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Genetic Analysis of the Susceptibility of Mouse Cytomegalovirus to Acyclovir

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Eight independently derived mouse cytomegalovirus (MCMV) mutants resistant to acyclovir (ACV) were obtained by the sequential plating of wild-type virus in increasing concentrations of ACV. Results of complementation studies among these eight mutants suggest that all had mutations within the same or closely associated genes. A ninth MCMV mutant resistant to phosphonoacetate (PAA) derived by plating wild-type virus in the presence of 100 μg of PAA per ml displayed coresistance to ACV and was unable to complement any of the ACV-derived mutants. Recombination experiments among all combinations of the nine MCMV mutants were performed and supported the complementation data in that no recombination could be detected. Seven of the eight ACV-resistant mutants demonstrated cross-resistance to PAA and hypersensitivity to aphidicolin. The one mutant not coresistant to PAA was more susceptible to PAA than was the parent virus. Only a few mutants demonstrated coresistance when the mutants were tested against 9-β-D-arabinofuranosyladenine (ara-A). The ACV mutant that demonstrated increased susceptibility to PAA was 30-fold more susceptible to ara-A but remained unchanged in susceptibility to aphidicolin. Two of the parent-mutant combinations were selected for DNA synthesis analysis in the presence of ACV (5 μM). A significant decrease in DNA synthesis was demonstrated for both parent viruses, and there was little effect on mutant virus DNA synthesis at the same drug concentration. These results suggest that susceptibility of MCMV to ACV is confined to a product of a single gene and that a mutation of this gene can lead to an altered phenotype when compared with parent virus in susceptibility of DNA synthesis to PAA, ara-A, and aphidicolin, drugs that are known to inhibit DNA polymerase activity.

The nucleoside analog acyclovir {9-[(2-hydroxyethoxy)-methyl]guanine; ACV} was first shown to be an effective inhibitor of herpes simplex virus (HSV) type 1 in vitro in 1977 (11). That initial study by Elion et al. demonstrated that inhibition of normal cell growth required a 3000-fold greater concentration of ACV than was needed to inhibit HSV replication. For ACV to exert its anti-HSV effect, it must be converted to its triphosphate form (acyclo-GTP) (27). HSV DNA polymerase activity is 30-fold more sensitive to acyclo-GTP than is cellular DNA polymerase activity (11, 13). It appears that a viral-coded enzyme, thymidine kinase (TK), phosphorylates ACV to its monophosphate form much more efficiently than does cellular TK (11, 14). Cellular enzymes then convert the monophosphate to its triphosphate form (18).

By using ACV-resistant HSV mutants in genetic studies, Coen and Schaffer confirmed the importance of the HSV-coded TK and DNA polymerase for the anti-HSV activity of ACV (6). Complementation and recombination studies with three phenotypically different ACV-resistant mutants proved resistance to ACV could result from mutations in two separate genes (6).

Because the viral TK appeared to be necessary for the phosphorylation of ACV leading to accumulation of acyclo-GTP in infected cells and expression of its anti-HSV effect, it was not surprising to find that human cytomegalovirus, a herpesvirus that does not encode for a TK, is much less sensitive to ACV (7). In contrast, the finding that mouse cytomegalovirus (MCMV), another herpesvirus lacking a TK (10, 21), is almost as sensitive to ACV in vitro and in vivo as is HSV was unexpected (2, 28). Even when grown in TK-negative host cells, the replication of MCMV is sensitive to ACV, indicating a mechanism not dependent on cellular or viral TK for converting ACV to its active form (2). Because drug-resistant mutants have proved useful in elucidating the mechanism of action of ACV in the HSV system (6), mutants of MCMV resistant to ACV have been isolated and used to study the mechanism of action of ACV in the MCMV system. The data presented are consistent with the hypothesis that a single gene (or closely linked genes) confers susceptibility of MCMV to ACV and a mutation in that gene can result in an altered phenotype in susceptibility to phosphonoacetate (PAA), 9-β-D-arabinofuranosyladenine (ara-A), and aphidicolin (Aph), along with increased resistance to ACV at the level of DNA replication.

MATERIALS AND METHODS

Tissue cultures. All tissue cultures were performed in mouse embryo fibroblast (MEF) monolayers which were prepared from 18- to 20-day-old ICR embryos followed by a 30-min trypsinization (1× trypsin-EDTA; Flow Laboratories, Inc.) at 37°C. Cells were seeded into 75-cm^2 (CoStar) flasks in minimal essential medium (MEM) containing 10% newborn calf serum (NCS) and gentamicin (35 μg/ml) and grown to confluency.

Virus. The Smith strain of MCMV (ATCC VR-194) was obtained from the American Type Culture Collection. Stock virus was prepared by inoculating confluent MEF in 75-cm^2 flasks with wild-type virus at a multiplicity of infection (MOI) of 0.05 to 0.1. When nearly all the cells showed a cytopathic effect (CPE), the virus stock was harvested by freezing at −70°C and thawing at 37°C in a water bath, and clarification was performed by centrifugation at 600 × *g* for

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5 min at 4°C before portions were taken and stored at −70°C. Titers ranged from 10^6 to 10^7 PFU/ml.

