonoacetic acid (PAA) sensitivity (31). Although the HCMV polymerase has not been studied as extensively, it also possesses polymerase activity, 3'-5' exonuclease activity, and aphidicolin and PAA sensitivity (58,63,71). The genetic location of both enzymes has been mapped to the long unique region of each genome and the nucleotide sequences have been deduced (28,58,71,96). Both polymerases show areas of considerable amino acid homology to the DNA polymerase protein of other another herpes virus, Epstein-Barr virus, as well as human DNA polymerase α, yeast DNA polymerase I, and several animal and bacterial polymerase enzymes (49,58,71,72,73,124,126). Recent successful cloning and expression of the HSV DNA polymerase (31,55,56) will allow further characterization of the enzyme. Unfortunately, attempts at expressing the HCMV DNA polymerase in expression vectors have been unsuccessful.

The second class of anti-herpesvirus agents are the nucleoside analogs including acyclovir (ACV) and ganciclovir (GCV). These agents are substrates for the virus-encoded thymidine kinase (TK). Monophosphorylation of the nucleoside analogs by the viral TK is the essential first step in their activation. Following conversion of the mono-phosphorylated nucleoside analogs to their triphosphate forms by host cellular kinases, the latter become preferential substrates for the herpesvirus-specific DNA polymerases. Incorporation

of the triphosphorylated analogs into viral DNA results in disruption of further DNA polymerase activity.

The HSV TK gene has been mapped to the long unique region of the genome (84) and has been cloned (51) and sequenced (70). HCMV does not encode a virus-specific thymidine kinase which can be separated from host cell activity (41,128). However, studies have shown that HCMV-infected cells are capable of monophosphorylating GCV (8). Conversely, two GCV-resistant mutants of HCMV lack this activity (7,76). Whether the virus encodes some type of GCV-specific kinase activity or whether it stimulates the activity of a cellular enzyme which is responsible for GCV-monophosphorylation has yet to be determined.

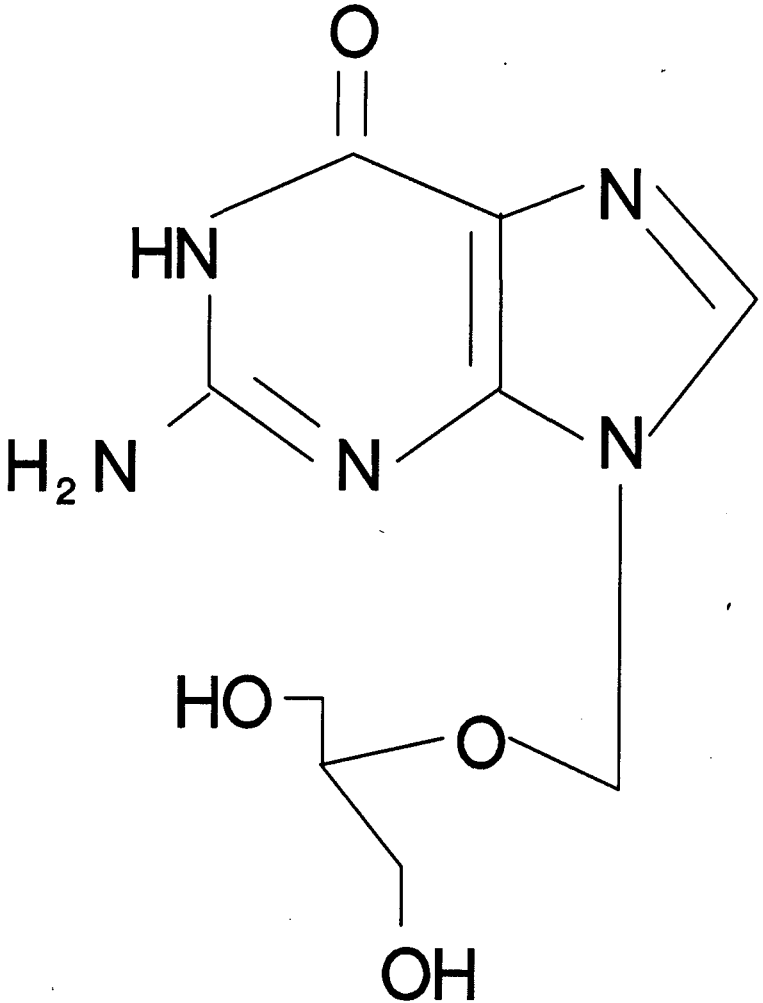
Viral resistance to the nucleoside analogs can occur through several mechanisms. Mutations in the HSV TK gene can induce truncated inactive TK polypeptides following premature termination at a stop codon (42). These TK^- HSV mutants therefore lack the ability to monophosphorylate ACV. A second class of ACV-resistant HSV induced a mutated TK which retained the ability to phosphorylate thymidine (42). The mutant viral TK was found to have a changed substrate specificity preventing monophosphorylation of the drug and resulting in resistance to one or more nucleoside analogs. Also, mutations in the viral DNA polymerase may result in an enzyme which no longer recognizes the triphosphorylated analog. This class of mutants, however, remain susceptible to the DNA polymerase

inhibitors.

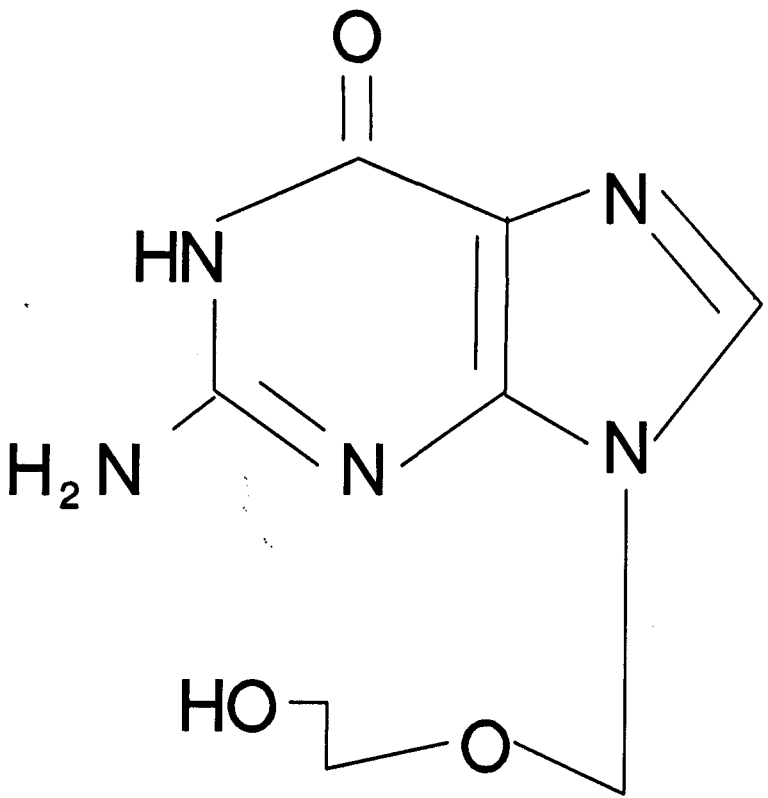
Resistance to the DNA polymerase inhibitors including phosphonoacetic acid (PAA) and phosphonoformic acid (PFA) is mediated specifically through mutations in the viral DNA polymerase gene. Mutants of this class show cross-resistance to the nucleoside analogs. These mutants display wild-type levels of phosphorylation of the nucleoside analogs. However, the mutation in the DNA polymerase enzyme prevents incorporation of the nucleoside triphosphate.

Anti-herpesvirus agents. ACV, an acyclic analogue of 2'-deoxyguanosine (Figure 1), has been extensively reviewed in the literature (36,59,62,99,123). ACV has potent anti-HSV activity in vitro and is sufficiently safe for human use. HSV infection induces a novel viral TK activity that is essential for the phosphorylation of ACV to ACV-monophosphate, the first step in the selective activation of ACV. Normal cellular enzymes do not monophosphorylate ACV to any significant degree. ACV-monophosphate is further phosphorylated to the diphosphate and triphosphate forms by cellular kinases. Once incorporated into the growing viral DNA chain, ACV-triphosphate prevents further chain elongation because of the absence of a 3'-hydroxyl group (Figure 1). Also, ACV cannot be excised from the DNA because the terminated chain is not a suitable substrate for the viral DNA polymerase-associated 3'-exonuclease activity (36). Therefore, the enzyme complex is inactivated and is no longer available to catalyze any

Figure 1. Structures of acyclovir and ganciclovir.



Ganciclovir



Acyclovir

further reactions (36). ACV-triphosphate is a more potent inhibitor of HSV DNA polymerase than of cellular DNA polymerase and therefore induces very little host cell cytotoxicity (36). ACV has very weak in vitro activity against HCMV (20,46,123) possibly due to the fact that HCMV infection induces only modest levels of ACV-triphosphate (8).

GCV, a recently developed acyclic analog of guanosine (Figure 1), is a potent inhibitor of HSV and HCMV replication (8,43,46,79,83,108). Like ACV, inhibition of viral replication by GCV is due to the selective inhibition of the HCMV DNA polymerase by GCV-triphosphate, with the host cell polymerase being much less sensitive (8,46,79). As mentioned above, HCMV does not encode a virus-specific TK. GCV anabolism studies have shown that levels of phosphorylated products of GCV increase in HCMV infected cells (8). Therefore, GCV must be monophosphorylated by a virus-encoded or virus-induced cellular enzyme. Cellular kinases phosphorylate the drug to the active triphosphate form. Although similar in structure to ACV, GCV possesses a 3'-hydroxyl group allowing incorporation of the active form into the growing viral DNA chain (Figure 1). GCV-triphosphate is probably not a strict chain terminator, but may act by disrupting the conformation and integrity of the DNA helix (59). GCV is currently the only FDA-approved drug for the treatment of AIDS-related HCMV retinitis. However, GCV has a narrow therapeutic-toxic ratio and often causes reversible

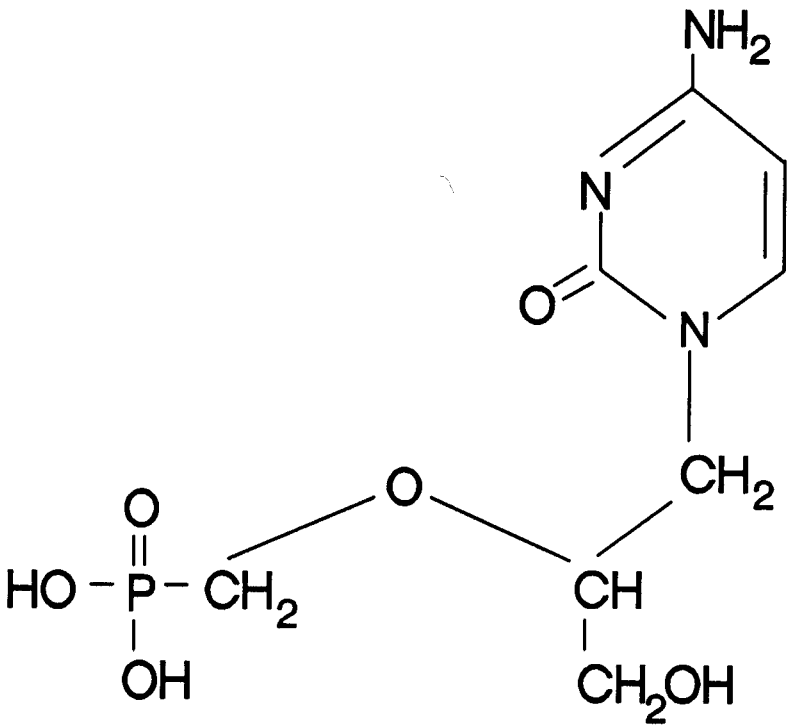
neutropenia (59).

AraA and AraT are nucleoside analogs with some activity against all members of the human herpesvirus group, but less activity against HCMV or EBV than against HSV or varicella zoster virus (59). Both inhibit viral DNA synthesis at concentrations below those required to inhibit host-cell DNA synthesis. In contrast to ACV and GCV, AraA and AraT are first monophosphorylated by cellular enzymes. They are further phosphorylated to their active triphosphate forms by cellular kinases. Therefore, since they are not dependent on the activity of the viral TK, they are active against TK^- mutants of HSV.

HPMPA ((S)-9-(3-hydroxy-2-phosphonylmethoxypropyl)-adenine) and HPCMC ((S)-1-(3-hydroxy-2-phosphonylmethoxy-propyl)cytosine are phosphonate derivatives of acyclic analogues of adenosine and cytosine, respectively (Figure 2). In vitro, both show selectivity against a broad spectrum of DNA viruses including HSV, HCMV, and EBV (29,90,105,109). HPMPA and HPMPC do not require phosphorylation by a viral-encoded kinase to express antiviral activity (105), and, therefore, are effective against TK^- strains of HSV (29,90). These agents are diphosphorylated by cellular enzymes and the diphosphoryl derivatives interfere with the viral DNA polymerase reaction.

PFA and PAA (Figure 3) are pyrophosphate (PPi) analogs which have significant anti-HSV and anti-HCMV activity in

Figure 2. Structure of HPMPC and HPMPA.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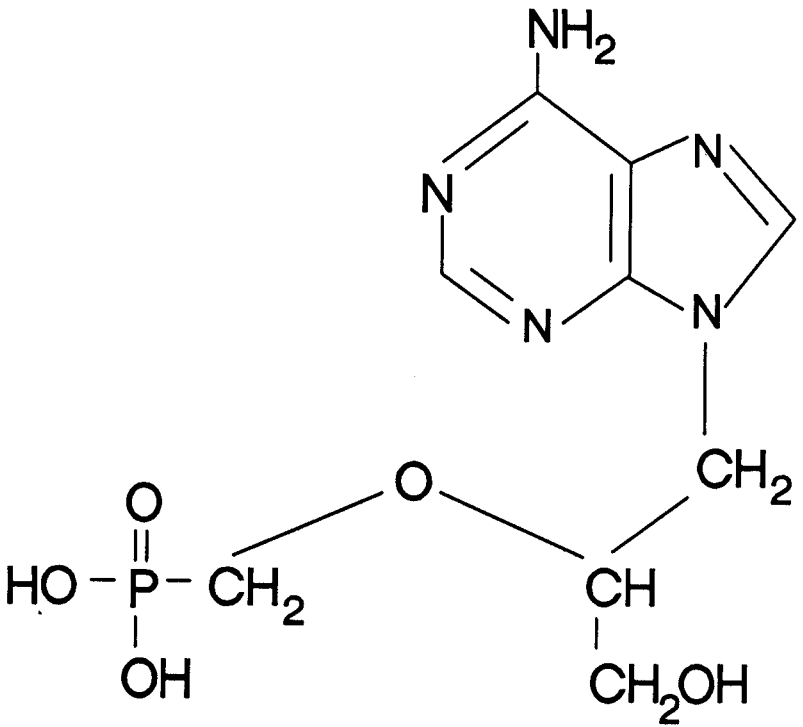
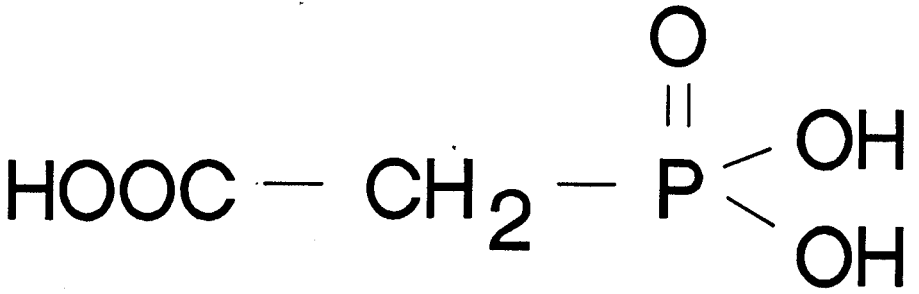
N
O
N
CH2
O
O
HO – P – CH2
CH
OH
CH2OH

HPMPC

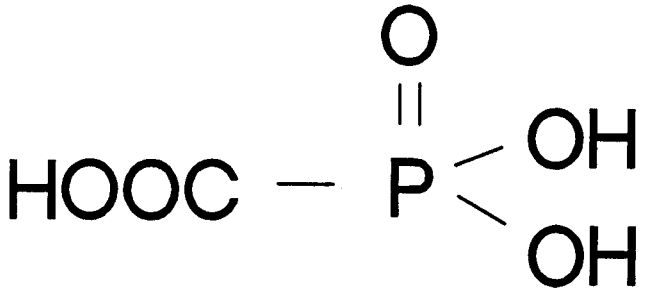

NH2
N
N
N
N
CH2
O
O
HO – P – CH2
CH
OH
CH2OH

HPMPA

Figure 3. Structure of PAA and PFA.



Phosphonoacetic acid (PAA)



Phosphonoformic acid (PFA)

vitro and in vivo (59,62,123). Studies have shown that PFA blocks the PPi binding site of the viral DNA polymerase preventing cleavage of pyrophosphate from the deoxynucleotide triphosphates (dNTPs) (17,91). Formation of the PFA-enzyme complex prevents release from, or translocation along, the template primer. Further studies suggest that PFA and pyrophosphate bind to distinct sites within the proposed PPi binding site (50) on the enzyme. PFA selectively inhibits viral polymerases at concentrations which do not affect host-cell polymerases. Ongoing clinical studies with PFA have shown that PFA may be effective and safe in the treatment of AIDS-related HCMV retinitis (17).

Aphidicolin, a fungal tetracyclic diterpenoid, is a specific inhibitor of the DNA α polymerase family. This agent also has potent anti-herpesvirus activity (53,57). In vitro, its inhibitory action stems from its presumed interaction with the nucleotide-binding domain. Several aphidicolin-resistant vaccinia virus mutants encode DNA polymerase enzymes which contain a substituted amino acid within a conserved domain thought to be involved in nucleotide binding (118). The clinical application of aphidicolin is limited by its detrimental effect on cellular DNA synthesis (53) and therefore may be more useful for studying the in vitro interactions between the viral DNA polymerase and dNTPs.

The activity of anti-herpes group drugs is determined using the plaque reduction assay (PRA; 125). In this assay,

cell monolayers are inoculated at a certain multiplicity of infection (MOI) to yield a countable number of plaques. The monolayers are then incubated in various dilutions of the antiviral agent. The IC_{50} or ED_{50} is defined as the concentration of drug which results in a 50% decrease in the number of plaques as compared to the control wells. Antiviral susceptibility testing of HSV using the PRA is rapid and reproducible. However, due to the slow growing nature of HCMV, antiviral susceptibility testing of HCMV using the PRA can take up to two weeks per assay. The need exists for a more rapid testing procedure for HCMV.