Individual clones (parents) of the original wild-type stock were prepared by a double plaque purification procedure. Original stock virus was inoculated onto MEF in tissue culture trays with 24 wells (TC 24; CoStar) to give 0.1 to 1.0 PFU per well. After 1 h of adsorption at 37°C, the wells were washed twice with MEM and overlayed with 0.9% methylcellulose containing MEM with 15 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer–5% NCS–35 μg of gentamicin per ml. After incubation for 1 week at 37°C in 5% CO_2 the plates were examined with an inverted microscope. Virus was harvested from wells containing a single plaque with a Pasteur pipette and directly inoculated into a 25-cm^2 flask of MEF. These individual clones were incubated until a 100% CPE was seen, at which time the clones were frozen and thawed, and the plaque purification procedure was repeated. The resulting double plaque-purified clones (parents) were inoculated onto 75-cm^2 flasks of MEF and incubated until a 100% CPE was seen. The cells were then frozen and thawed, and portions were taken and stored at −70°C.

Drugs. ACV (Burroughs Wellcome Co.) was dissolved in sterile water at a concentration of 10^{-2} M and passed through a 0.2-μm filter, and portions were taken and stored at −70°C. PAA (Abbott Laboratories) was dissolved in sterile water to give a 10-mg/ml stock solution which was filtered, and portions were taken and stored at −70°C. ara-A (Sigma Chemical Co.) was dissolved in water at a concentration of 10^{-3}M, filter sterilized, and stored at −70°C. Aph (Sigma) was dissolved at a concentration of 1 mg/ml in dimethyl sulfoxide and diluted into media for plaque reduction analysis.

Measurement of viral replication. Most analyses of viral replication were assessed by a one-step growth cycle. Duplicate TC 24 wells were inoculated at an MOI of 3 with 0.1 ml of virus. After a adsorption for 1 h, the wells were washed twice, and 1 ml of MEM with 2% NCS was added (with or without antiviral compound). After 45 h at 37°C in 5% CO_2, the plates were stored at −70°C until titrated. Plates stored at −70°C after the one-step growth cycle were thawed at 37°C in a water bath. The duplicate wells were then pooled and clarified by centrifugation at 600 × *g* for 5 min at 4°C. Portions of 0.1 ml of 10-fold dilutions of the virus supernatants were inoculated onto TC 24 wells of MEF in quadruplicate. After a adsorption for 1 h, the supernatant was aspirated, the wells were overlayed with 0.9% methylcellulose containing MEM with 15 mM HEPES buffer–5% NCS–35 μg of gentamicin per ml, and the plates were incubated at 37°C in 5% CO_2 for 5 days. The plates were then inverted to remove the overlay, and the monolayer was fixed in absolute ethanol for 5 min and stained with 0.5% methylene blue for 5 min. After staining, the monolayers were rinsed with water and air dried, and the plaques were counted, resulting in a virus titer expressed in PFU per milliliter.

Sensitivity to Aph was determined by plaque reduction analysis. Confluent MEF cells in TC 24 wells were infected in quadruplicate with 50 PFU per 0.1 ml of MCMV parent or mutant virus. After adsorption for 1 h, the supernatant was aspirated, and the cells were overlayed with the methylcellulose solution described above that contained various concentrations of Aph. The plates were incubated for 5 days at 37°C in 5% CO_2 and were fixed and stained as described above. The percentage of survivors was calculated by taking the number of plaques with drug per the number of plaques without drug times 100.

Drug-resistant mutant isolation. To select ACV-resistant mutants, parent stocks were propagated in 25-cm^2 flasks of MEF (MOI, 0.1 to 1.0) in a step-wise manner in graduated amounts of ACV. Virus was first grown in the presence of 1 μM ACV with progeny virus successively grown in 5, 10, and 25 μM ACV. Virus mutants able to replicate in 25 μM ACV were then double plaque-purified, and portions were taken and frozen at −70°C.

Isolation of the temperature-sensitive mutant. The temperature sensitive mutant was obtained by mutagenizing the stock virus with UV light exposure for 1 min at a distance of 20 cm from a 30-W germicidal UV light (26). This treatment diminished viral infectivity by 99.9%. The UV-treated virus stock was then diluted, inoculated in 96-well microtiter plates, and incubated at 34°C in 5% CO_2 to give a positive CPE in approximately 30% of the wells. Cells from wells showing a CPE were scraped with a Pasteur pipette and, along with the medium, were adsorbed on replicate 96-well plates containing MEF monolayers, with one used for growth at 34°C and the other used for growth at 40°C. Virus in wells demonstrating a CPE at 34°C and no CPE in corresponding 40°C wells were picked and grown in small stock in 25-cm^2 flasks at 34°C. After a 100% CPE at 34°C, titers of progeny virus were determined at 34 and 40°C. Virus showing at least a 100-fold lower titer at 40°C were double plaque-purified and retested for temperature sensitivity.

Complementation. Duplicate wells of MEF TC 24 cultures were coinfected with 0.1 ml of two independently derived drug-resistant mutants at as high an MOI as could consistently be achieved (MOI, 3) for each mutant to ensure that most cells were coinfected. With each experiment, titers of input virus were determined to be sure of the MOI. After adsorption for 1 h at 37°C, the wells were washed twice, and MEM containing 5 μM ACV was added for the remaining 45 h of the one-step growth cycle, at which time the plates were frozen at −70°C until titration. Titers of progeny virus then were determined in the absence of ACV. As a measure of complementation, the fold reduction method of Coen and Schaffer (6) was used. Fold reduction was equal to the PFU per milliliter of single mutant infections in ACV divided by the PFU per milliliter of mixed infections in ACV. The numerator in this equation when two ACV mutants were used was the PFU per milliliter of the less resistant mutant. When a mutant and parent were used, the numerator was the PFU per milliliter of the mutant. Mixed infections in the absence of ACV were also performed to control for viral interference. As per Coen and Schaffer, reproducible twofold or more reductions were considered positive for complementation.