Resistance to antiviral drugs. The numerous drug-resistant mutants of HSV reported are either laboratory-derived mutants or clinically isolated strains. All provide excellent models for the study of drug-enzyme interactions. Of most concern are the increasing numbers of drug-resistant HSV-1 and HSV-2 being isolated from immunocompromised patients (6,13,23,40,75,102). ACV resistance typically becomes apparent during or after a period of antiviral therapy. The infection may be resolved after administration of PFA if the ACV-resistant virus is a TK^- mutant (13,40,75,102). However, treatment with viral DNA polymerase inhibitors may be ineffective if the mutation lies in the viral DNA polymerase gene (23).

Sequence analysis of the TK and DNA polymerase genes of HSV drug-resistant mutants has suggested that point mutations

in these genes may be responsible for drug resistance (12,71,120). Marcy et al. (80) successfully constructed drug-resistant recombinant HSV-1 which contained point mutations in the viral DNA polymerase gene. Gibbs et al. (71) mapped the location of nine HSV DNA polymerase mutants. They proposed that a dNTP binding site and a PPi binding site lie in the carboxyl terminal portion of the HSV DNA polymerase protein. This area shows significant homology to other human DNA α polymerase-like proteins.

To date, there have been only two reports describing resistance to GCV in clinical isolates of HCMV. In order to determine the prevalence of resistance of HCMV to GCV in patients with AIDS, Drew et al. (33) performed serial cultures of patients receiving GCV for HCMV retinitis. Of 31 isolates tested prior to therapy, all were GCV-sensitive. Thirteen patients were culture-positive after 3 or more months of treatment. Five of these isolates were GCV-resistant (ED_{50} >12 uM). Three other GCV-resistant clinical isolates from immunosuppressed patients have been described (39). However, the mechanism of resistance of these GCV-resistant isolates was not determined in either study.

Only, three laboratory-derived drug-resistant HCMV isolates have been reported. Two GCV-resistant mutants were isolated by passage of wild-type AD169 in increasing concentrations of GCV (7,76). Analysis of phosphorylation products of GCV in virus-infected cells showed the isolates

to be deficient in kinase activity. Since the isolates were not cross-resistant to the viral DNA polymerase inhibitors, this suggested that the mutation responsible for drug resistance was in the gene encoding kinase activity. D'Aquila and Summers (28) isolated PAA-resistant HCMV mutants by passage of wild-type AD169 in increasing concentrations of PAA. These mutants had PAA-resistant DNA polymerase activity in vitro suggesting that mutations responsible for PAA resistance were in the HCMV DNA polymerase gene.

Prevention. Strategies for vaccine development have centered on the use of live attenuated viral vaccines and viral subunit vaccines (25,54). However, concern exists about the long-term safety of any attenuated live virus. Mutations restoring the pathogenicity of the virus could lead to disease. Also, the transforming properties of both HSV and HCMV sequences may be of concern. Active research continues in the development of safe subunit vaccines. HCMV sero-negative transplant recipient patients and HCMV sero-negative women of child-bearing age would greatly benefit from a preventative vaccine (113).

Strain variation may play an important role in HCMV vaccine design. James et al. (66) demonstrated antigenic variation among HCMV isolates using monoclonal antibodies and an indirect ELISA. Restriction endonuclease patterns of clinical isolates also demonstrate strain variation (15,34,122) and that patients may shed several different

strains of HCMV simultaneously (22,110). Therefore, effective vaccine design should be targeted to conserved areas of the virus.

MATERIALS AND METHODS

Tissue culture cells. Low passage human foreskin fibroblasts (HFF) were purchased from Bartels (Bellevue, WA). Confluent monolayers of HFF in 150 cm^2 tissue culture flasks were passaged 1:2 and grown to confluency in growth medium (Eagle's minimal essential medium (EMEM) containing amphotericin B (2.5 μg/ml) and gentamicin (50 μg/ml) and supplemented with 2 mM L-glutamine, 5% fetal calf serum (FCS) and 5% NuSerum (92; Collaborative Research, Inc., Lexington, MA)). The cells were then held on maintenance medium (EMEM containing amphotericin B (2.5 μg/ml) and gentamicin (50 μg/ml) and supplemented with 2 mM L-glutamine and 1% FCS). HFF cells greater than passage 11 were discarded.

Monolayers of HFF in 24-well tissue culture treated plates were prepared by harvesting cells from a confluent 150 cm^2 flask, resuspending the cells in 100 ml of growth medium, and adding 1 ml of cell suspension to each well of 4 plates. The plates were incubated at 37°C in 4% CO_2. Monolayers of HFF in 96-well tissue culture treated plates were prepared in a similar manner with the following modifications. Cells were resuspended in 75 ml of growth medium and the 60 inner wells of 6 plates were seeded with 0.2 ml of cell suspension. All outer wells were filled with 0.2 ml of sterile water.

Monolayers of HFF in 100 mm tissue culture dishes were

prepared by harvesting the cells from a confluent 150 cm^2 flask, resuspending the cells in 40 ml of growth medium, and adding 10 ml of cell suspension to each of 4 dishes.

African green monkey kidney cells (Vero cells) were propagated and maintained in a similar manner. For propagation, Vero cells were split 1:5. Preparation of multiwell plates and tissue culture dishes was identical to that of HFF cells except that a confluent 75 cm^2 flask of Vero cells was used to seed 4 24-well plates, 6 96-well plates, or 4 100 mm dishes.

Viruses. HCMV strain AD169 (ATCC VR-538) was used throughout this study. Medium from a confluent monolayer of HFF cells in a 150 cm^2 flask was removed and replaced with 3 ml of stock virus diluted in maintenance medium (MOI = 0.01-0.001). After a 2 h absorption period at 37°C, the inoculum was replaced with maintenance medium. At approximately 10 % CPE, the medium was replaced with fresh maintenance medium. At 3 days past 100% CPE, the culture fluid was removed, dimethyl sulfoxide (DMSO; Kodak, Rochester, NY) and FCS were added to a final concentration of 10% and aliquots were frozen at -70°C.

HCMV isolated from heart, bone marrow, or kidney transplant patients at LUMC were obtained from the clinical microbiology laboratory. The initial isolation of the virus was done in tubes of HFF. Most commonly, the monolayers showed only one to a few areas of CPE. The medium from these

tubes was aspirated, the monolayers were scraped with a sterile plastic 1 ml pipet, and the cells were resuspended in 0.4 ml of maintenance medium. Two low passage tubes of HFF (Bartels, Bellevue, WA) were inoculated with 0.2 ml of cell suspension and incubated for 2 h at 37°C to allow viral adherence. The tubes were then fed with 2 ml maintenance medium containing 1% FCS and incubated at 37°C for five weeks. The medium was changed weekly. If the CPE did not reach 100% after 5 weeks, the cells were again passed in a similar manner. At 100% CPE, the fluid was diluted 1:100 in maintenance medium and 0.2 ml of the diluted virus suspension was used to inoculate new HFF tubes. When the CPE in the tubes reached 100% CPE, the fluid was harvested, FCS and DMSO were added to 10%, and 0.4 ml aliquots were frozen at -70°C.

Wild-type HSV-1 strain KOS and HSV-2 ATCC VR-734 stock virus was prepared by inoculating confluent monolayers of Vero cells in 75 cm^2 flasks with 2 ml of virus in maintenance medium at an MOI of 0.005. Flasks were incubated at 37°C for 1 h. The inoculum was then replaced with 15 ml of maintenance medium. The flasks were incubated at 37°C for 3-4 days until 100% CPE was observed. The culture fluid was collected, DMSO and FCS were added to 10%, and 0.5 ml aliquots of the fluid were stored at -70°C.

Antiviral agents. Acyclovir (ACV) was a gift from Burroughs-Wellcome, Research Triangle Park, NC. Ganciclovir (GCV) was a gift from Burroughs-Wellcome, Research Triangle

Park, NC, and Syntex, Palo Alto, CA. Phosphonoformic acid (PFA), phosphonoacetic acid (PAA), adenine 9-ß-D-arabinofuranoside (AraA), thymine-1-D-arabinofuranoside (AraT), and aphidicolin (Aph) were obtained from Sigma Chemical Co., St. Louis, MO. (S)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine (HPMPC) and (S)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine (HPMPA) were gifts from Bristol-Myers Squibb (Wallingford, CT). All antiviral agents were resuspended in distilled water and filter sterilized (0.2 μm). Stocks were stored at -20°C.

Plaque dilution assay. The plaque dilution assay used in this study was a modification of the method of Wentworth and French (125). For the HCMV plaque dilution assay, 10-fold dilutions of stock virus were made in maintenance medium. The medium from each well of monolayers of HFF in 24-well tissue culture plates was aspirated and replaced with 0.1 ml of the appropriate virus dilution. Each dilution was run in quadruplicate. After a 2 h absorption at 37°C in 5% CO_2, viral inoculum was removed and replaced with 1 ml of maintenance medium containing 0.3% agarose. Plates were incubated at 37°C in 5% CO_2 for 1 week. A second overlay of maintenance medium containing 0.3% agarose was added to each well and plates were incubated an additional week. Plaques were counted microscopically and the virus titer was determined.

The HSV plaque dilution assay was identical to the HCMV plaque assay with the following modifications. Confluent Vero

cell monolayers in 24-well tissue culture plates were inoculated with dilutions of stock HSV. After a 1 h absorption at 37°C in 5% CO_2, viral inoculum was removed and replaced with 1 ml of maintenance medium containing 15% methylcellulose (Sigma Chemical Co., St. Louis, MO). The plates were incubated at 37°C in 5% CO_2 for 2 to 4 days post-inoculation until sufficient plaque formation was visible. The cells were fixed by replacing the medium with 0.5 ml methanol and incubating the plates for 3 min at room temperature. The methanol was removed and the plates were allowed to dry at room temperature. The monolayers were stained with 0.5 ml of 1% methylene blue for 10 min at room temperature. The dye was aspirated and the plates were dried prior to microscopic examination.

Percent survival assay. Monolayers of HFF or Vero cells in 24-well cell culture plates were inoculated with virus as in the plaque dilution assay. After virus adherence, monolayers were overlaid with the appropriate medium with or without antiviral compound. Plates were incubated until substantial plaque formation was observed in the control wells without antiviral drug. The percent survival is defined as (titer of virus in the presence of drug)/(titer of virus in the absence of drug) X 100.

Plaque reduction assay. The plaque reduction assay was performed as in the plaque dilution assay with the following modifications. HFF or Vero cell monolayers were inoculated

with a virus dilution sufficient to yield approximately 20 plaques per well. After the virus adherence, monolayers were overlaid with the appropriate medium with or without dilutions of the antiviral compound. Plates were incubated until substantial plaque formation was observed in the control wells without drug. The IC_{50} was defined as the concentration of drug which resulted in a 50% or greater decrease in virus titer as compared to the no drug control.

Viral DNA isolation. For extracellular virus DNA isolation, stock virus was grown as described above in 150 cm^2 tissue culture flasks or 490 cm^2, 850 cm^2, or 1700 cm^2 tissue culture roller bottles. Culture fluid was transferred to 250 ml centrifuge bottles and centrifuged in a Sorvall GSA rotor at 5,000 x g for 10 min at 4°C. The supernatant was transferred to sterile 40 ml Oak Ridge tubes and centrifuged at 25,000 x g in a Sorvall SS34 rotor at 4°C for 90 min. The supernatants were decanted and the pellets were resuspended in 0.5 ml DNA buffer (0.4 M sodium chloride (NaCl), 0.5 mM Tris-HCl pH 8, 0.1 M ethylenediaminetetraacetic acid (EDTA) containing 500 ug/ml heat-treated RNase, 1.0% sodium dodecyl sulfate (SDS) and 0.5% Sarkosyl. The tube was then incubated for 2 h at 37°C. After addition of pronase (1 mg/ml), the tube was incubated overnight at 37°C.

The fluid was transferred to 1.5 ml microcentrifuge tubes. The DNA was extracted once by the addition of an equal volume of phenol saturated with Tris-HCl pH 8.0 followed by

centrifugation for 5 min at 12,000 x g. The upper aqueous layer was transferred to a new microcentrifuge tube and extracted with and equal volume of chloroform:isoamyl alcohol (24:1). The upper aqueous layer was again transferred to a new microcentrifuge tube. The DNA was precipitated by the addition of 2 volumes 95% ethanol (EtOH) and incubation at -20°C overnight. The DNA was pelleted by centrifugation at 12,000 x g for 10 min at 4°C. The DNA pellets were resuspended in 20 ul TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA). DNA quantitation was determined by agarose gel electrophoresis using known DNA standards.

Cell-associated viral DNA was isolated using a modified Hirt extraction (35). Stock virus was grown in 150 cm^2 flasks as described above. The culture fluid was removed and extracellular virus was isolated from the fluid as described above. The monolayer was washed with 5 ml phosphate buffered saline (PBS). One milliliter of lysis solution (0.01 M Tris pH 8.0, 10 mM EDTA, 0.5% SDS) was added per flask and the flasks was incubated at 37°C for 15 min. The solutions were pooled and transferred to 15 ml Corex tubes. After addition of 5 M NaCl to a final concentration of 1 M, the tubes were incubated at 4°C overnight. The tubes were spun at 25,000 x g at 4°C in the SS34 rotor. The supernatant was transferred to a new Corex tube and extracted with an equal volume of phenol saturated with 10 mM Tris-HCl pH 8.0. The tubes were spun at 8,000 x g for 10 min. The upper aqueous layers were

transferred to new Corex tubes and extracted with an equal volume of chloroform:isoamyl alcohol (24:1). Again, the upper aqueous layer was transferred to a new Corex tube, two volumes of 95% EtOH were added, and the DNA was precipitated at -20°C overnight. The DNA was collected by centrifugation for 30 min at 25,000 x g at 4°C. The fluid was decanted and the pellets were dried. The DNA was resuspended in 1 ml of TE. DNA concentration was determined as above.

Cell-associated viral DNA was also isolated using an alternate method. Stock virus was grown as described above. The culture supernatant was centrifuged at 5,000 x g as described above for extracellular virus isolation. However, the cell pellet was saved. Any remaining adherent cells in the flasks were harvested by trypsinization and added to the tubes containing the initial cell pellets. Cells were pelleted at 5,000 x g. The fluid was removed and the cells were resuspended in 10 ml of lysis solution (1% SDS, 500 ug/ml RNase A, 0.45% sodium deoxycholate, in TE), transferred to 40 ml Oak Ridge tubes, and incubated at 37°C for 2 h. After addition of 0.1 ml proteinase K (20 mg/ml), the tubes were incubated overnight at 37°C. The volume of each tube was adjusted to 25 ml with TE and 32.5 g of cesium chloride (CsCl) was added and mixed. The DNA solution was centrifuged in the Vti 50 rotor at 40,000 rpm at room temperature for 20 h in a Beckman ultracentrifuge. Fractions (0.5 ml) were collected in microcentrifuge tubes. The DNA in fractions with a

refractive index of 1.4007-1.4030 was precipitated by the addition of 1/10 volume of 3 M sodium acetate pH 5.2 and 2 volumes of 95% EtOH. After overnight incubation at -20°C, tubes were centrifuged at 12,000 x g for 10 min. The fluid was decanted and the DNA pellets were dried. DNA concentration was determined as before.

ELISA for HCMV late nuclear antigen. Confluent monolayers of HFF in 96-well tissue culture treated plates were prepared as previously described. The configuration of the plates consisted of six uninfected cell control wells, six virus control wells, and six wells per drug concentration (eight concentrations per plate). The growth medium was removed from the wells and 50 µl of maintenance medium was added to the cell control wells and 50 µl virus inoculum was added to the remaining wells. The virus was allowed to adsorb for 2 h at 37°C and 5% CO_2. The viral suspension was removed and 0.2 ml of maintenance medium was added to the cell control and virus control wells. Increasing concentrations of the antiviral agent diluted in maintenance medium was added to the remaining wells. The plates were then incubated at 37°C in 5% CO_2.

At various times, plates were removed and the medium was aspirated. One hundred microliters of blocking solution (0.5% bovine serum albumin (BSA) in PBS) was added to each well and the plates were incubated at room temperature for 30 min. Blocking solution was aspirated and the cells were fixed by

adding 0.1 ml ethanol:acetone (95:5) per well and incubating the plates at -20°C for 30 min. Each well was washed 5 times with 0.2 ml of wash solution (PBS containing 0.5% BSA and 0.05% Tween 20). One hundred microliters of monoclonal anti-CMV late nuclear antigen (DuPont, Wilmington, DE) diluted 1:200 in PBS containing 10% goat serum, 1% FCS, and 0.05% Tween 20 was added to each well and the plates were incubated at 37°C for 1 h. The primary antibody solution was aspirated and the cells were washed as before. One hundred microliters of horseradish peroxidase conjugated goat anti-mouse IgG (Bio-Rad Laboratories, Richmond, CA) diluted 1:500 in PBS containing 10% goat serum and 0.05% Tween 20 were added to the wells and plates were incubated at 37°C for 2 h. The secondary antibody solution was aspirated, the cells were washed as before, and 0.2 ml of substrate solution (2,2'-azinobis(3-ethylbenzthiazoline sulfonic acid) (AEBS; Sigma, St. Louis, MO; 1 mg/ml), 0.01% hydrogen peroxide in phosphate-citrate buffer pH 4.8) was added to each well. The plates were incubated at room temperature for approximately 15 to 20 min. The plates were then mixed (Shaking Apparatus, Arthur H. Thomas Co., Philadelphia, PA) and the optical density (OD) of each well were read against a distilled water blank using a Dynatech MR580 Microelisa Auto Reader set at a wavelength of 450 nm. In some cases, TMBlue (3,3',5,5'-tetramethyl-benzidine) substrate solution (Transgenic Sciences, Inc., Worcester, MA) was used for detection with the following

modifications. The primary antibody was diluted a final concentration of 1:600, and the OD_{630} was determined after 5-10 min incubation at room temperature. In comparison to AEBS, TMBlue required no preparation and is non-mutagenic. In addition, the color change of this substrate occurred faster than AEBS.

The average OD of the six identical wells for each drug concentration was calculated and the change in OD as compared to the uninfected cell control was then calculated. The percent change in OD was calculated as follows: (average sample OD)/(average virus control OD - average cell control OD) x 100. The inhibitory concentration (IC_{50}) was defined as the concentration of antiviral agent which produced a 50% reduction in the OD of the colored substrate solution.

ELISA for HSV-2 antigen. The in situ ELISA for the quantitation of HSV-2 antigen was performed as previously described for HCMV with the following modifications. Microtiter plates were seeded with Vero cells. HSV-2 ATCC VR-734 was used as the control virus. The virus was allowed to adhere for 1 h. The plates were incubated 2 to 3 days until substantial plaque formation was observed. The primary antibody was HSV-II(IgG1) Cell Line 1 (DuPont Company, Wilmington, DE) used at a final dilution of 1:200.