Recombination. Recombination studies were performed as described for complementation, except that ACV was not present during the one-step growth cycle but was added in the overlay during virus titration. Based on the work of Coen and Schaffer with HSV (6), some, if not all, of the MCMV-resistant mutants should be only partially resistant to ACV, producing smaller plaques with an increasing concentration of drug. An indication of recombination (double mutated virus) should be an increased plaque size from the progeny of a mixed infection as compared with the plaque size of individual mutants at an increased ACV concentration (50 μM). The recombination frequencies (RF) were calculated as follows: RF = (PFU per milliliter in 50 μM ACV)/(PFU per milliliter in the absence of ACV) × 2 × 100. The factor of two is needed to compensate for an equal number of reciprocal recombinants (which would be wild-type virus and would not be detected). As a positive control for

recombination, temperature-sensitive mutants were used. A one-step growth cycle at 34°C was performed with an ACV-resistant mutant alone and a mixed infection of an ACV-resistant mutant and a temperature-sensitive mutant. Titers of progeny virus then were determined at 34 and 40°C with or without 5 μM ACV. Any significant decrease in titer seen in the mixed infection at 40°C in the presence of 5 μM ACV compared with ACV-resistant mutant alone with titers determined at 40°C in 5 μM ACV should be due to a recombination between the ACV and the temperature-sensitive mutants.

Measurement of DNA synthesis. Cellular and viral DNA synthesis was measured by a modification of the method of Overby et al. (23). Confluent MEF cells in tissue culture trays with six wells (40-mm diameter wells; Costar) were irradiated with 3000 rads (cesium source; Gammacell 40; Atomic Energy of Canada Limited). The cells were either mock infected or infected in triplicate 24 h later with 0.2 ml of MCMV at an MOI of 3. After adsorption for 1 h, the cells were washed twice with MEM–2% NCS before the addition of 3 ml of medium with or without antiviral compounds. At various intervals after infection, the cells were pulse-labeled with [^{3}H]thymidine (10 μCi/ml; New England Nuclear Corp.) for 30 min. After the labeling period, the cells were washed three times with cold phosphate-buffered saline. After the third wash, 3 ml of phosphate-buffered saline added to each well, and the monolayers were disrupted with a rubber policeman. The phosphate-buffered saline-cell mixture was then transferred into a 15-ml centrifuge tube, and trichloroacetic acid was added to a final concentration of 5%. The mixture was frozen, thawed once, and passed through a membrane filter (pore size, 0.45 μm). The filter was then washed in three separate baths of 5% trichloroacetic acid, air dried, and counted in a liquid scintillation counter.

DNA isolation. Confluent MEF cells in 25-cm^2 flasks were infected at an MOI of 3 with either parent or mutant virus. At 45 h postinfection, the DNA was extracted from the cells essentially by the method of Mounts et al. (20). After removal of the growth medium, the cells were lysed by the addition of 0.5 ml of 0.6% sodium dodecyl sulfate in 10 mM EDTA (pH 7.5)–0.15 NaCl. After 10 min at room temperature, proteinase K (Sigma Chemical Co.) was added at a concentration of 100 μg/ml. After overnight incubation at 37°C, the DNA was deproteinized by sequential extractions with phenol and chloroform-isoamyl alcohol (24:1). The samples were then extensively dialyzed against 0.15 M NaCl–10 mM Tris-hydrochloride (pH 7.4)–1 mM EDTA for 48 h at 4°C. The DNA was then precipitated overnight at −20°C by increasing the salt concentration to 0.3 M NaCl and by adding an equal volume of isopropanol. The DNA was then pelleted by a 15-min spin in an Eppendorf centrifuge. The supernatant was removed and the pellet was air dried and suspended in 200 μl of 10 mM Tris-hydrochloride–1 mM EDTA (pH 7.5).

Purification of a MCMV DNA probe. Flasks of MCMV-infected MEF cells showing 100% CPE were frozen at −70°C. After thawing in a 37°C water bath, the suspensions were centrifuged at 600 × *g* for 15 min. The supernatant was then concentrated to 5 ml with immersible CX-30 ultrafilters (Millipore Corp.). To remove detectable traces of host DNA, the concentrated supernatant was treated with pancreatic DNase (50 μg/ml for 15 min at 37°C; Sigma). The purified virions were pelleted by centrifugation at 20,000 rpm with a SS34 rotor in an RC-5 superspeed centrifuge (Sorvall, Du Pont). The virions were then suspended in 1 ml of a solution containing 0.6% sodium dodecyl sulfate, 10 mM EDTA (pH 7.5), 0.15 M NaCl, and proteinase K (100 μg/ml; Sigma) and incubated overnight at 37°C. The DNA was then extracted as described above. MCMV DNA fragments of approximately 10 kilobase pairs (kb) were generated by partial digestion of viral DNA with *Mbo*I. These were cloned into the *Bam*HI site of plasmid pBR322 and transfected into *Escherichia coli* HB101 (1). A colony of the ampicillin-resistant, tetracycline-sensitive transfectants was selected for use as a virus-specific probe. This pBR322 recombinant contained a 9-kilobase-pair insert which was specific for MCMV DNA in that it failed to hybridize to human cytomegalovirus DNA or MEF total DNA. For hybridization, the plasmid containing the 9-kilobase pair MCMV DNA was nick translated with [^{32}P]deoxycytidine triphosphate with the Amersham nick translation kit, resulting in a specific activity of 5 × 10^7 cpm/μg of DNA.