HPLC analysis of GCV anabolism in HCMV-infected HFF. HFF were seeded into 75 cm^2 tissue culture flasks and incubated until confluent. The flasks were inoculated with

2.5 ml of HCMV diluted in maintenance medium at an MOI of 1. Virus was allowed to absorb at 37°C for 2 h. The inoculum was removed and replaced with 15 ml of maintenance medium. After 3 days incubation at 37°C, the medium in each flask was replaced with 7.5 ml of maintenance medium containing 235 μl ^{3}H-GCV (approximately 1 x 10^{7} cpm; a gift from Syntex, Palo Alto, CA) and unlabelled GCV (25 μM final concentration). To ensure the sterility of the labelled medium, the ^{3}H-GCV was first added to 10 ml of medium. The solution containing the radiolabelled GCV was then filter sterilized (0.45 μm filter) and brought to volume with the remaining medium and GCV. The flasks were incubated an additional 24 h at 37°C.

The medium was removed from the flasks and the cells were treated with 0.5 ml of 0.5% trypsin for 10 min at 37°C. The cells were resuspended in 3 ml of growth medium and transferred to a 15 ml conical tube. The flasks were washed with an additional 3 ml of growth medium and the wash fluid was added to the harvested cells. The tubes were centrifuged in a Beckman TJ-6 tabletop centrifuge for 10 min at 2000 rpm at room temperature. All but 0.5 ml of fluid was removed and the tube was vortexed to resuspend the cells. The cells were then transferred to microcentrifuge tubes. One milliliter of phosphate buffered saline (PBS) was added to the tubes. The tubes were centrifuged at 12,000 x g for 5 min at 4°C. The fluid was removed and the cells were washed twice with 1 ml of PBS in a similar manner. PBS was added to the tubes to a

final volume of 200 μl. The tubes were then vortexed to resuspend the cells. Sixty-five microliters of 2.0 M perchloric acid was added to each tube. The tubes were vortexed and centrifuged for 30 min. The supernatant containing the cell extracts was transferred to a new microcentrifuge tube and placed on ice prior to HPLC analysis or stored at -70°C.

Phosphorylated products of GCV metabolism were separated by HPLC using the method of Lurain (76). The mobile phase was 0.02 M KH_2PO_4 pH 3.65 run at a flow rate of 0.3 ml/min. Briefly, 10 μl of GCV standard (100 μM GCV, 100 μM GCV-monophosphate) was injected and run through a Partisil 10 OD3 250 x 4.6 mm reverse phase HPLC column (Phenomenex, Rancho Palos Verdes, CA). One hundred microliters of the perchloric acid extracts of infected cells was injected and fractions were collected in scintillation vials at 0.3 min intervals for 15 min. Seven milliliters of scintillation fluid (Ultima Gold, Packard Instrument Company, Meriden, CT) was added to each vial and the radioactivity of each vial was measured for 2 min using an LKB 1214 Rackbeta liquid scintillation counter.

Bacterial plasmid DNA Isolation. Miniprep plasmid DNA isolation was performed using a modified alkaline lysis protocol (78). Two ml of Luria Broth (Gibco BRL, Grand Island, NY) containing the appropriate selective antibiotic was inoculated with a single bacterial colony and incubated overnight at 37°C with shaking. The culture was transferred

to a 2 ml microcentrifuge tube and the cells were pelleted by centrifugation at 12,000 x g for 1 min. The medium was removed and cells were resuspended in 0.1 ml ice-cold lysis solution containing 50 mM glucose, 10 mM EDTA, and 25 mM Tris-HCl pH 8.0. The tube was incubated for 5 min at room temperature. Two hundred microliters of a freshly prepared solution of 0.2 N NaOH, 1% SDS was added and the contents were mixed by gentle inversion. Following incubation for 5 min at room temperature, 0.15 ml of ice-cold 5 M potassium acetate solution pH 4.8 was added followed by incubation on ice for 10 min. The tube was centrifuged for 5 min at 12,000 x g and the supernatant was transferred to a fresh tube. An equal volume of phenol saturated with 50 mM Tris pH 8.0:chloroform: isoamyl alcohol (50:48:2) was added and tubes were centrifuged at 12,000 x g for 5 min. The upper aqueous layer was transferred to a fresh tube. Plasmid DNA was precipitated by the addition of 2 volumes of ice-cold 95% EtOH. DNA was pelleted by centrifugation at 12,000 x g for 10 min. The supernatant was decanted and the pellet was washed with 1 ml of 70% EtOH. The DNA pellet was dried and resuspended in 30 μl of TE containing RNase (20 μg/ml). DNA concentration was estimated using agarose gel electrophoresis with known DNA standards.

For large-scale isolation of plasmid DNA, 200 ml of CircleGrow medium (Bio101, LaJolla, CA) containing the appropriate selective antibiotic was inoculated with an

isolated colony of E. coli and incubated at 37°C with shaking overnight. The bacteria were pelleted by centrifugation at 5,000 x g for 10 min in the Sorvall GSA rotor. The pellet was resuspended in 5 ml of a solution containing 50 mM glucose, 25 mM Tris-HCl pH 8.0, and 10 mM EDTA. The fluid was transferred to 40 ml Oak Ridge tubes and an additional 5 ml of the same solution containing 10 mg/ml of lysozyme was added. After incubation for 5 min at room temperature, 20 ml of 0.2 N NaOH/1% SDS was added followed by incubation for 5 min at room temperature. Fifteen milliliters of ice-cold 5 M potassium acetate solution (pH 4.8) were added, the contents were mixed by vigorous shaking and incubated on ice for 10 min. The tube was centrifuged in the Sorvall SS34 rotor for 30 min at 25,000 x g. The supernatant was transferred to two 30 ml Corex tubes (18 ml/tube), 12 ml of isopropanol was added, and the tubes were incubated at room temperature for 20 min. The tubes were centrifuged in the SS 34 rotor at 8,000 x g for 30 min at room temperature. The pellets were resuspended in 3 ml of TE and pooled. The volume was adjusted to 6.6 ml with TE. Cesium chloride (7.245 g) was added followed by 0.54 ml of 10 mg/ml ethidium bromide. The DNA solution was divided between three 3.5 ml Quik-Seal tubes which were then centrifuged in the Sorvall TL100.3 rotor at 300,000 x g for 5 h. Plasmid bands were harvested from the tubes by side puncture. The ethidium bromide was extracted with isopropanol saturated with 10X SSC (3 M NaCl, 0.3 M

sodium citrate, pH 7.0). The DNA was precipitated by the addition of an equal volume of TE and then 2 volumes of 95% EtOH followed by incubation at -20°C for 1 h. DNA was pelleted in microcentrifuge tubes by centrifugation at 12,000 x g for 10 min. The DNA pellets were resuspended in 0.2 ml of TE. DNA concentration was determined spectrophotometrically (78).

Agarose gel electrophoresis. Viral and bacterial plasmid DNA samples were analyzed on horizontal 0.7% agarose gels containing 0.12 μg/ml ethidium bromide in buffer containing 89 mM Tris base, 89 mM boric acid, and 2 mM disodium EDTA (final pH 8.0).

Cloning of restriction fragments. pUC18 vector DNA was linearized with EcoRI and treated with calf alkaline phosphatase (CIP) (Boehringer Mannheim, Indianapolis IN). HCMV genomic DNA was digested with EcoRI, ethanol precipitated, and redissolved in distilled water and stored at -20°C. HSV genomic DNA was digested with EcoRI and fragments were separated by agarose gel electrophoresis. The band of interest was excised and the DNA was electroeluted and purified by ethanol precipitation. Ligation of 0.5 μg vector and 0.5 μg insert DNA was carried out in 60 mM Tris-HCl pH 8.0, 33 mM NaCl, 10 mM $MgCl_2$, 1 mM ATP, and 1 unit of T_4 ligase (Stratagene, LaJolla, CA) in a total volume of 50 μl for 2 h at room temperature. Control ligations contained only CIP-treated or CIP-untreated vector DNA.

Competent cells were prepared as follows. A 1 ml Luria broth preculture was inoculated with a single colony of E. coli DH5α and incubated overnight at 37°C with shaking. Two hundred microliters of the overnight culture was added to 19.8 ml of Luria broth and the culture was incubated for 2-3 h at 37°C with shaking. The bacteria were pelleted by centrifugation in a Sorvall SS34 rotor at 3,000 x g for 5 min at 4°C. Pelleted cells were resuspended in 5 ml ice-cold 50 mM $CaCl_2$ and held on ice for 20 min. The bacteria were pelleted as before, gently resuspended in 1 ml ice-cold 50 mM $CaCl_2$, and placed on ice until use.

Ligation products were transformed into competent E. coli DH5α cells. The ligation mixes were added to 100 μl competent cells in prechilled 17 x 100 mm tubes. The suspensions were held on ice for 1 h followed by 42°C incubation for 2 min. One ml of Luria broth prewarmed at 37°C was added to the tube. Cultures were incubated for 1 h at 37°C with shaking. Four aliquots (0.25 ml) were plated on Luria agar plates containing 100 μg/ml ampicillin. Plasmid DNA from resultant colonies was isolated, digested with various restriction endonucleases, and analyzed by agarose gel electrophoresis.

Preparation of labelled probes. Plasmid DNA was labelled with ^{32}P by random priming (Multiprime Labelling Kit; Amersham, Arlington Heights, IL) according to the manufacturer's instructions. Unincorporated nucleotides were separated from the labelled probes using spin columns (5'-->3', Inc., West

Chester, PA).

Southern hybridizations. Agarose gels were depurinated in 0.4 M HCl for 10 min at room temperature, denatured in 0.5 M NaOH/1.0 M NaCl for 30 min, and neutralized in 0.5 M Tris-HCl/1.5 M NaCl (final pH 7.4) for 30 min. All incubations were performed at room temperature. The gels were then blotted overnight onto Nytran (Scheicher and Schuell, Keene, NH) with 10X SSPE (1X SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, 1 mM EDTA). After briefly rinsing the membranes in 2X SSPE, the DNA was UV crosslinked to the membrane using the UV Stratalinker (Stratagene, La Jolla, CA) set on auto crosslink. The membranes were prehybridized in 1% SDS, 6X SSPE, 10X Denhardt's solution, and 50 μg/ml denatured salmon sperm DNA for 1-2 h at 42°C. The prehybridization fluid was replaced with solution containing 50% formamide, 1% SDS, 6X SSPE, 50 μg/ml of denatured salmon sperm DNA, and 10 ng denatured probe DNA.

The membranes were washed twice with 2X SSPE for 15 min at room temperature, twice with 2X SSPE/0.1% SDS for 15 min at 65°C, and twice with 0.1% SSPE at room temperature. Membranes were placed on Whatman 3M paper, covered with plastic wrap and placed in X-ray cassettes with Crones Lightning Plus intensifying screens (DuPont, Wilmington, DL) and Kodak X-OMAT AR film. Cassettes with held at -70°C overnight.

DNA sequence analysis. DNA sequence analysis performed

using the Sanger dideoxy termination method (103). DNA sequencing reactions were performed using the T7 DNA sequencing kit (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) or the TaqTrack sequencing system (Promega Corporation, Madison, WI) according to the manufacturer's instructions. Reaction products were electrophoresed through a 0.4 mm 6% polyacrylamide/7.56 M urea gel at 60 watts.

Site-directed in vitro mutagenesis. In vitro mutagenesis was performed using the Altered Sites Mutagenesis System (Promega Corporation, Madison, WI) according to the manufacturer's instructions. For the mutagenesis reaction, a synthetic oligonucleotide complementary to a region of the HCMV DNA polymerase was synthesized at the LUMC Macromolecular Facility. The sequence of the 16-mer is as follows: 5' GTTCTGTGACAGCGG. The underlined base corresponds to the mismatch of adenosine in the complementary strand at nucleotide position 3048 in the HCMV AD169 sequence (71). Incorporation of the mismatched base was confirmed by DNA sequence analysis.

Transfection of mammalian cells. The procedure of Cai et al. (11) was followed for transfection of HFF and Vero cells. DNA for transfection was precipitated by addition of the following components in the following order: sterile distilled water to final volume, salmon sperm DNA (40 μg/ml), plasmid DNA (6.6 μg/ml), HSV-1 KOS or HCMV AD169 DNA (0.2 μg/ml), calcium chloride (final concentration = 0.25 M), and

one-tenth volume of 2X Hepes buffered saline pH 7.04 (HBS). The mixture was allowed to sit at room temperature for 30 min. During this time, a slightly cloudy precipitate formed. The medium was removed from HFF or Vero cell monolayers in 100 mm dishes and replaced with 3 ml/plate of the CaCl-DNA precipitate. After 30 min incubation at 37°C in 5% CO_2, 7 ml of growth medium was added to the monolayers.

After 4 h incubation at 37°C in 5% CO_2, the cells were shocked with glycerol as follows. The medium was replaced with 3 ml of maintenance medium containing 15% glycerol and monolayers were incubated at room temperature for 1 min. The shock medium was removed and the cells were washed twice with maintenance medium. The monolayers were then overlaid with 10 ml of growth medium with or without antiviral drug and incubated at 37°C in 5% CO_2 until confluent. The medium was replaced weekly with maintenance medium until substantial plaque formation was observed.

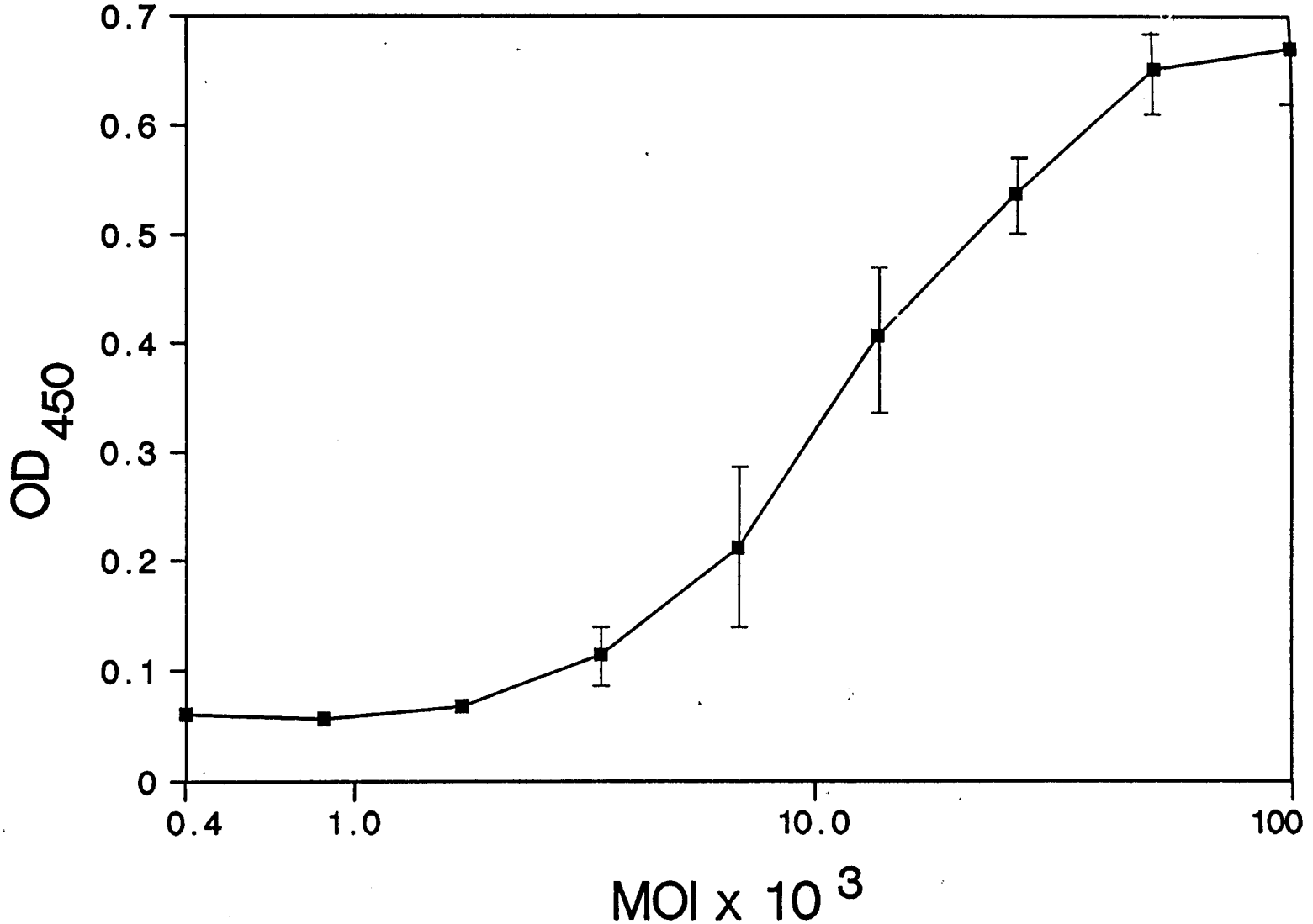
RESULTS

Development of an in situ ELISA for a late HCMV antigen. Recent studies have described the development of in situ ELISA assays for the quantitation of HSV (2,97) and VZV (5) antigens in infected monolayers. We have adapted these protocols for the quantitation of an HCMV late nuclear antigen.

The VZV protocol described by Berkowitz and Levin (5) was used with following modifications. First, a primary monoclonal antibody against a HCMV late nuclear antigen was chosen rather than an early antigen since synthesis of a late antigen was strictly dependent on viral DNA synthesis and therefore was directly related to virus reproduction. Second, preliminary experiments showed that fixation of cell monolayers with ethanol:acetone (95:5) was far superior to fixation with glutaraldehyde.

Initial experiments were performed to determine the optimum virus inoculum concentration. Monolayers of HFF in 96-well microtiter plates were inoculated with increasing 2-fold concentrations of HCMV strain AD169. The ELISA was performed when the CPE in the wells containing the highest concentration of virus (MOI = 0.1) reached 100% (4+). At that time, 25% CPE was observed in the monolayers inoculated with the lowest concentration of virus (MOI = 3.9×10^{-4}). The relationship between MOI and OD_{450} is shown in Figure 4. A low

Figure 4. Relationship between MOI and OD_{450} for detection of a late HCMV antigen in the ELISA assay.


0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
OD 450
0.4
1.0
10.0
100
MOI x 10 3

level of viral antigen was detected in monolayers infected with the lowest concentration of virus and with the least CPE. A linear relationship between MOI and OD_{450} existed between an MOI of 3.1×10^{-3} and 2.5×10^{-2}. These inoculum concentrations resulted in 25 to 50% CPE (2-3+). At the higher MOI (≥ 0.05) and therefore the higher CPE, the monolayers began to disintegrate resulting in a loss of viral antigen. This was reflected in a plateau in the OD_{450} readings. These results indicated that optimum results were obtained when the CPE involved 25-50% of the infected monolayer (2-3+ CPE).

Adaptation of the ELISA for HCMV susceptibility testing. Experiments were performed to determine the effect of MOI on susceptibility testing of HCMV AD169 using the ELISA assay. Four 96-well plates seeded with HFF were inoculated with HCMV AD169 at four different MOI. The monolayers in each plate were then incubated in the presence or absence of various concentrations of GCV, ACV, PAA, or PFA. The ELISA was performed when the CPE in the virus control wells (medium without drug) reached 2 to 3+ (4 to 7 days post-infection). The relationship between the IC_{50} and MOI for GCV susceptibility is shown in Figure 5. At the lowest MOI tested (0.005), the IC_{50} for GCV was 1.6 μM. Increasing the MOI ten-fold to 0.05 increased the IC_{50} to 25 μM. Therefore, the IC_{50} value of GCV was MOI dependent. Similar results were obtained for PFA, PAA, and ACV (Table 1). Therefore, in order to

Figure 5. Relationship between MOI and the IC_{50} for GCV using the ELISA assay. MOI = 0.005 (open triangle), MOI = 0.01 (open diamond), MOI = 0.025 (X), MOI = 0.05 (closed squares).

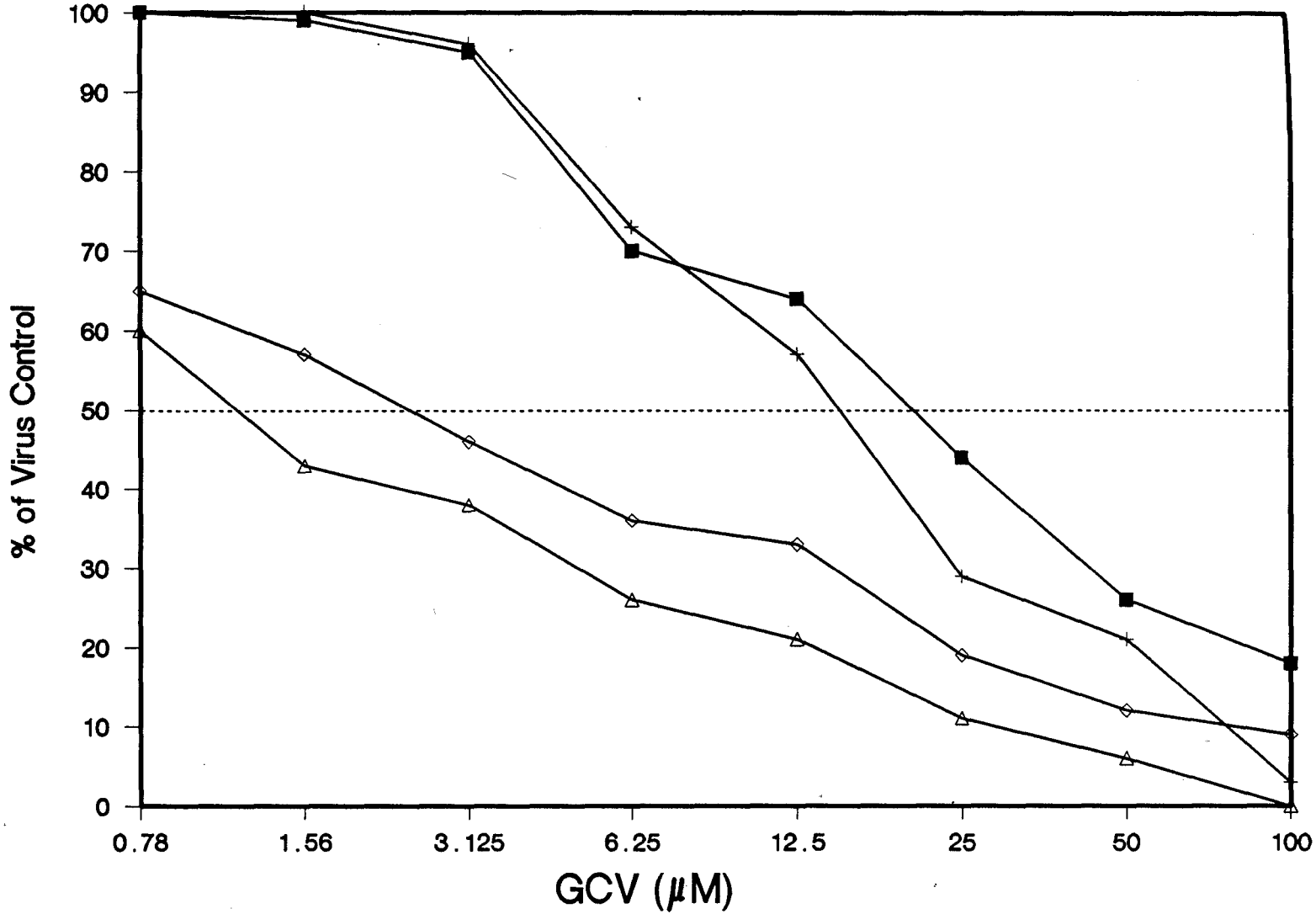

100
90
80
70
60
50
40
30
20
10
0
% of Virus Control
0.78
1.56
3.125
6.25
12.5
25
50
100
GCV (μM)

Table 1. Effect of MOI on IC_{50} values for HCMV strain AD169 using the ELISA.

	IC_{50} (μM)			
MOI	GCV	ACV	PFA	PAA
0.005	1.6	50	62.5	18
0.012	3.1	50	62.5	18
0.025	25	>100	250	45
0.05	25	>100	250	45

standardize the assay, an MOI of 0.012 was chosen for use in further studies. At this MOI, the IC_{50} value of HCMV strain AD169 as determined by the ELISA approximated the IC_{50} value for GCV using the plaque reduction assay (20,43,123, Tables 2 and 4).

To further test the accuracy of the ELISA for susceptibility testing, susceptibility profiles of two GCV-resistant laboratory-derived mutants of HCMV strain AD169 were determined using the ELISA and the PRA. These results are summarized in Table 2. Both mutants were previously shown to be deficient in GCV-kinase activity and therefore remained susceptible to DNA polymerase inhibitors (76). The ELISA clearly demonstrated that the mutants were susceptible to PAA, PFA, and aphidicolin and hypersensitive to AraA and AraT. The data show that though the numerical values of the IC_{50} may differ between the two assays, resistance can be determined using either assay.

Also, an additional experiment revealed that regardless of the inoculum size used, resistance can be detected using the ELISA. At an MOI of 0.015, the IC_{50} values for AD169 and D6/3/1 were 3.1 μM and 100 μM, respectively. When the MOI was increased to 0.06, the IC_{50} values for AD169 and D6/3/1 increased to 50 μM and 200 μM, respectively. Therefore, the inoculum size does not affect the relative IC_{50} results when the test virus and control virus AD169 are used at the same MOI.

Table 2. HCMV susceptibility testing using the ELISA and PRA.

Assay and virus tested[a]	IC_{50} (μM)						
	GCV	ACV	PAA	PFA	AraA	AraT	Aph
ELISA							
AD169	6.2	200	45	150	100	1000	3.8
D6/3/1[b]	200	150	90	150	25	150	3.8
D1/3/4[b]	200	250	90	200	25	150	3.8
PRA							
AD169	6.2	150	180	300	125	>1000	5
D6/3/1	200	150	180	300	50	300	5
D1/3/4	200	150	180	300	50	100	5

[a]MOI = 0.012

[b]Laboratory derived GCV-resistant mutants of HCMV strain AD169 (76).

Collection and growth of HCMV clinical isolates from LUMC. Clinical isolates of HCMV from transplant patients at LUMC were collected from the clinical microbiology laboratory over a 27 month period. During that time, GCV therapy for HCMV disease was available for these patients. We hoped to collect pre-treatment, during-treatment, and post-treatment isolates. Any virus isolated during- or post-treatment would theoretically be GCV-resistant.

Most isolates from the clinical microbiology laboratory displayed very little CPE in the initial isolation tubes. Cells from these tubes were harvested by scraping and used to inoculate fresh HFF tubes. Cultures were incubated for 5 weeks. This process was repeated until the virus became extracellular (100% CPE) and aliquots of the culture fluid were stored at -70°C. Virus from the clinical isolates became extracellular between pass 3 and pass 11, with an average of approximately 6-8 passes. Eighty-three clinical isolates were collected in this manner. All virus strains were isolated from patients prior to any GCV therapy. All patients remained virus-free during therapy and post-treatment or expired during treatment.

Initial screening of GCV-susceptibility of clinical HCMV isolates. In order to perform GCV susceptibility testing of these isolates, the titer of each virus stock was determined. Plaque dilution assays were performed on all frozen stocks. Of the 83 isolates screened, 9 isolates grew to high enough

titer ($\geq 10^3$ pfu/ml) to be screened for GCV susceptibility using the ELISA assay.

The nine isolates were tested for susceptibility to GCV using the ELISA assay. Eight of the isolates appeared to be GCV-sensitive as compared to the AD169 control (Table 3) while the ninth, H51856, appeared to be resistant (IC50 = 50 μM). Multiple testing of isolate H51856 yielded similar results. GCV-resistant strain H51856, cultured from a bronchial wash specimen, and GCV-sensitive strain H51853, cultured from a blood specimen, were collected from the same patient on the same day. Both specimens were obtained prior to GCV treatment for HCMV disease. No virus was isolated from the patient during or post-treatment. These two isolates were chosen for further investigation for two reasons. First, isolate H51856 was a source of GCV-resistant HCMV. Second, because isolates H51856 and H51853 were isolated from the same patient on the same day, comparisons could be made between the two strains.

Several studies have shown that clinical isolates of HSV were actually mixtures of both drug-resistant and drug-sensitive strains (18,37,81,94). Therefore, to determine the percentage of GCV-resistant virus in strain H51856, a percent survival assay was performed. Monolayers of HFF were inoculated with ten-fold dilutions of H51856 and incubated in the presence and absence of 12.5 μM GCV. Comparison of titers in the absence of GCV (6×10^4 pfu/ml) and in the presence of GCV ($< 1 \times 10^2$ pfu/ml) revealed that less than 1% of the

Table 3. GCV susceptibility testing of HCMV clinical isolates using the ELISA.

	IC_{50} (μM)	
Clinical isolate	Test	AD169 control
S43096	6.2	6.2
H46893	1.6	3.1
S42651	1.6	6.2
T1840	12.5	6.2
W66730	1.6	3.1
S15165	<0.8	3.1
M25527	3.1	3.1
H51853	3.1	6.2
H51856	50	6.2

population was GCV-resistant. In order to purify the GCV-resistant subpopulation of H51856, two rounds of plaque purification were performed in the presence of 12.5 μM GCV. Two clones, D13 and D16, were chosen for further study. Both clones exhibited growth characteristics (e.g. growth rate, titer) similar to wild-type HCMV strain AD169 (data not shown).

Antiviral susceptibility profiles of GCV-resistant H51856 clones D13 and D16. The plaque reduction assay (PRA) and the ELISA assay were performed to determine the antiviral susceptibility profiles of H51856 clones D13 and D16. Both the PRA and the ELISA confirmed that the two clones were GCV-resistant (Table 4). Using the PRA, both clones showed only a two-fold increase in resistance to the DNA polymerase inhibitor phosphonoformic acid (PFA). However, susceptibility testing using the ELISA revealed that D13 and D16 were indeed PFA-resistant (IC_{50} = 625 μM and 1250 μM, respectively). The ELISA IC_{50} values for PFA were at least 4-fold higher for the GCV-resistant clones as compared to wild-type HCMV strain AD169. Similar results were obtained with another DNA polymerase inhibitor, phosphonoacetic acid (PAA). These results indicate that GCV-resistance was due to either a mutation in the viral DNA polymerase gene alone or in conjunction with a mutation in the ability of the virus to monophosphorylate the nucleoside analog GCV. Further testing was performed using the nucleoside analogs HPMPA and HPMPC.

Table 4. Antiviral susceptibility profile of H51856 clones.

Assay and virus tested	IC_{50} (μM)				
	GCV	PFA	PAA	HPMPA	HPMPC
ELISA					
AD169	6.2	156	45	<0.06	<0.06
D13	>200	625	360	N.D.[a]	N.D.
D16	>200	1250	360	8	0.5
PRA					
AD169	6.2	200	125	0.25	0.12
D13	50	400	N.D.	2	4
D16	50	800	250	4	1

[a]N.D. = not done

since these two compounds are monophosphate nucleoside derivatives, viral-induced kinase activity is not necessary for activation of the drugs (90). The drugs are phosphorylated to the active triphosphate form by cellular kinases. Clones D13 and D16 were resistant to HPMPA and HPMPC in both the PRA and ELISA assays (Table 4) suggesting that the mutation conferring GCV-resistance is probably at the level of the viral DNA polymerase gene.

GCV anabolism in HCMV-infected HFF. Two GCV-resistant laboratory mutants of strain AD169, BW B759^{r}D100 and D6/3/1, have been reported (7,76). Analysis of infected cell extracts showed that both were unable to phosphorylate GCV at levels comparable to wild-type HCMV strain AD169. However, both remained sensitive to the viral DNA polymerase inhibitors PFA and PAA. These observations suggested that a mutation in the gene involved in monophosphorylation of GCV was responsible for drug resistance. Therefore, similar GCV anabolism studies were performed using HCMV strain AD169-, D13-, D16-, or D6/3/1-infected HFF. Two days post-infection, the cultures were incubated for an additional 24 h in the presence of ^{3}H-GCV. Infected cell extracts were analyzed for GCV anabolism products using HPLC.

Previous analysis of HPLC fractions of infected cell extracts showed that phosphorylated derivatives of ^{3}H-GCV eluted between fractions 5 and 15 while unphosphorylated ^{3}H-GCV eluted between fractions 30 and 40 (76). However, this

assay cannot resolve the individual phosphorylation products of GCV. Experiments here confirmed that little, if any, GCV phosphorylation occurred in mock infected cells (Figure 6). The peak height of phosphorylated GCV derivatives is half that of non-phosphorylated GCV. However, significant GCV phosphorylation was induced in wild-type AD169 infected HFF (Figure 6). The peak height of phosphorylated GCV derivatives is comparable, if not higher, than non-phosphorylated GCV. GCV anabolism profiles of clones D13 and D16 were nearly identical to the wild-type HCMV strain AD169 profile (Figure 6) suggesting that GCV-resistant clones induced wild-type levels of GCV phosphorylation.

In a second similar experiment, the induction of GCV phosphorylation by D13 and the GCV-kinase mutant D6/3/1 was compared (Figure 7). Strain D13 induced GCV phosphorylation at levels comparable to wild-type HCMV strain AD169. In contrast, strain D6/3/1 showed a decreased level of induction of GCV phosphorylation as compared to strain AD169 and strain D13. These data, taken in conjunction with the antiviral susceptibility profiles of D13 and D16, suggest that the virus-induced GCV-kinase activity is intact and that a mutation in the viral DNA polymerase gene is responsible for GCV-resistance.

Genomic location of the H51856 D13 and H51853 DNA polymerase genes. The HCMV DNA polymerase gene of HCMV strain AD169 is located on a 7.1 kb EcoRI M fragment (Figure 8). In

Figure 6. GCV phosphorylation products in mock-, AD169-, D13-, and D16-infected HFF. Phosphorylated derivatives of ^{3}H-GCV elute between fractions 5 and 15. Unphosphorylated ^{3}H-GCV elutes between fractions 30 and 40. The mobile phase was 0.02 M KH_2PO_4 pH 3.65 run at a flow rate of 0.3 ml/min through a Partisil 10 OD3 column.

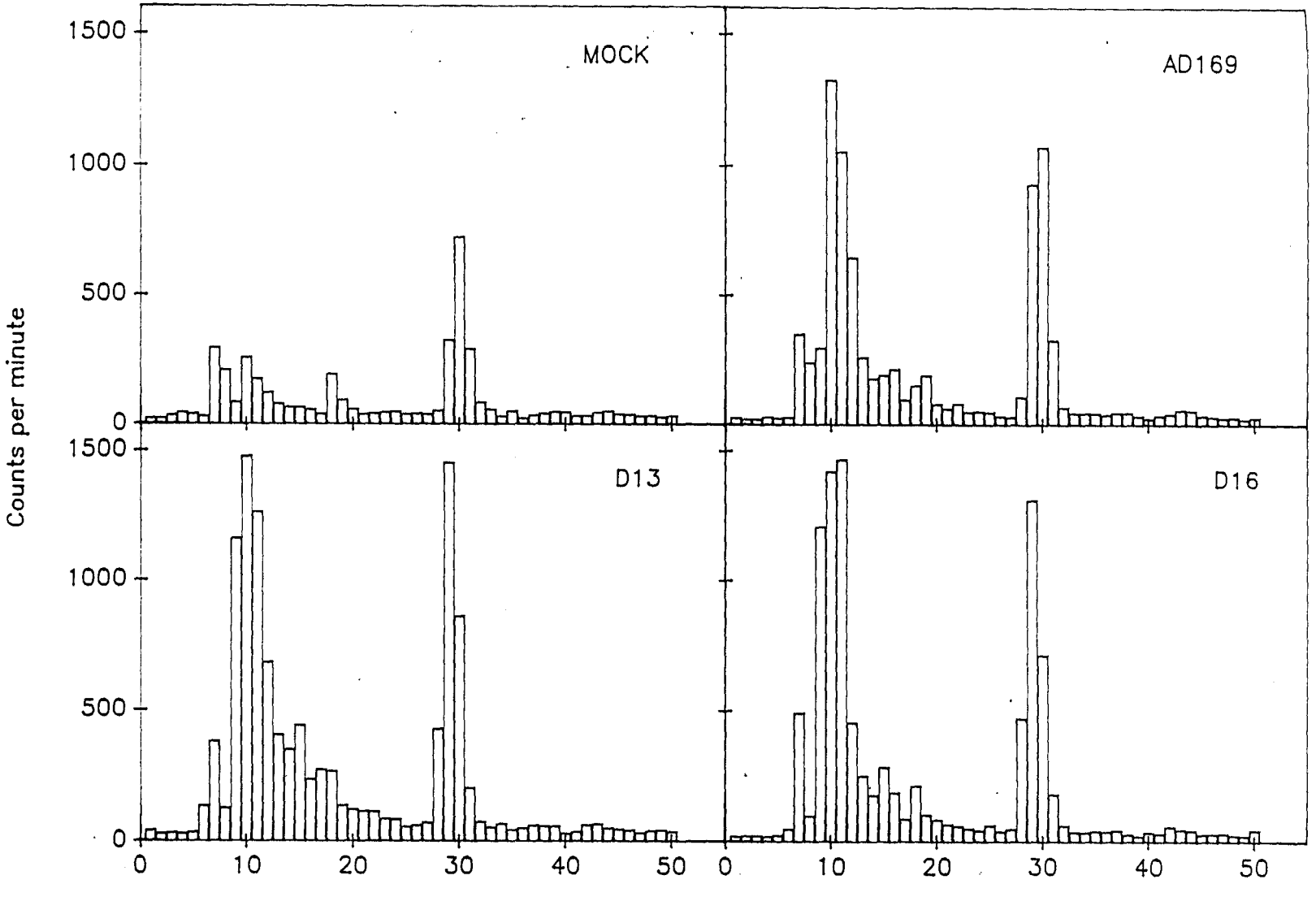

MOCK
AD169
D13
D16
1500
1000
500
0
0
10
20
30
40
50
Counts per minute
Fraction number

Figure 7. GCV phosphorylation products in AD169-, D13-, and D6/3/1-infected HFF. Phosphorylated derivatives of ^{3}H-GCV elute between fractions 5 and 15. Unphosphorylated ^{3}H-GCV elutes between fractions 30 and 40. HPLC conditions are described in Figure 6.