Hybridization. The DNA to be hybridized was treated with a one-tenth volume of 5 M NaOH for 10 min on ice. The samples were then neutralized with one volume of 3 M sodium acetate (pH 5.3). Serial twofold dilutions were made in 4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0]), and 100 μl of each sample was applied under vacuum (96-well Minifold; Schleicher & Schull, Inc.) to Biodyne transfer membrane (Pall Ultrafine Filtration Corp.) prewetted with 4× SSC. The filter was baked at 80°C for 2 h and prehybridized for 2 h at 67°C in 5 ml of a solution containing 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM sodium phosphate, and 1 M EDTA [pH 7.4]), 5× Denhardt solution (50× Denhardt solution is 1% Ficoll, 1% polyvinylpyrrolidone, 1% bovine serum albumen) (8), 2% sodium dodecyl sulfate, and 500 μg of denatured salmon sperm DNA. Hybridization was carried out for 48 h at 67°C in 4 ml of the same solution as described for prehybridization with the addition of 500 ng of ^{32}P-labeled probe DNA. The filter was washed three times in a solution containing 5 mM sodium phosphate, 1 mM EDTA, 0.2% sodium dodecyl sulfate (pH 7.0) at 37°C with agitation. After the final wash, the filter was air dried and exposed to Kodak XAR5 film with intensifying screens for 24 h at −70°C.

RESULTS

MCMV one-step growth curve. To determine the time of peak virus production in MEF-infected cells, growth curves were performed on representative parent and mutant combinations. Figure 1 illustrates a typical one-step growth curve which confirms previously published data (17, 24) that demonstrate peak virus production for MCMV occurs between 40 and 50 h postadsorption. Therefore, cultures for all one-step growth curves were frozen at 45 h postadsorption.

Isolation of drug-resistant mutants. The antiviral activity of ACV against eight independently derived ACV-resistant mutants (ACV mutant) and one PAA-derived mutant (PAA-5) is shown in Fig. 2. Although none of the mutants were completely resistant to ACV, they were more resistant than the parent virus, and this is best seen at concentrations of 5 μM ACV and higher. In Table 1, results of resistance of each parent and mutant to 5 μM ACV are shown. The mutants range from being 48 to 156 times more resistant to 5 μM ACV than the corresponding parent. The mutant obtained by growth in 100 μg of PAA (PAA-5) per ml was approximately 100-fold more resistant to PAA than was the parent virus (see Table 5). This mutant showed a degree of resistance to ACV that was similar to that for the ACV-derived mutants (Table 1).

Isolation of the temperature-sensitive mutants. The temperature-sensitive mutant used as a positive control for recom-

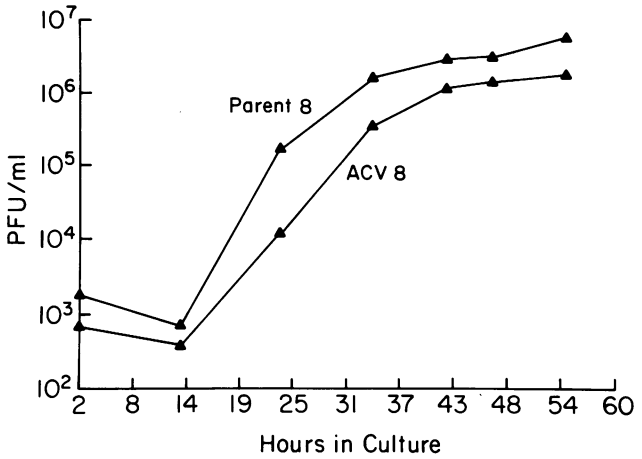

FIG. 1. One-step growth curve of a MCMV parent-mutant combination.

TABLE 1. ACV resistance phenotypes

Virus	No. of times tested	Percent survivors[a] in 5 μM ACV ± SD
Parent 1	4	0.35 ± 0.06
ACV-1	12	53 ± 19
Parent 3	3	0.043 ± 0.2
ACV-3	10	67 ± 20
Parent 5	2	0.55 ± 0.07
PAA-5	7	38 ± 18
Parent 6	2	0.8 ± 0.5
ACV-6	9	43 ± 24
Parent 8	2	0.7 ± 0.4
ACV-8	9	63 ± 22
Parent 9	4	0.9 ± 0.2
ACV-9	10	66 ± 25
Parent 11	4	1.25 ± 0.7
ACV-11	10	67 ± 24
Parent 12	3	1.0 ± 0.4
ACV-12	7	48 ± 16
Parent 13	5	0.48 ± 0.3
ACV-13	8	62 ± 26

[a] Percent survivor calculated by (PFU per milliliter with drug)/(PFU per milliliter without drug) × 100.

bination was inhibited by >99% at 40°C as compared with 34°C, and demonstrated no altered phenotype with regard to susceptibility to ACV and PAA (data not shown).

Complementation tests. Complementation tests can be used to determine the number of complementation groups which, when containing a mutation, can code for a gene product, resulting in drug resistance. This would give an estimate of the number of genes involved in determining the phenotype of drug sensitivity. Complementation studies were performed at least twice on all combinations of mutants. Results presented in Table 2 are examples of typical complementation experiments. Positive controls (parent-mutant) revealed small but reproducible complementation indices greater than 2, whereas the complementation indices of most mutants were less than 1. Even though the complementation indices of positive controls were small, these results are not unlike those reported by Coen and Schaffer (6) in their complementation studies with ACV-resistant HSV, especially when the MOI of the wild-type virus was less than 10. There was no indication of viral interference (experiment 1 samples 6 through 10 and experiment 2 samples 12 and 13, Table 2). The complementation data are summarized in Table 3.

Recombination studies. The incubation of ACV-resistant mutants with 50 μM ACV in the overlay resulted in either no plaque formation or small aborted plaques that were easily distinguished from typical resistant plaques seen at lower drug concentrations. If MCMV ACV-resistant mutants are analogous to HSV ACV-resistant mutants (6), then a double mutated virus (mutations in two different genes) grown in 50 μM ACV should produce resistant plaques that should be easily distinguished from a single mutated virus plaque.

The temperature-sensitive mutant was used as a positive control in all recombination experiments. After a one-step growth cycle at 34°C, the titers of either the ACV mutant

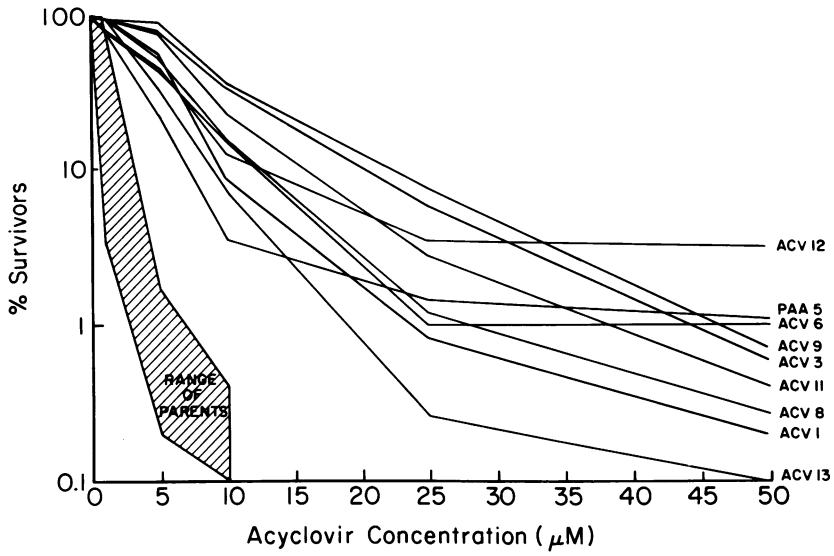

FIG. 2. Effect of various concentrations of ACV on virus yield from a one-step growth cycle for eight ACV-derived resistant mutants, one PAA-derived mutant, and parent virus.

TABLE 2. Complementation experiments

Complementation Exp. no.	Sample	Virus	Titer (PFU/ml) at the following ACV concn (μM): 0	5	CI[a]
1	1	PAA-5	5.4×10^5	2.7×10^5	
	2	ACV-9	5.1×10^5	4.9×10^5	
	3	ACV-12	6.0×10^5	2.1×10^5	
	4	ACV-13	4.7×10^5	4.5×10^5	
	5	Parent 5	1.3×10^5	1.0×10^2	
	6	PAA 5 + ACV-9	6.6×10^5	1.9×10^5	1.4
	7	PAA-5 + ACV-12	5.5×10^5	3.0×10^5	0.7
	8	PAA-5 + ACV-13	6.4×10^5	3.1×10^5	0.9
	9	ACV-9 + ACV-13	5.4×10^5	4.9×10^5	0.9
	10	ACV-9 + Parent 5	3.6×10^5	1.5×10^5	3.3
2	1	ACV-6	3.3×10^5	2.8×10^5	
	2	ACV-8	8.5×10^5	5.5×10^5	
	3	ACV-9	1.1×10^6	1.0×10^6	
	4	ACV-11	5.3×10^5	4.5×10^5	
	5	ACV-12	2.6×10^5	2.6×10^5	
	6	Parent 9	7.5×10^5	8.3×10^4	
	7	ACV-6 + ACV-8	ND[b]	5.2×10^5	0.5
	8	ACV-6 + ACV-11	ND	6.6×10^5	0.4
	9	ACV-6 + ACV-12	ND	3.4×10^5	0.8
	10	ACV-8 + ACV-11	ND	6.6×10^5	0.7
	11	ACV-8 + ACV-12	ND	7.3×10^5	0.4
	12	ACV-9 + ACV-11	1.3×10^6	8.8×10^5	0.5
	13	ACV-9 + ACV-12	7.5×10^5	6.9×10^5	0.4
	14	ACV-11 + ACV-12	ND	3.9×10^5	0.7
	15	ACV-9 + Parent 9	ND	1.2×10^5	8.3

[a] Complementation index.
[b] ND, Not done.

alone or the ACV mutant plus temperature-sensitive mutant progeny virus were determined at 40°C in the presence of 5 μM ACV. A decrease in titer of the mixed mutant infection (ACV mutant plus temperature-sensitive mutant) compared with the ACV mutant alone was assumed to be due to recombination (viral interference was not observed).

A typical recombination experiment is illustrated in Table 4. No fully resistant plaques were observed at 50 μM ACV, whereas there was a reduction in titer when the temperature-sensitive mutant was allowed to recombine with an ACV mutant. All combinations of mutants were tested for recombination, and no recombination was detected among any of the eight ACV-resistant mutants or between any of the ACV mutants and the PAA-derived mutant. Recombination between the ACV mutants and the temperature-sensitive mutants resulted in reduction in the titer from 1.9- to 6.25-fold.