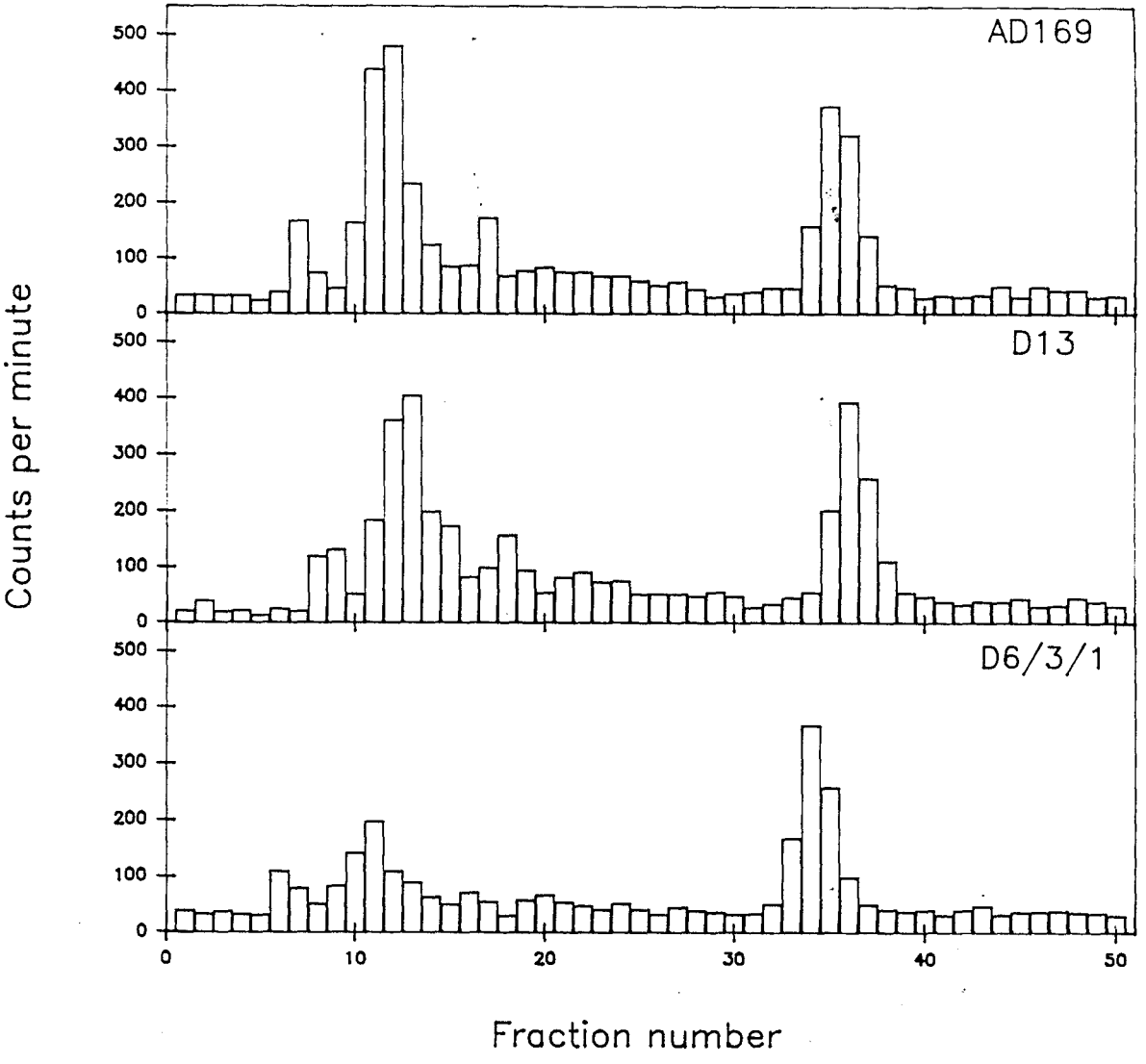
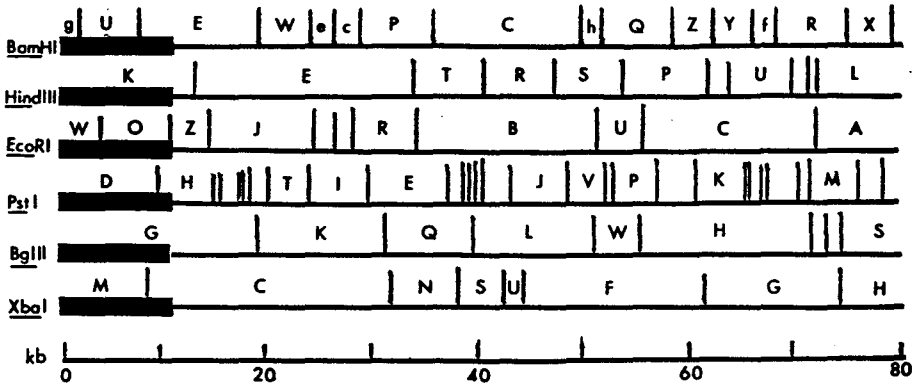
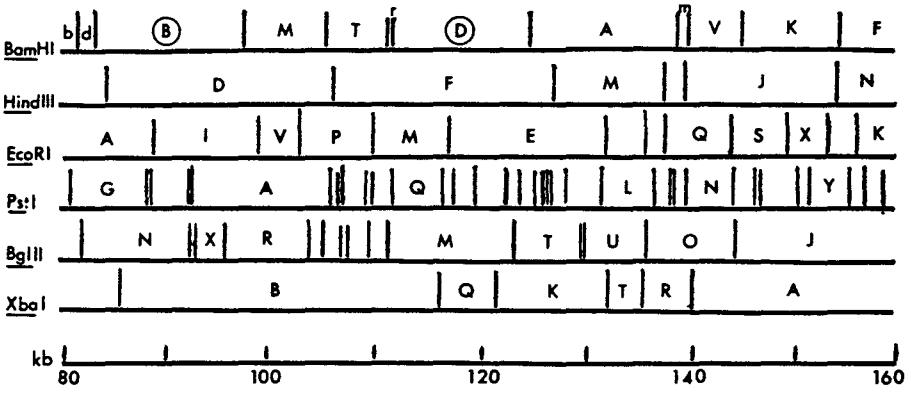
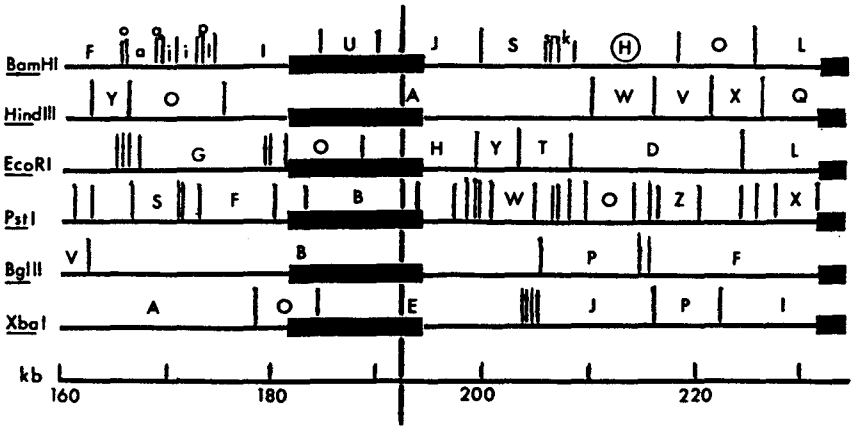

AD169
D13
D6/3/1
Counts per minute
Fraction number
0
100
200
300
400
500
10
20
30
40
50

Figure 8. Restriction endonuclease map of HCMV strain AD169 (reproduced with permission from reference 76).


BamHI
HindIII
EcoRI
PstI
BglII
XbaI
kb
0
20
40
60
80


BamHI
HindIII
EcoRI
PstI
BglII
XbaI
kb
80
100
120
140
160

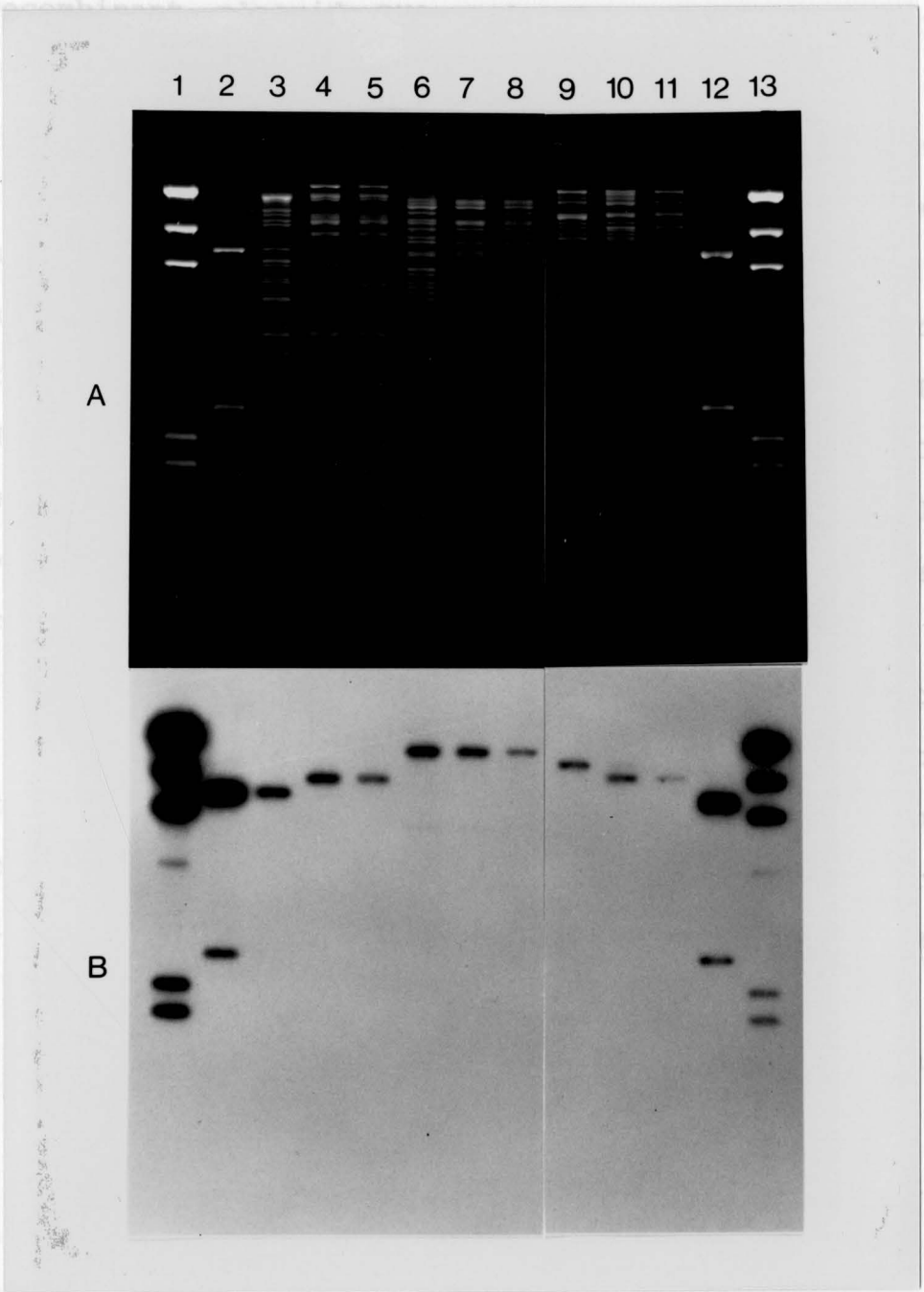

BamHI
HindIII
EcoRI
PstI
BglII
XbaI
kb
160
180
200
220

order to locate the HCMV DNA polymerase genes from the GCV-resistant clone D13 and its corresponding GCV-sensitive strain H51853, EcoRI digests of extracellular viral DNA were analyzed by agarose gel electrophoresis (Figure 9A). Comparison of EcoRI restriction digest profiles between D13 and H51853 showed very few differences (lanes 4 and 5). However, several differences were noted between the clinical isolates and wild-type strain AD169 (lane 3). Similar results were seen with BamHI digests (lanes 6 - 8). The BglII restriction digests of D13 and H51853 appeared identical (lanes 10 - 11) but slightly different than AD169 BglII digests (lane 9). The digested DNA was then transferred by Southern blotting and probed with pEcoM, a pUC18-derived plasmid construct containing the AD169 EcoRI M fragment. The probe hybridized to the 2.6 kb vector DNA band and to the EcoM fragment of pEcoM (Figure 9B, lane 2). The probe hybridized to one EcoRI viral fragment each in the pEcoM, AD169, D13, and H51853 digests (Figure 9B, lanes 3 - 5). The size of the D13 and H51853 hybridizing fragments were approximately 1 kb larger than the AD169 EcoRI M fragment. Since the probe hybridized to only one fragment in the D13 and H51853 digests, the viral DNA polymerase gene must lie within this fragment.

Cloning of the D13 and H51853 DNA polymerase genes. Genomic DNA from strains D13 and H51853 was digested with EcoRI, ligated with EcoRI-digested pUC18 vector plasmid DNA, and transformed into E. coli DH5α. Transformant plasmid DNA

Figure 9. Agarose gel (A) with corresponding Southern blot (B) of restriction endonuclease digested AD169, GCV-resistant D13, and GCV-sensitive H51853 viral DNA. Probe was pEcoM and lambda DNA. Sizes of the fragments in lanes 1 and 13 are 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, and 0.6 kb.

DNA:	Lambda	Lanes 1 and 13
	pEcoM	Lanes 2 and 12
	AD169	Lanes 3, 6, and 9
	D13	Lanes 4, 7, and 10
	H51853	Lanes 5, 8, and 11
Enzymes:	HindIII	Lanes 1 and 13
	EcoRI	Lanes 2 - 5, 12
	BamHI	Lanes 6 - 8
	BglII	Lanes 9 - 11


1 2 3 4 5 6 7 8 9 10 11 12 13
A
B

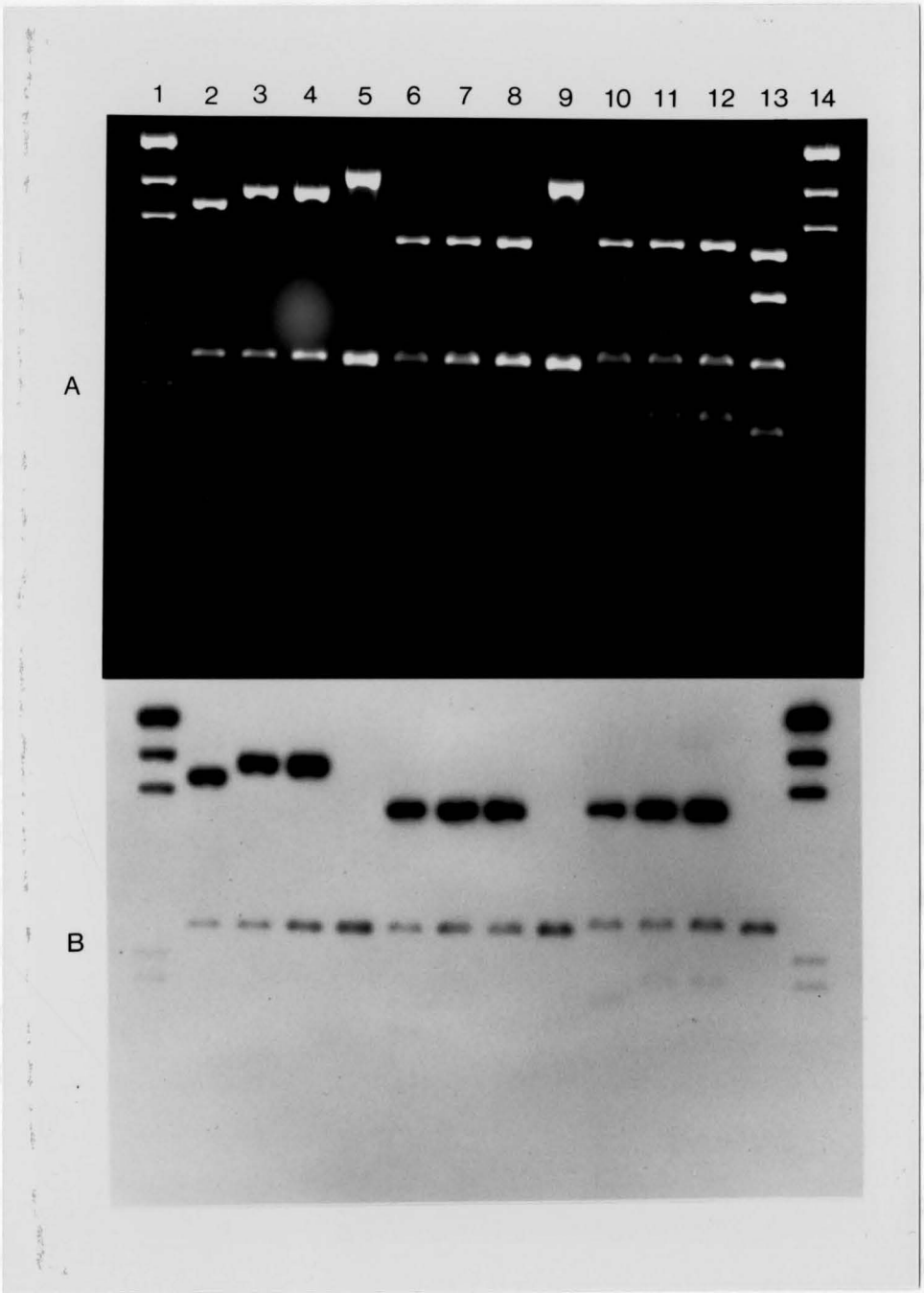
was isolated and digested with EcoRI and analyzed by agarose gel electrophoresis. Those clones with inserts approximately 8 kb in size were selected and further analyzed.