Susceptibility to antiviral compounds. The nine MCMV parent-ACV-resistant mutant combinations were tested for susceptibility to the antiviral compound PAA (Table 5). The PAA-derived, ACV-resistant mutant PAA-5 was 50 to 100 times more resistant to PAA than was its parent. Seven of the eight ACV-derived mutants demonstrated increased resistance to PAA. One ACV mutant (ACV-1) demonstrated no increased resistance to 100 μg of PAA per ml and was slightly more susceptible to 50 μg/ml than was its parent. To further examine the difference between the susceptibility of ACV-1 and its parent to PAA, a dose-response experiment was performed. Results presented in Fig. 3A show mutant ACV-1 to be more sensitive to PAA than was its corresponding parent, especially at lower concentrations of drug. The dose of drug which inhibited 50% of virus (50% effective dose) for parent virus was approximately 15 μg/ml, whereas mutant ACV-1 had a 50% effective dose of <1 μg/ml.

All nine parent-mutant combinations were tested for susceptibility to the antiviral compound ara-A (Table 5). Two of the nine mutants (ACV-9 and ACV-12) were slightly more

TABLE 3. Average Complementation index

Virus	Avg complementation index for the following mutants[a]: A3[b]	PAA-5	A6	A8	A9	A11	A12	A13	Parent
A1	1 ± 0.6	0.4 ± 0.3	0.5 ± 0.4	0.6 ± 0.1	0.7 ± 0.3	0.8 ± 0.6	0.9 ± 0.1	0.5 ± 0.1	3.7 ± 1.5
A3		0.9 ± 0.3	0.7 ± 0.1	1.3 ± 0.5	0.3 ± 0.3	0.6 ± 0.5	1.2 ± 0.9	0.3 ± 0.1	4.1 ± 1.9
PAA-5			0.3 ± 0.1	0.6 ± 0.1	1.2 ± 0.3	0.5 ± 0.1	0.4 ± 0.4	0.5 ± 0.5	3.3 ± 0.8
A6				0.8 ± 0.3	1.2 ± 0.6	0.8 ± 0.5	0.9 ± 0.1	1.3 ± 0.6	2.8 ± 0.9
A8					0.7 ± 0.6	0.7 ± 0.1	0.6 ± 0.3	0.8 ± 0.3	2.9 ± 0.7
A9						0.8 ± 0.5	0.9 ± 0.7	0.9 ± 0.5	5.5 ± 4
A11							0.7 ± 0.1	1 ± 0.2	4.8 ± 2.1
A12								0.9 ± 0.5	2.8 ± 0.7
A13									3.6 ± 1.8

[a] Average of at least two separate complementation experiments.
[b] A, ACV-resistant mutant.

TABLE 4. Recombination titers

Virus	Titer (PFU/ml) at the following ACV conc. (μM):			Reduction[b]
	0	5	50	
ACV-11	1.8×10^5		$<10^2$	
ACV-12	3.2×10^5		$<10^2$	
ACV-13	3.8×10^5		$<10^2$	
ACV-11 × ACV-12	4.0×10^5		$<10^2$	
ACV-11 × ACV-13	7.0×10^5		$<10^2$	
ACV-12 × ACV-13	4.8×10^5		$<10^2$	
Controls[a]				
ACV-11		1.5×10^5		
ACV-12		3.3×10^5		
ACV-13		3.6×10^5		
ACV-11 × ts6		8.0×10^4		1.9
ACV-12 × ts6		1.1×10^5		3.0
ACV-13 × ts6		1.8×10^5		2.0

[a] One-step growth at 34°C without ACV; titers were determined at 40°C with 5 μM ACV in overlay.

[b] (PFU per milliliter of titer of ACV mutant determined at 40°C in 5 μM ACV)/(PFU per milliliter of titer of ACV mutant × temperature sensitive mutant 6 determined at 40°C in 5 μM ACV).

resistant to ara-A as compared with parent virus. In contrast, mutant ACV-1 was more than 50-fold more susceptible to 10 μM ara-A than was the parent virus. A dose-response experiment was then performed (Fig. 3B). Mutant ACV-1 was much more susceptible to ara-A than was its parent, with the difference best seen at higher concentrations of drug. The dose of drug that inhibited 90% of virus for parent 1 was greater than 15 μM, whereas the 90% effective dose for ACV-1 was approximately 2 μM.

The PAA-derived mutant (PAA-5), as well as all the ACV-derived mutants coresistant to PAA, were much more susceptible to Aph than were parent viruses (Table 5). In contrast, the ACV-resistant mutant demonstrating increased susceptibility to PAA and ara-A (ACV-1) was not hypersensitive to Aph.

Effect of ACV, PAA, and ara-A on MCMV parent and ACV-resistant mutant DNA synthesis. Irradiated MEF cells were used in DNA synthesis experiments to depress background thymidine incorporation because of cellular DNA synthesis. Viral titers from a one-step growth cycle (45 h) are shown in Table 6 and confirm that the use of MEF cells irradiated with 3000 rads did not affect viral replication. These results also demonstrate that this amount of irradiation had no effect on the antiviral action of ACV.

The kinetics of DNA synthesis in irradiated MEF cells infected with MCMV were analyzed with one of the parent viruses. By using a 30-min pulse at various intervals, the incorporation of [^{3}H]thymidine was followed from 4 to 31 h post-virus adsorption. [^{3}H]thymidine incorporation began approximately 12 h postadsorption, peaked at approximately 20 h, and fell to background levels within 30 h (Fig. 4). These

TABLE 5. Sensitivity to antiviral compounds

Virus	Percent survivors at the indicated concn of the following antiviral compounds:					
	PAA		Ara-A		Aph	
	50 μg/ml	100 μg/ml	1 μM	10 μM	0.1 μg/ml	0.5 μg/ml
Parent 1	0.5	0.06	75	21	100	21
ACV-1	0.1	0.06	15	0.4	100	70
Parent 3	0.2	0.1	93	42	100	41
ACV-3	39	14	100	60	35	<0.5
Parent 5	0.7	0.6	55	4.5	100	23
PAA-5	79	32	72	4.7	83	<0.4
Parent 6	0.6	0.6	72	53	100	10
ACV-6	63	7	76	14	100	<0.8
Parent 8	0.8	0.1	45	6.8	94	26
ACV-8	33	3	29	7.3	100	<0.5
Parent 9	0.7	0.1	57	1.3	100	39
ACV-9	21	4.8	75	12	89	<0.3
Parent 11	0.6	0.5	85	12	100	27
ACV-11	46	11	50	11	59	<0.4
Parent 12	0.3	0.05	75	7.3	100	29
ACV-12	30	4.1	72	24	99	<0.4
Parent 13	0.9	0.3	73	10	99	68
ACV-13	34	1.5	57	11	100	<0.4

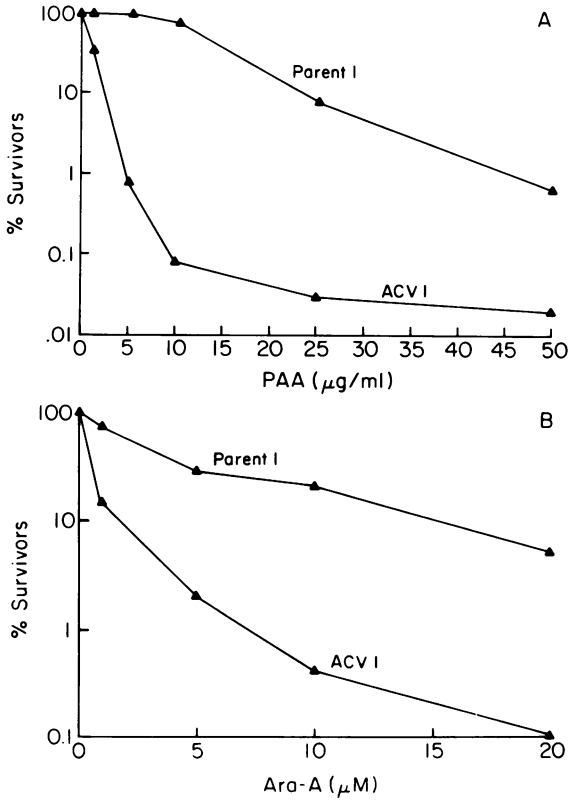

FIG. 3. Comparison between parent 1 and ACV-1 in susceptibility to PAA and ara-A.

TABLE 6. MCMV replication in irradiated cells

Virus	3000 rads	Titer (PFU/ml) (percent survivors[a]) in the following ACV concn (μM):	
		0	5 μM ACV
Parent 1	−	1.5×10^6	4.3×10^3 (0.3)
Parent 1	+	1.5×10^6	5.0×10^3 (0.3)
ACV-1	−	1.8×10^6	8.3×10^5 (47)
ACV-1	+	8.3×10^5	4.2×10^5 (51)
Parent 11	−	3.5×10^5	6.3×10^3 (1.8)
Parent 11	+	2.7×10^5	5.3×10^3 (1.9)
ACV-11	−	1.7×10^5	8.5×10^4 (50)
ACV-11	+	1.2×10^5	9.3×10^4 (78)

[a] Percent survivors was calculated by (PFU per milliliter with drug)/(PFU per milliliter without drug) × 100.

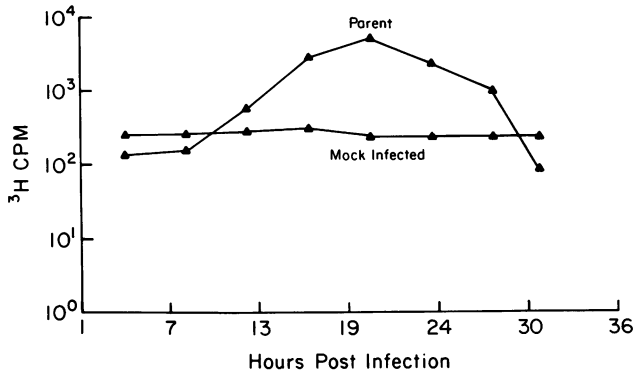

FIG. 4. [^{3}H]thymidine incorporation over time in irradiated MEF cells infected with MCMV.

data are consistent with results reported by Moon et al. (19), who demonstrated that MCMV DNA synthesis is initiated 10 to 14 h postadsorption and peaks between 18 and 25 h. The low levels of [^{3}H]thymidine incorporation seen in mock-infected cells were consistent throughout the DNA synthesis experiments and were not included in all figures describing DNA synthesis.