Recombinant plasmid DNA and pEcoM plasmid DNA were digested with EcoRI, EcoRI and BamHI, or EcoRI and BglII and separated by agarose gel electrophoresis (Figure 10A). Digestion with EcoRI alone should yield a 2.7 kb linear vector fragment and insert viral DNA. Viral inserts from two plasmids, pD13M (lane 3) and p53POL (lane 4), comigrated at approximately 8 kb, slower than the 7.1 kb HCMV strain AD169 EcoRI M fragment in pEcoM (lane 2). A third plasmid, p170, harbored a D13 EcoRI insert larger than pD13M and p53POL (lane 5). Double restriction digests with EcoRI and BamHI (lanes 6-9) and EcoRI and BglII (lanes 10-13) revealed that pD13M and p53POL shared several common fragments with pEcoM while p107 showed very little similarity with pD13M, p53POL and pEcoM.

The digested DNA was then transferred by Southern blotting and probed with pEcoM (Figure 10B). The probe hybridized to the 2.7 kb vector DNA and to the AD169 EcoRI M insert in pEcoM (lane 2). The probe also hybridized to the vector DNA and to the two larger inserts in pD13M and p53POL (lanes 3 and 4). However, the probe hybridized only to the vector DNA in p107 (lane 5). Analysis of hybridization patterns of double restriction digests (lanes 6-13) using restriction maps of HCMV strain AD169 (Figure 8) confirmed that pD13M and p53POL harbored an EcoRI insert which encoded

Figure 10. Agarose gel (A) with corresponding Southern blot (B) of restriction endonuclease digested pEcoM and plasmid clones containing D13 EcoRI inserts. Probe was pEcoM and lambda DNA. Sizes of the fragments in lanes 1 and 14 are 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, and 0.6 kb.

DNA:	Lambda	Lanes 1 and 14
	pEcoM	Lanes 2, 6, and 10
	pD13M	Lanes 3, 7, and 11
	p53POL	Lanes 4, 8, and 12
	p107	Lanes 5, 9, and 13
Enzymes:	HindIII	Lanes 1 and 14
	EcoRI	Lanes 2 - 5
	EcoRI/BamHI	Lanes 6 - 9
	EcoRI/BglII	Lanes 10 - 13


1 2 3 4 5 6 7 8 9 10 11 12 13 14
A
B

the viral DNA polymerase gene. The slight differences in restriction and hybridization patterns between the recombinants pD13M and p53POL and the wild-type pEcoM in the double digests were probably due to the additional 1 kb of viral DNA in the pD13M and p53POL viral EcoRI inserts. Since the pEcoM probe hybridized to only one insert band in EcoRI digests of genomic D13 and H51853 DNA, this suggested that pD13M and p53POL EcoRI inserts encoded the entire viral DNA polymerase gene. Therefore, pD13M and p53POL were chosen for further study.

Sequence analysis of the D13 and H51853 DNA polymerase genes. Previous studies have demonstrated that DNA polymerase drug-resistant mutants of HSV and vaccinia virus contain nucleotide base substitutions in specific conserved areas of the viral DNA polymerase gene (49,118). All HSV mutations were mapped to one region, the proposed deoxynucleotide triphosphate (dNTP) and pyrophosphate (PPi) binding sites (50). Significant homology exists between the HSV, EBV, and HCMV DNA polymerase proteins at these two sites (71). Therefore, sequence analysis was performed on these homologous areas of the DNA polymerase genes of GCV-resistant clone D13 and the GCV-sensitive strain H51853. Based on the published sequence of the AD169 DNA polymerase gene (71), the homology to the HSV dNTP and PPi binding sites occurs between bases 2145 and 3503. The published sequence of HCMV AD169 and the sequence data for HCMV GCV-resistant clone D13 and GCV-

sensitive clone H51853 are shown in Figure 11. Out of 1358 bases, only 21 changes were observed in D13 and 21 in H51853. Seventeen of the 21 changes were shared in both strains. Analysis of the amino acid sequence generated by these nucleotide sequences is shown in Figure 12. In HSV-1, the proposed dNTP and PPi binding sites lie on a continuous stretch of the DNA polymerase protein. However, the HCMV DNA polymerase protein contains several additional stretches of amino acids which separate the homology to the HSV dNTP and PPi binding sites. Of the shared nucleotide changes, only two changes would result in altered amino acids when compared to GCV-sensitive wild-type HCMV strain AD169. At amino acid number 685, there is a conservative asparagine (N) to serine (S) change and at position number 691, there is a conservative threonine (T) to serine (S) substitution. Of the non-shared nucleotide changes, two substitutions in the GCV-sensitive strain H51353 polymerase gene would result in adjacent non-conservative amino acid changes. Glutamine (Q) (position 651) and aspartic acid (D) (position 652) would be replaced with histidines (H). The wild-type glutamine and aspartic acid at positions 651 and 652 are not conserved in the HSV or EBV polymerase proteins (71). Most strikingly, only one non-shared amino acid substitution was observed in the GCV-resistant clone D13, a relatively non-conservative alanine (A) to threonine (T) substitution at amino acid position 885. This mutation is located between two areas of homology with

Figure 11. Nucleotide sequence of the proposed dNTP and PPi binding sites of the HCMV AD169, D13 and H51853 DNA polymerase genes. The right most number of the nucleotide position is positioned above the corresponding base. Base substitutions in the D13 and H51853 genes are shown below the AD169 sequence.

```
        2148                                                                                                2239
AD169   TCGCCCAACTATAAGCTCAACACTATGGCCGAGCTTTACCTGCGGCAACGCAAGGATGACCTGTCTTACAAGGACATCCCGCGTTGTTTCGTGGC
D13M                                                                                C
53POL                                                                               C

        2243                                                                                                2334
AD169   TAATGCCGAGGGCCGCGCCCAGGTAGGCCGTTACTGTCTGCAGGACGCCGTATTGGTGCGCGATCTGTTCAACACCATTAATTTTCACTACGAGG
D13M
53POL

        2338                                                                                                2429
AD169   CCGGGGCCATCGCGCGGCTGGCTAAAATTCCGTTGCGGCGTGTCATCTTTGACGGACAGCAGATCCGTATCTACACCTCGCTGCTGGACGAGTGC
D13M                            G                           T
53POL                           G                           T

        2433                                                                                                2524
AD169   GCCTGCCGCGATTTTATCCTGCCCAACCACTACAGCAAAGGTACGACGGTGCCCGAAACGAATAGCGTTGCTGTGTCACCTAACGCTGCTATCAT
D13M                                                                                C               C
53POL                                                                                               C

        2528                                                                                                2619
AD169   CTCTACCGCCGCTGTGCCCGGCGACGCGGGTTCTGTGGCGGCTATGTTTCAGATGTCGCCGCCCTTGCAATCTGCGCCGTCCAGTCAGGACGGCG
D13M                                                                                        G
53POL                                                                                       G       TC

        2623                                                                                                2714
AD169   TTTCACCCGGCTCCGGCAGTAACAGTAGTAGCAGCGTCGGCGTTTTCAGCGTCGGCTCCGGCAGTAGTGGCGGCGTCGGCGTTTCCAACGACAAT
D13M                                                                                                    G
53POL                                                                                                   G

        2718                                                                                                2809
AD169   CACGGCGCCGGCGGTACTGCGGCGGTTTCGTACCAGGGCGCCACGGTGTTTGAGCCCGAGGTGGGTTACTACAACGACCCCGTGGCCGTGTTCGA
D13M            T       GC                  A
53POL           T       GC                  A
```

```
        2813                                                                                                2904
AD169   CTTTGCCAGCCTCTACCCTTCCATCATCATGGCCCACAACCTCTGCTACTCCACCCTGCTGGTGCCGGGTGGCGAGTACCCTGTGGACCCCGCCG
D13M    T                                                                                   C         T
53POL   T                                                                                   C         T

        2908                                                                                                2999
AD169   ACGTATACAGCGTCACGCTAGAGAACGGCGTGACCCACCGCTTTGTGCGTGCTTCGGTGCGCGTCTCGGTGCTCTCGGACCTGCTCAACAAGTGG
D13M                                                                                T
53POL                                                                               T

        3003                                                                                                3094
AD169   GTTTCGCAGCGGCGTGCCGTGCGCGAATGCATGCGCGAGTGTCAAGACCCTGTGCGCCGTATGCTGCTCGACAAGGAACAGATGGCGCTCAAAGT
D13M
53POL

        3098                                                                                                3189
AD169   AACGTGCAACGCTTTCTACGGTTTTACCGGCGTGGTCAACGGTATGATGCCGTGTCTGCCCATCGCCGCCAGCATCACGCGCATCGGTCGCGACA
D13M          T
53POL         T                                   C

        3193                                                                                                3284
AD169   TGCTAGAGCGCACGGCGCGGTTCATCAAAGACAACTTTTCAGAGCCGTGTTTTTTGCACAATTTTTTTAATCAGGAAGACTATGTAGTGGGAACG
D13M                    A                     C
53POL

        3288                                                                                                3379
AD169   CGGGAGGGGGATTCGGAGGAGAGCAGCGCGTTACCGGAGGGGCTCGAAACATCGTCAGGGGGCTCGAACGAACGGCGGGTGGAGGCGCGGGTCAT
D13M                                    A                                       G       A
53POL                                                                           G       A

        3373                                                                                                3474
AD169   CTACGGGGACACGGACAGCGTGTTTGTCCGCTTTCGTGGCCTGACGCCGCAGGCTCTGGTGGCGCGTGGGCCCAGCCTGGCGCACTACGTGACGG
D13M
53POL                                                                                       T
```

```
        3478                        3503
AD169   CCTGTCTTTTTGTGGAGCCCGTCAAGCTG
D13M
53POL
```

Figure 12. Deduced amino acid sequence of the proposed dNTP and PPi binding site of the HCMV DNA polymerase protein. The right most number of the amino acid position is positioned above the corresponding amino acid. The homology to the HSV dNTP and PPi binding site is shown by a line above the AD169 sequence. Amino acid substitutions in the D13 and H51853 proteins are shown below the AD169 sequence. Amino acid substitutions responsible for drug resistance in HSV are shown above the AD169 sequence (49).

alanine	A	leucine	L
arginine	R	lysine	K
asparagine	D	methionine	M
aspartic acid	B	phenylalanine	F
cysteine	C	proline	P
glutamine	Q	serine	S
glutamic acid	E	threonine	T
glycine	G	tryptophan	W
histidine	H	tyrosine	Y
isoleucine	I	valine	V

```
HSV-1                                                  T                  545
AD169   SPNYKLNTMAELYLRQRKDDLSYKDIPRCFVANAEGRAQVGRYCLQDAVL
D13M
53POL

HSV-1                 K        V                                           595
AD169   VRDLFNTINFHYEAGAIARLAKIPLRRVIFDGQQIRIYTSLLDECACRDF
D13M
53POL

HSV-1                                                                      645
AD169   ILPNHYSKGTTVPETNSVAVSPNAAIISTAAVPGDAGSVAAMFQMSPPLQ
D13M
53POL

HSV-1                                                                      695
AD169   SAPSSQDGVSPGSGSNSSSSVGVFSVGSGSSGGVGVSNDNHGAGGTAAVS
D13M                                                    S      S
53POL        HH                                         S      S

HSV-1      G                          N                               745
AD169   YQGATVFEPEVGYYNDPVAVFDFASLYPSIIMAHNLCYSTLLVPGGEYPV
D13M
53POL

HSV-1                                                                      795
AD169   DPADVYSVTLENGVTHRFVRASVRVSVLSELLNKWVSQRRAVRECMRECQ
D13M
53POL

HSV-1    T                  M        M                                  S845
AD169   DPVRRMLLDKEQMALKVTCNAFYGFTGVVNGMMPCLPIAASITRIGRDML
D13M
53POL

HSV-1                                                                      895
AD169   ERTARFIKDNFSEPCFLHNFFNQEDYVVGTREGDSEESSALPEGLETSSG
D13M                                                    T
53POL

HSV-1                                                                      945
AD169   GSNERRVEARVIYGDTDSVFVRFRGLTPQALVARGPSLAHYVTACLFVEP
D13M
53POL

HSV-1
AD169   VKL
D13M
53POL
```

the proposed PPi binding site in HSV.

Isolation of a GCV-resistant clinical strain of HSV-2. An isolate of HSV-2 cultured from patient W.C. was obtained from the clinical microbiology laboratory at LUMC. HSV-2 strain H6472 was isolated from a patient who was being treated with GCV for AIDS-related HCMV retinitis (Dr. J. Paul O'Keefe, personal communication). Isolation of HSV-2 during GCV treatment suggested that strain H6472 might be resistant to GCV. Stock virus was prepared and aliquots were stored at -70°C. The antiviral susceptibility profile of the non-plaque purified virus was determined using the plaque reduction assay. Initial results using non-plaque purified virus revealed that strain H6472 was GCV- and ACV-resistant and PFA-sensitive as compared to reference strain HSV-1 KOS (Table 5). The virus was twice plaque purified and clone CD1/1/1 was further characterized.

Antiviral drug susceptibility testing of HSV-2 clone CD1/1/1. Antiviral drug susceptibility testing was performed on HSV-2 clone CD1/1/1 using the PRA. HSV-2 ATCC strain VR-734 was used as the control virus. CD1/1/1 was both GCV- and ACV-resistant and PFA-sensitive (Table 6), confirming the results obtained using non-plaque purified virus. The susceptibility profile of CD1/1/1 was also determined using an ELISA assay for HSV-2. Though the numerical values for the IC50s of GCV, ACV, and PFA were different than those obtained with the PRA, the susceptibility profile of CD1/1/1 remained

Table 5. Antiviral drug susceptibility profile of HSV-2 strain H6472.

	IC_{50} (μM)	
Drug	HSV-1 KOS	HSV-2 H6472
GCV	12.5	>500
ACV	6.2	>50
PFA	100	50

Table 6. Antiviral drug susceptibility profile of HSV-2 strain H6472 clone CD1/1/1.

	IC_{50} (μM)			
	PRA		ELISA	
Drug	ATCC 74-3	CD/1/1/1	ATCC 74-3	CD1/1/1
GCV	1.6	500	0.03	15.6
ACV	1.6	125	0.2	1000
PFA	100	50	125	125

the same. This susceptibility profile showing resistance to the nucleoside analogs and sensitivity to viral DNA polymerase inhibitors suggested that drug resistance was due to a mutation in the viral TK gene.

Site-directed in vitro mutagenesis of the HCMV AD169 DNA polymerase gene. Analysis of the nucleotide sequence of the viral DNA polymerase gene of HSV mutants resistant to DNA polymerase inhibitors has provided insight into the structure and function of the gene. Likewise, the effect of specific mutations on the expression of the cloned HSV polymerase gene has also been very useful. By introducing a specific mutation in the HCMV DNA polymerase gene followed by recombination of the mutated gene into the wild-type genome, we hoped to construct a recombinant drug-resistant strain of HCMV. A single base substitution was incorporated into the AD169 DNA polymerase gene using site directed mutagenesis. The AD169 EcoRI M fragment was excised from pEcoM and inserted into the plasmid vector pSelect-1. Single strand template DNA template was prepared. An 18-mer oligonucleotide 5'-GGCGCACAGTGTCTTGAC-3' containing a single guanine to thymine mismatch (underlined base) was annealed to the single strand template DNA and synthesis of the complementary strand was completed. Based on the published AD169 DNA polymerase nucleotide sequence (71), the resultant complementary sequence with the cytosine to adenine substitution at nucleotide 3048 (see Figure 11) should encode a mutant enzyme with a threonine

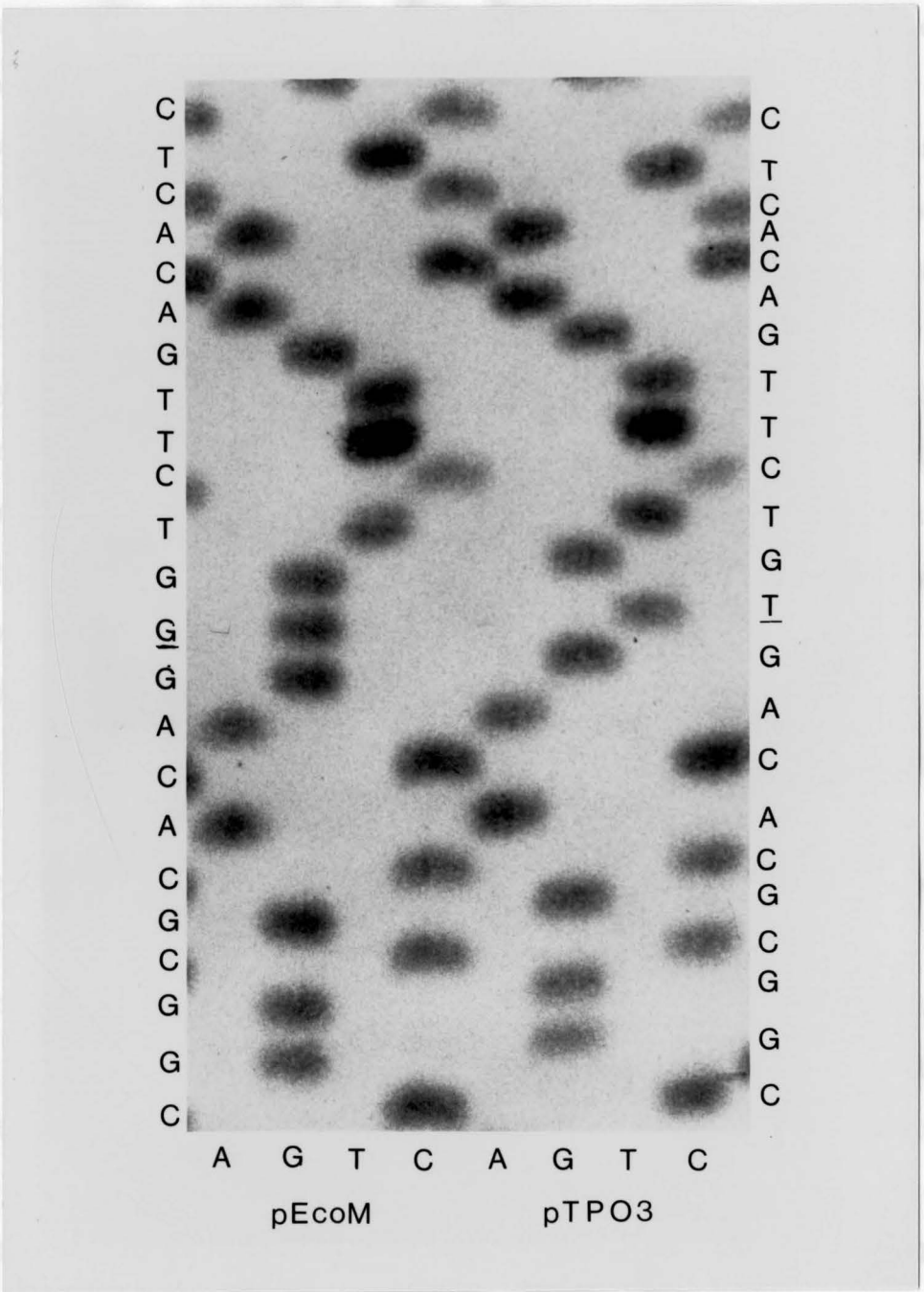
(T) substituted for proline (P) at amino acid 797 (see Figure 12). This mutation in the proposed dNTP and PPi binding site in the HSV DNA polymerase gene is thought to be responsible for ACV and PAA resistance in HSV (49). The mutagenesis reaction yielded several hundred clones. Sequence analysis of 10 clones revealed that approximately 25% of the clones had incorporated the mismatch. Plasmid pTPO3 was chosen for further study. Figure 13 shows the substitution of guanine for thymine at nucleotide number 3048 in the viral polymerase gene encoded on plasmid pTPO3.

Marker transfer between pTPO3 and AD169 DNA. Previous work has shown that recombination can take place between viral sequences on plasmids and wild-type HSV DNA when both DNAs are co-transfected into permissive cells (11). We attempted to perform similar experiments in hopes of constructing a drug-resistant HCMV.

Preliminary studies showed that calcium phosphate-mediated transfection of HFF with a plasmid encoding resistance to G418 yielded a very low percentage of G418-resistant HFF cells. However, Vero cells transfected in a similar manner yielded a much higher percentage of G418-resistant cells. Therefore, since transfection of HFF was much less efficient than that of Vero cells, we anticipated a very low yield of recombinant drug-resistant HCMV.

In several experiments, monolayers of HFF were co-transfected with EcoRI-linearized pTPO3 and HCMV strain AD169

Figure 13. Autoradiogram of the sequence of pTPO3 showing the G to T substitution (underlined bases) at nucleotide number 3048.


C T C A C A G T T C T G G G A C A C G G C G G C
C T C A C A G T T C T G T G A C A C G C G G G C
A G T C A G T C
pEcoM
pTPO3

genomic DNA. The monolayers were then incubated with medium alone, medium containing 12.5 μM GCV or 600 μM PFA and monitored for the appearance of plaques. All transfections were unsuccessful, even those selected for in the absence of any drug.

Recently, a second strategy was used for transfection of HFF. Slightly subconfluent monolayers of HFF were transfected with EcoRI-linearized pTPO3. After several days incubation in growth medium when the cells reached confluency, the monolayers were infected with HCMV strain AD169 at an MOI of 1. One day post-infection, the cells were harvested by trypsinization and used to infect fresh HFF monolayers at an MOI of approximately 0.1. The infected monolayers were incubated in maintenance medium plus 0.3% agarose with and without 12.5 μM GCV. Substantial plaque formation was observed in the presence and absence of GCV. Several plaques from the plate containing GCV were picked and pooled in 1.2 ml of maintenance medium. Six tubes of HFF were inoculated with 0.2 ml of virus suspension. Half were incubated in the presence of 12.5 μM GCV. To date, several plaques can be seen in all tubes. However, the progression of plaque formation in tubes with GCV has been much slower than in tubes without GCV.

Marker transfer between pTPO3 and HSV-1 DNA. pTPO3 contained a nucleotide substitution in the HCMV AD169 DNA polymerase gene at a region which shows significant homology

to the HSV DNA polymerase gene (71). Therefore, we attempted marker transfer experiments in Vero cells using EcoRI-linearized pTPO3 and wild-type HSV-1 KOS genomic DNA. Drug-resistant virus was selected in the presence of 500 μM PFA. Several plaques were observed and picked for further characterization.

Antiviral susceptibility testing using the PRA confirmed that clone PFAR5 was resistant to the DNA polymerase inhibitor PFA (Table 7). The virus was also cross-resistant to the nucleoside analog ACV. This susceptibility profile suggested that a mutation in the viral DNA polymerase was responsible for drug resistance. However, at this point, it was not known whether the mutation was due to recombination event or whether it was a spontaneous event.

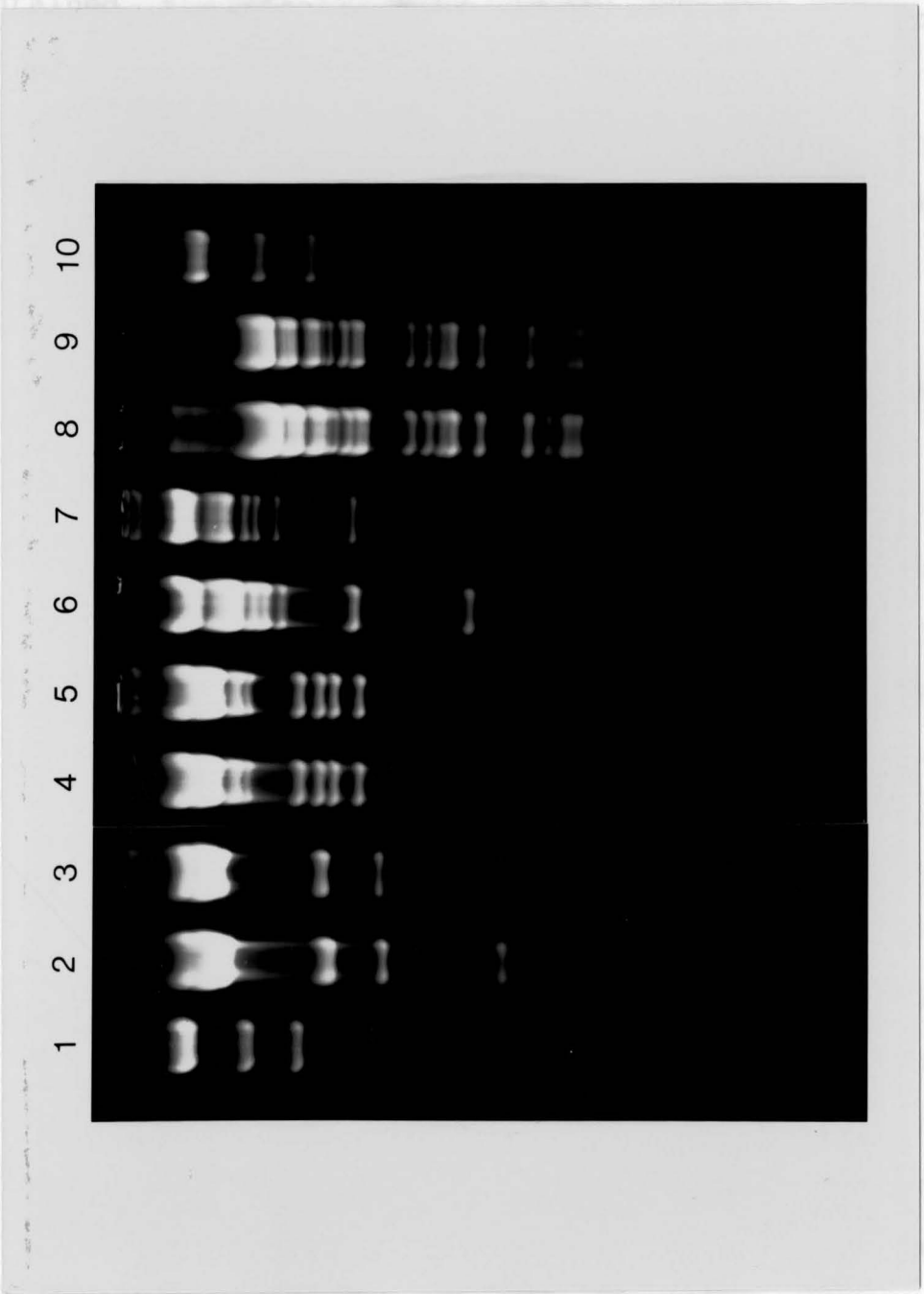
Cloning of the HSV-1 strain PFAR5 DNA polymerase gene. The DNA polymerase gene of HSV-1 strain PFAR5 was cloned for further analysis. Clone PFAR5 viral DNA was isolated and digested with EcoRI, BglII, HindIII, and BamHI. The restriction enzyme profile of strain PFAR5 was identical to wild-type KOS (Figure 14). The carboxyl terminal portion of the HSV-1 KOS DNA polymerase protein is encoded on the EcoRI M fragment (50,51). This area of the protein shares maximum homology with human DNA polymerase α-like enzymes and contains the proposed HSV dNTP and PPi binding sites (49,50). Therefore, the PFAR5 EcoRI fragment migrating at the same position was excised, purified, and cloned into the pUC18

Table 7. Antiviral susceptibility profile of HSV-1 strain PFAR5.

	IC_{50} (μM)	
Drug	PFAR5	KOS
PFA	1000	125
PAA	800	200
ACV	25	6.2
HPMPA	6.2	3.1

Figure 14. Agarose gel of restriction endonuclease digested HSV-1 KOS and PFAR5. Sizes of the fragments in lanes 1 and 10 are 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, and 0.6 kb.

DNA:	Lambda	Lanes 1 and 10
	KOS	Lanes 2, 4, 6, 8
	PFAR5	Lanes 3, 5, 7, 9
Enzymes:	HindIII	Lanes 1, 6, 7, 10
	EcoRI	Lanes 2 - 3
	BglII	Lanes 4 - 5
	BamHI	Lanes 8 - 9


1
2
3
4
5
6
7
8
9
10

vector. Restriction analysis of transformant plasmid DNA and pSG17, a pBR325 derived clone containing the HSV-1 KOS EcoRI M fragment, revealed that two clones, pPFAR5M1 and pPFAR5M11, contained the EcoRI M fragment inserted in opposite orientations (data not shown).

Sequence analysis of the PFAR5 DNA polymerase gene. In order to locate the mutation in the PFAR5 DNA polymerase gene that might be responsible for drug resistance, the cloned PFAR5 DNA polymerase gene was then sequenced. Analysis was performed specifically on the areas thought to be involved in dNTP binding and PPi binding (amino acids 535-696 and 696-924, respectively, according to the published sequence of the KOS DNA polymerase gene (50). Mutations responsible for drug resistance of nine known HSV mutants have been mapped to this area. The published nucleotide sequence of this region of the HSV-1 KOS DNA polymerase gene and the deduced sequence of PFAR5 are shown in Figure 15. Nucleotide changes were observed at positions 2276, 2532, and 2534. There is also a triple deletion at positions 2295-2297. The amino acid sequences of HSV-1 KOS and PFAR5 deduced from the nucleotide sequence are shown in Figure 16. PFAR5 has a relatively non-conservative threonine (T) to alanine (A) substitution at position 566, a substitution found in the ACV-resistant, PAA-resistant mutant PFA^{r}2 (48). However, PFA^{r}2 also contained a conservative change from alanine (A) to valine (V) at position 605, a change not seen in PFAR5. PFAR5 shows two adjacent

Figure 15. Nucleotide sequence of the proposed dNTP and PPi binding sites of the HSV-1 KOS and PFAR5 DNA polymerase genes. The right most number of the nucleotide position is positioned above the corresponding base. Base substitutions and deletions in the PFAR5 gene are shown below the KOS sequence.

```
        2186                                                                                                2278
KOS     CTCTCGAGCTACAAGCTCAACGCCGTGGCCGAAGCCGTCCTGAAGGACAAGAAGAAGGACCTGAGCTATCGCGACATCCCCGCTACTACGCCACC
PFAR5                                                                                                    G

        2282                                                                                                2373
KOS     GGGCCCGCGCAACGCGGGGTGATCGGCGAGTACTGCATACAGGATTCCCTGCTGGTGGGCCAGCTGTTTTTTAAGTTTTTGCCCCATCTGGAGCT
PFAR5                   ---

        2377                                                                                                2468
KOS     CTCGGCCGTCGCGCGCTTGGCGGGTATTAACATCACCCGCACCATCTACGACGGCCAGCAGATCCGCGTCTTTACGTGCCTGCTGCGCCTGGCCG
PFAR5

        2472                                                                                                2563
KOS     ACCAGAAGGGCTTTATTCTGCCGGACACCCAGGGGCGATTTAGGGGCGCCGGGGGGGAGGCGCCCAAGCGTCCGGCCGCAGCCCGGGAGGACGAG
PFAR5                                                                                  A G

        2567                                                                                                2658
KOS     GAGCGGCCAGAGGAGGAGGGGGAGGACGAGGACGAACGCGAGGAGGGCGGGGGCGAGCGGGAGCCGGAGGGCGCGCGGGAGACCGCCGGCCGGCA
PFAR5

        2662                                                                                                2753
KOS     CGTGGGGTACCAAGGGGCCAAGGTCCTTGACCCCACTTCCGGGTTTCACGTGAACCCCGTGGTGGTGTTCGACTTTGCCAGCCTGTACCCCAGCA
PFAR5

        2757                                                                                                2848
KOS     TCATCCAGGCCCACAACCTGTGCTTCAGCACGCTCTCCCTGAGGGCCGACGCAGTGGCGCACCTGGAGGCGGGCAAGGACTACCTGGAGATCGAG
PFAR5

        2852                                                                                                2943
KOS     GTGGGGGGGCGACGGCTGTTCTTCGTCAAGGCTCACGTGCGAGAGAGCCTCCTCAGCATCCTCCTGCGGGACTGGCTCGCCATGCGAAAGCAGAT
PFAR5

        2947                                                                                                3038
KOS     CCGCTCGCGGATTCCCCAGAGCAGCCCCGAGGAGGCCGTGCTCCTGGACAAGCAGCAGGCCGCCATCAAGGTCGTGTGTAACTCGGTGTACGGGT
PFAR5
```

```
        3042                                                                                                3133
KOS     TCACGGGAGTGCAGCACGGACTCCTGCCGTGCCTGCACGTTGCCGCGACGGTGACGACCATCGGCCGCGAGATGCTGCTCGCGACCCGCGAGTAC
PFAR5

        3137                                                                                                3228
KOS     GTCCACGCGCGCTGGGCGGCCTTCGAACAGCTCCTGGCCGATTTCCCGGAGGCGGCCGACATGCGCGCCCCCGGGCCCTATTCCATGCGCATCAT
PFAR5

        3232                                                                                                3323
KOS     CTACGGGGACACGGACTCCATATTTGTGCTGTGCCGCGGCCTCACGGCCGCCGGGCTGACGGCCATGGGCGACAAGATGGCGAGCCACATCTCGC
PFAR5

        3327                        3352
KOS     GCGCGCTGTTTCTGCCCCCCATCAAACTC
```

Figure 16. Deduced amino acid sequence of the proposed dNTP and PPi binding site of the HSV-1 KOS and PFAR5 DNA polymerase proteins. The right most number of the amino acid position is positioned above the corresponding amino acid. Amino acid substitutions and deletions in the PFAR5 protein are shown below the KOS sequence. Amino acid substitutions responsible for drug resistance in HSV are shown above the KOS sequence (49). Amino acid codes are listed in Figure 12.

```
                                                A                 584
KOS       LSSYKLNAVAEAVLKDKKKDLSYRDIPAYYATGPAQRGVIGEYCIQDSLL
PFAR5                                           A     -

                        K       V                           634
KOS       VGQLFFKFLPHLELSAVARLAGINITRTIYDGQQIRVFTCLLRLADQKGF
PFAR5

                                                            684
KOS       ILDPTQGRFRGAGGEAPKRPAAAREDEERPEEEGEDEDEREEGGGEREPE
PFAR5                     HE

                      G                               N     734
KOS       GARETAGRHVGYQGAKVLDPTSGFHVNPVVVFDFASLYPSIIQAHNLCFS
PFAR5

                                                          . 784
KOS       TLSLRADAVAHLEAGKDYLEIEVGGRRLFFVKAHVRESLLSILLRDWLAM
PFAR5

                        T                   M       M       834
KOS       RKQIRSRIPQSSPEEAVLLDKQQAAIKVVCNSVYGFTGVQHGLLPCLHVA
PFAR5

                  S                                         884
KOS       ATVTTIGREMLLATREYVHARWAAFEQLLADFPEAADMRAPGPYSMRIIY
PFAR5

                                                      924
KOS       GDTDSIFVLCRGLTAAGLTAMGDKMASHISRALFLPPIKL
PFAR5
```

non-conservative substitutions, a histidine (H) for a proline (P) at position 651 and a glutamic acid (E) for lysine (K) at position 652. PFAR5 also has a triple nucleotide deletion in frame resulting in the deletion of glycine (G) at position 572.

DISCUSSION

Development of the in situ ELISA for HCMV antiviral susceptibility testing. With the recent reports of the development of in situ ELISA assays for the determination of antiviral susceptibility of HSV (2,97), VZV (5) and respiratory syncytial virus (68), we modified this technique for use with HCMV. Data presented in this study demonstrated that the HCMV ELISA is rapid and reproducible, and showed good correlation with the standard plaque reduction assay.

The ELISA assay objectively measures the level of a late HCMV nuclear antigen synthesized during a productive infection. The amount of this gamma protein is dependent on viral DNA synthesis (106) and, therefore, is directly proportional to the amount of viral replication. In contrast, the PRA measures the ability of an infectious particle to produce additional infectious progeny, which, in turn, infect adjacent cells forming a viral plaque. Plaque formation is determined visually and therefore is a subjective measurement. However, in the PRA, GCV-sensitive strains of HCMV tend to form single enlarged cells in wells containing concentrations of GCV higher than the IC_{50}. The infection does not spread to adjacent cells. It is difficult to determine the endpoint of the PRA because the amount of viral replication within single cells cannot be determined. Therefore, the ELISA appears to

be the more accurate assay for the measurement of inhibition of viral replication by GCV.

The ELISA assay was more rapid than the PRA. IC_{50} values obtained in the PRA after 2 weeks incubation were obtained after only 1 week incubation in the ELISA. Also, if the MOI of the control and test virus was increased equivalently, the assay could be read in 3 to 4 days. Although the IC_{50} values of the control and test strains increased with the shortened assay, the relative relationship between sensitive and resistant strains remained the same.

The ELISA assay also detected resistance in cases where the PRA gave ambiguous results. GCV-resistant HCMV clones D13 and D16 were only 2-fold more resistant to the DNA polymerase inhibitors PFA and PAA as determined by the PRA (Table 4). Further testing using the ELISA showed that, in fact, both were clearly resistant to PFA and PAA (Table 4). Initial evaluation of the ELISA assay using the GCV-resistant kinase mutants D6/3/1 and D1/3/4 did not show this phenomenon since both clones were PFA- and PAA-sensitive (Table 2). No HCMV DNA polymerase mutants were available for testing during the initial studies.

The ELISA assay was also able to detect a very small subpopulation of drug-resistant virus. GCV resistance in clinical strain H51856 was first detected using the ELISA assay (Table 3). A percent survival assay performed to determine the percent of the population resistant to GCV

revealed that less than 1% of the population was GCV-resistant. If the PRA had been used to screen the clinical isolates for drug resistance, only 20 to 40 infectious particles would have been used to inoculate a well (MOI = 0.001). Therefore, out of 80 to 160 possible plaques (4 wells per drug dilution), only 0 - 1 drug-resistant plaques would have been detected in the PRA. Calculations done to determine the IC_{50} for GCV in the PRA would have ignored this small number of GCV-resistant plaques and resulted in an IC_{50} equivalent to wild-type HCMV strain AD169. Therefore, since the ELISA is inoculated using a 10-fold higher MOI (0.012), drug-resistant plaques may be detected more easily and more reliably than in the PRA. Also, progeny virus produced in the PRA do not spread farther than adjacent cells because of the semi-solid agarose overlay medium. In the ELISA, drug-resistant progeny virus may spread to other areas of the monolayer for further infection and replication since the overlay is a liquid medium. Since the MOI of the ELISA can also be increased to allow shorter assay times, the amount of the drug-resistant sub-population per well would be increased.

A definite drawback of the ELISA as well as the other assays described here is the requirement of extracellular virus for inoculation of cell monolayers. Numerous passages over a several month period are required for HCMV to become extracellular. Multiple passage may inadvertently cause selection of a sub-population of virus. Therefore, although

the ELISA assay may not be appropriate for the rapid screening of HCMV clinical isolates for drug resistance within the clinical microbiology laboratory setting, it would be a valuable assay for use in research and development. From the data presented in this study, the ELISA assay should supplement the PRA for the screening of the anti-HCMV activity of new antiviral agents.

Telenti and Smith (118) recently described a rapid method to screen for GCV-resistance in HCMV. Shell vials containing MRC-5 cells are infected with extracellular HCMV in the presence of 20 μM GCV and incubated for 4 days. The coverslips are stained by an indirect fluorescent antibody procedure using a mouse monoclonal antibody to an early nuclear HCMV protein. Although this procedure is rapid, it appears to require extracellular virus, the measurement is subjective and the results are not quantitative. Also, detection of an early nuclear antigen measures only immediate or early events in the virus life cycle. Therefore, the assay cannot distinguish between an abortive infection with limited viral gene expression and true viral DNA replication.

An enzyme immunocytochemical staining assay has been reported to determine HCMV infectivity (86). Although similar to the ELISA described here, monolayers in 24-well plates are stained with an alkaline phosphatase-conjugated antibody to a late viral antigen. The results are read visually using a microscope. Antiviral susceptibility testing using this

procedure would therefore be cumbersome and subjective.

Four other assays recently developed for antiviral susceptibility testing of herpesviruses also measure DNA synthesis. Gadler et al. (47) employed a conventional DNA hybridization procedure using ^{32}P-labeled probes which measured the effects of antiviral compounds on HCMV replication. HCMV-infected cell monolayers in 24- or 96-well plates were incubated in the presence or absence of antiviral drugs. The cells were then harvested and placed on nitrocellulose filters. Following cell lysis and DNA denaturation, the filters were hybridized with HCMV-specific probes. Hybridization was detected by autoradiography. A similar assay was described for VZV (114). However, drawbacks of procedures which use ^{32}P-labeled probes include the short half-life of the label and the many tedious manipulations involved. Swierkosz et al. (117) modified this procedure for use with ^{125}I-labeled HSV probes and 96-well plates. The advantages of ^{125}I-labeled probes are a longer half-life and that hybridization can be measured objectively and more quickly using a gamma counter rather than by autoradiography. A HCMV hybridization assay for susceptibility testing also used ^{125}I-labeled probes (26). HFF in 24-well plates were infected with HCMV at a high MOI and incubated for 3-5 days until 3-4+ CPE was reached. The cells were lysed, DNA wicked onto membranes, and hybridized with a ^{125}I-labeled HCMV DNA probe. Although all three procedures show good correlation to the PRA and are

more rapid, all involve the use of radioactivity.

Characterization of GCV-resistant clinical isolate HCMV strain H51856. GCV susceptibility testing of 9 clinical isolates of HCMV revealed that strain H51856 was GCV-resistant. This virus was isolated from the patient prior to any antiviral therapy. Parris and Harrington (94) have shown that HSV variants resistant to high concentrations of acyclovir exist in clinical isolates prior to antiviral therapy and speculated that these ACV-resistant strains may be selected for during antiviral therapy. No similar studies have been conducted to date to determine the prevalence of GCV-resistant sub-populations in HCMV clinical isolates. Plaque dilution assays done here demonstrated that, in fact, H51856 contained a sub-population of GCV-resistant virus which comprised <1% of the total population. Erice et al. (39) described a GCV-resistant HCMV isolated prior to GCV treatment. However, plaque dilution assays were not performed to determine if the isolate was actually a mixture of GCV-resistant and GCV-sensitive virus. In this study, no drug-resistant virus was isolated from the patient during or post-treatment. Evidently, the small GCV-resistant subpopulation may not have been selected during therapy and may have been cleared naturally by the patient's immune system.

Because of the life-threatening nature of the infection and because of promising in vitro susceptibility data, no controlled studies have been performed to determine the

efficacy of GCV treatment during HCMV disease in transplant patients. In fact, in certain clinical cases, HCMV infection has resolved without the administration of GCV. In this study, the patient who harbored strain H51856 which contained a GCV-resistant subpopulation remained culture-negative following GCV therapy. Thus, importance of the GCV-resistant subpopulation during therapy is questionable.

Reports of ACV-resistant HSV isolated from patients who received long term ACV therapy suggest that antiviral therapy may select for the drug-resistant virus (6,23,40,75,102). Two reports have described the isolation of clinical strains of GCV-resistant HCMV from patients receiving GCV therapy although the biochemical and genetic basis of GCV resistance in these strains was not been determined (33,39). However, Erice et al. (39) also described a GCV-resistant clinical strain of HCMV (IC_{50} = 14.4 μM) isolated from a patient prior to GCV therapy. After 79 days of treatment, the HCMV isolated from the patient had become significantly more resistant (IC_{50} = 30.5 μM). Restriction endonuclease patterns revealed no discernable differences between the two strains.

Restriction endonuclease analysis of GCV-resistant D13 and GCV-sensitive H51853 showed only very minor differences between the two strains (Figure 9). Since HCMV strains H51856 and H51853 were isolated from the same patient from different sites on the same day, their strikingly similar restriction patterns suggests that the GCV-resistant strain may have

arisen from mutation of the GCV-sensitive strain. Several studies have used restriction endonuclease patterns to study the epidemiology of HCMV in AIDS patients (15,22,34,111,122). However, use of restriction endonuclease patterns for epidemiological purposes suffers from subjective interpretation. How many differences are necessary to call two strains unique? Huang et al. (64) demonstrated that HCMV isolates share 80-90% homology in their DNA content which would allow for considerable differences in sequence homology and restriction pattern. Also, it has not been determined if strain variation plays a role in drug resistance. Work is in progress to isolate and analyze the GCV-sensitive subpopulation of H51856. It will be interesting to see if the restriction pattern of this virus is more similar to clone D13 than to strain H51853.

GCV anabolism studies were performed to determine if the GCV-resistant clones D13 and D16 were able to phosphorylate GCV. Results showed that both clones were able to phosphorylate GCV at wild-type levels (Figures 6 and 7). These results in combination with the antiviral susceptibility patterns (Table 4) suggested that a mutation in the viral DNA polymerase gene was responsible for GCV resistance. This was very surprising since the majority of clinically isolated ACV-resistant HSV strains are TK mutants (12) and not polymerase mutants. TK mutants may be more common since mutations in the viral TK gene are non-lethal (24). In contrast, mutations in

the viral DNA polymerase gene which result in a pol$^-$ phenotype are lethal.

Sequence analysis of the GCV-resistant and GCV-sensitive HCMV DNA polymerase genes was performed on areas which show significant amino acid homology to the proposed dNTP and PPi binding sites of the HSV-1 DNA polymerase gene thought to be important in drug susceptibility (Figure 11). When compared to the wild-type HCMV strain AD169 sequence, only 4 of the 21 nucleotide changes in the GCV-sensitive H51853 DNA polymerase gene resulted in altered amino acids (Figure 12). Two of these changes were shared by the GCV-resistant D13 DNA polymerase gene (positions 685 and 691). The other two would result in adjacent histidine substitutions at positions 651 - 652. The amino acids at HCMV positions 651 - 652 are not conserved in the HSV-1 or EBV sequences (69) and may not be critical for enzyme function and drug susceptibility. Three of the 21 nucleotide changes in the GCV-resistant D13 DNA polymerase gene would result in altered amino acids, the two shared by H51853 (positions 685 and 691) and a unique alanine (A) to threonine (T) substitution at HCMV amino acid position 884. Since the A to T substitution at position 884 is the only unique substitution seen in the GCV-resistant polymerase gene, this amino acid change may be responsible for drug resistance in HCMV clone D13. Amino acid mutations resulting in a greatly changed secondary structure may produce a non-functional protein and hence be a lethal mutation. Therefore,

since the GCV-resistant enyzme is functional, the threonine substitution does not change or only slightly changes the secondary structure of the protein.

This non-conservative change occurs in a 24 amino acid region (amino acids 880 - 904) of the HCMV polymerase protein not homologous to any sequences in the HSV-1 and EBV DNA polymerase proteins (71). These additional amino acids divide the region of the HCMV protein homologous to the HSV-1 dNTP and PPi binding sites (50,71). Gaps such as these are identified by alignment of the HSV, HCMV, and EBV sequences to account for the different lengths of the three polypeptides and to generate maximum alignment of homologous amino acids. Two other additional amino acid regions also exist, both which also divides the region homologous to the dNTP and PPi binding sites. The actual dNTP and PPi binding sites in the HCMV enzyme may therefore be larger than in the HSV enzyme. Also, these non-homologous regions may be important for the structural integrity or protein folding of the HCMV enzyme. Analysis of mutants with small deletions in these areas or isolation of drug-resistant virus with mutations in these areas would provide valuable information.

Several experiments should be performed to confirm that the A to T substitution at position 884 is responsible for drug resistance. Sequence analysis of the remaining areas of the D13 and H51853 polymerase genes should be completed to determine if any other amino acid changes exist that are

unique to the GCV-resistant enzyme. Marker rescue experiments can be carried out to construct recombinant HCMV. Monolayers of HFF should be co-transfected with EcoRI-linearized pD13M DNA and wild-type HCMV strain AD169 genomic DNA and incubated in the presence of GCV or PFA. Sequence analysis of drug-resistant progeny would reveal if recombination actually occurred and if the A to T substitution was present. If the mutation is absent, then some other amino acid change in the D13 enzyme may have been transferred or a spontaneous mutation may have occurred. Also, site-directed mutagenesis should be performed on plasmid pEcoM introducing the A to T substitution at amino acid position 884. This plasmid construct can then be used in same type of marker rescue experiments described above. Since the A to T substitution would be the only amino acid change in the mutagenized wild-type sequence, progeny which are drug-resistant should contain this specific change. Conversely, similar experiments constructing revertants back to the original sequence encoding the alanine at position 884 should be drug-resistant.

Once the GCV-sensitive subpopulation of H51856 is purified, sequence analysis of the DNA polymerase gene should be performed. One would expect that the nucleotide sequence should be nearly identical to the GCV-resistant clone D13 gene since they are subpopulations of the same isolate. Comparison of the these two sequences as well as the H51853 DNA polymerase sequence may provide more evidence as to which

amino acid is responsible for drug susceptibility.

Biochemical analysis of the D13 enzyme should also be undertaken. In vitro assays have been described to measure the function of crudely purified HCMV DNA polymerase in the presence and absence of specific DNA polymerase inhibitors (8,46,79). Preliminary studies would assess the activity of the wild-type AD169 and D13 enzymes in the presence of PFA or PAA. One would expect the AD169 enzyme to be inhibited in the presence of PFA while the D13 enzyme should exhibit wild-type levels of activity in the presence of the inhibitor. Once expression vectors systems are developed for the HCMV DNA polymerase, the AD169 and D13 polymerase genes could then be cloned into plasmid expression vectors. Site directed mutagenesis would be performed on both plasmids yielding a wild-type AD169 protein with an A to T substitution at position 884 and D13 protein with T to A substitution at position 884. The in vitro expressed enzymes from all four constructs would then be assayed for activity in the presence and absence of DNA polymerase inhibitors. If the A to T substitution is responsible for drug resistance, then the enzyme expressed by the mutagenized AD169 construct containing the A to T substitution should be resistant to PFA. Conversely, the mutagenized D13 construct enzyme containing the corrected amino acid should be inhibited by PFA. Similar experiments have been done analyzing the role of specific amino acid residues in the HSV-1 TK gene (65) and the HSV-1

DNA polymerase gene (32,80,82).

D'Aquila and Summers (28) have reported the isolation and characterization of laboratory-derived PAA-resistant mutants of HCMV. Since these mutants had PAA-resistant DNA polymerase activity in vitro, these workers hypothesized that mutations in the DNA polymerase gene may be responsible for drug resistance. These mutants were isolated by incubating HCMV strain AD169 in increasing concentrations of PAA thereby increasing the possibility that several mutations may have been introduced (61). However, since sequence analysis was not performed on the PAA-resistant viral polymerase genes, the site of the mutation(s) is unknown.

Characterization of a GCV-resistant clinical isolate of HSV-2. A clinical strain of HSV-2 was isolated from a patient receiving GCV for AIDS-related HCMV retinitis. PFA treatment was eventually initiated and the HSV-2 and HCMV infections resolved. Other studies have reported isolation of ACV-resistant HSV following ACV therapy which resolved following administration of PFA (13). Antiviral susceptibility testing of the initial isolate (Table 4) and a plaque purified clone (Table 5) revealed that the virus was resistant to GCV and ACV but not cross-resistant to PFA. This susceptibility profile suggested that GCV and ACV resistance was due to a mutation in the viral TK gene. Since the majority of drug resistant HSV strains are TK mutants (24), and these mutations have been characterized by sequence analysis (12,65,70), we chose not

to characterize this isolate any further.

Mutagenesis and marker transfer of the HCMV DNA polymerase gene. A guanine to thymine substitution was introduced in the HCMV DNA polymerase gene at nucleotide 3048 by site directed mutagenesis (Figure 13) which would result in a proline (P) to threonine (T) substitution in the viral enzyme. Sequence analysis of PAA-resistant, ACV-resistant HSV-1 strain PAA^{r}C polymerase gene revealed a proline to threonine substitution at the homologous HSV position (49). Since the proline residue at this position is conserved between HSV-1, HSV-2, HCMV, and EBV as well as in human DNA polymerase α and yeast DNA polymerase I (49,121), it may serve a critical function in enzyme activity and antiviral drug susceptibility. Marker rescue experiments using the mutagenized plasmid pTPO3 were performed in order to construct recombinant drug-resistant HCMV virus. Numerous attempts were unsuccessful. This was not surprising for two reasons. First, preliminary experiments showed that the efficiency of HFF transfection in our laboratory is very low. In general, the efficiency of transfection of primary cells is 10 to 100-fold less than of cell lines (45). Secondly, the large size of the HCMV genome makes it difficult to isolate intact viral DNA. The isolation procedure may compromise the integrity of the viral chromosome thereby rendering the DNA non-infectious.

Recently, the transfection procedure was modified and several GCV-resistant plaques have been isolated. Monolayers

of fibroblasts were transfected with plasmid DNA followed by infection with wild-type virus at an MOI of 1. This modification avoided the preparation and use of infectious viral DNA. The infected cells were then used to infect fresh monolayers of HFF. Plaques were selected in the presence of GCV. Once plaque purified, the antiviral susceptibility pattern of the clones will be determined. If recombination was successful, progeny virus should be GCV- and PFA-resistant. Sequence analysis will then reveal if the progeny are wild-type derived recombinant virus encoding the guanine to thymine substitution.

Marker transfer experiments were also performed using the HCMV derived plasmid pTPO3 and wild-type HSV KOS DNA since significant homology exists between the two viral DNA polymerase genes. One PFA-resistant plaque was observed and purified and clone PFAR5 was further characterized. The antiviral susceptibility profile revealed that clone PFAR5 was resistant to PFA, PAA, and ACV, and marginally resistant to HPMPA (Table 6). This phenotype suggested that a mutation in the viral pol gene was responsible for drug resistance. Sequence analysis of the dNTP and PPi binding sites suggested that PFAR5 was a spontaneous mutant since the nucleotide sequence (Figure 15) and theoretical amino acid sequence (Figure 16) were nearly identical to the parental HSV-1 KOS sequence. Several amino acid changes may be responsible for drug resistant phenotype. PFAR5 has a triplet deletion of

glycine at amino acid position 572. Since this deletion would not result in a frameshift, one would expect a nearly wild-type length protein. However, the 3-dimensional configuration of the folded protein may be altered and prevent recognition and binding of the antiviral drug and substrate. A threonine (T) to alanine (A) substitution was seen at position 566. The T residue at this position in not conserved in HSV-2 (121), but surprisingly, an A residue is found in HSV-1 strain 17 (96), HCMV AD169 (71), and EBV (71). This variability suggests that the T or A residues at this position are not responsible for drug resistance. A proline (P) to histidine (H) and a lysine (K) to glutamic acid (E) substitution were seen positions 651 and 652, respectively. Amino acids at these positions are conserved in HSV-2 (96) and HSV-1 strain 17 (96) but not in HCMV or EBV (71). These substitutions alone or combined may be responsible for drug resistance. Marker rescue experiments similar to those described for GCV-resistant HCMV clone D13 using the cloned PFAR5 DNA polymerase gene should be performed. Similar additional experiments using a mutagenized wild-type KOS gene containing the specific nucleotide substitutions seen in PFAR5 would yield valuable information.

Future design of new anti-herpesvirus agents. This study described a molecular analysis of the first reported clinically isolated drug-resistant DNA polymerase mutant of HCMV. A DNA polymerase mutant of HSV-1 was also described.

Further characterization of wild-type and mutant herpesvirus DNA polymerase genes should benefit in the design of more effective anti-herpesvirus agents. Characterization of more drug-resistant mutants and expression of mutated cloned enzymes will more clearly define the areas of the enzyme involved in drug interaction. Interactions of the DNA polymerase and template DNA with cellular proteins can also be studied in more detail. X-ray crystallography of the purified enzymes will shed light on the structure of the enzymes. Since the sequences of the enzymes have been determined, computer models of their structures could be correlated to the X-ray crystallography results. With the aid of computer modeling, it may now possible to design specific inhibitors targeted to unique sites of purified viruses or their associated enzymes (59).

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The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the Committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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