To determine the effect of 5 μM ACV on MCMV DNA synthesis, two MCMV parent-mutant combinations were studied by monitoring the levels of [^{3}H]thymidine incorporation in the absence and presence of 5 μM ACV over a period of 15 h. Results presented in Fig. 5A describe the effect of 5 μM ACV on MCMV clone parent 11. [^{3}H]thymidine incorporation peaked at 20 h with or without ACV present. However, counts per minute were decreased from 2200 at peak incorporation without drug to 640 in the presence of 5 μM ACV. In contrast to parent 11, the results shown in Fig. 5B reveal very little difference in the [^{3}H]thymidine incorporation in the absence or presence of ACV for the corresponding ACV-resistant mutant ACV-11. The time of peak [^{3}H]thymidine incorporation for ACV-11 is not as apparent as for parent virus. Virus yield from a one-step experiment (parent 11 and ACV-11, Table 6) run concurrently with the thymidine incorporation experiment is consistent with the DNA synthesis data. The percentage of survivors at 45 h postinfection for parent 11 in 5 μM ACV was 1.9 compared with 78 for ACV-11. [^{3}H]thymidine incorporation results for parent 1 and ACV-1 are presented in Fig. 5C and 5D, respectively. As with parent 11, cells infected with parent 1 in the presence of 5μM ACV exhibited reduced [^{3}H]thymidine incorporation compared with cells infected with parent virus in the absence of drug. In contrast, little difference was seen in [^{3}H]thymidine incorporation in cells infected with ACV-resistant mutant ACV-1.

Because ACV-resistant mutant ACV-1 has been shown to be much more susceptible to PAA and ara-A as compared with parent virus, [^{3}H]thymidine incorporation experiments were performed on parent 1 and ACV-1 in the presence of 50 μg of PAA per ml, 10μM ara-A, and no drug (Fig. 6). Peak [^{3}H]thymidine incorporation (20 h) in cells infected with parent 1 was greater when no drug was present (8400 cpm) compared with the presence of 10 μM ara-A (5200 cpm) or 50 of PAA per ml (2200 cpm). In cells infected with ACV-resistant mutant ACV-1, the presence of PAA resulted in a substantial inhibition of [^{3}H]thymidine uptake. In the presence of ara-A, [^{3}H]thymidine incorporation for ACV-1 showed the greatest amount of inhibition 20 h postinfection. After 20 h, [^{3}H]thymidine incorporation continued to rise until it equalled that of the control (no drug). This was most likely due to the deamination over time of ara-A to hypoxanthine arabinoside, a compound with much less antiviral activity than ara-A (22).

Virus-specific nature of DNA synthesis. To determine whether the differences in [^{3}H]thymidine incorporation seen between parent and mutant virus in the presence of antiviral compounds reflected actual differences in the amount of virus-specific DNA, a virus-specific probe was used in DNA

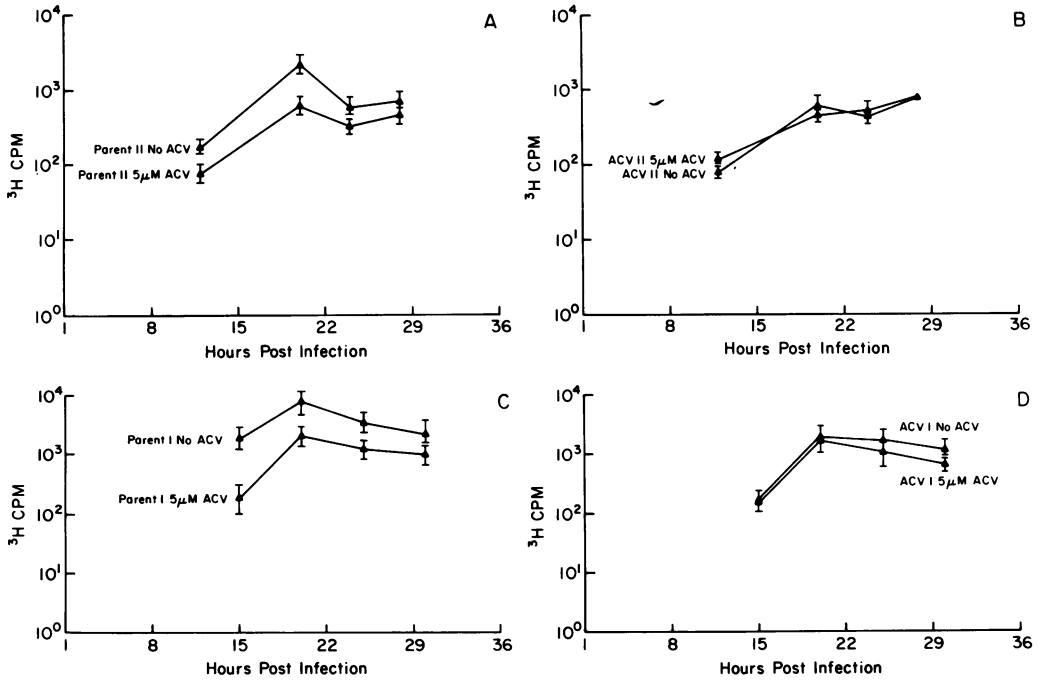

FIG. 5. Effect of ACV on [^{3}H]thymidine incorporation in MEF cells infected with parent or ACV-resistant mutant virus.

hybridization experiments. The presence of 5 μM ACV during the one-step growth cycle resulted in a decrease in virus-specific DNA for both parent viruses (column 2, rows A through D and rows E through H, Fig. 7A), whereas the same concentration had no effect on DNA synthesis for either of the ACV-resistant mutants (column 10, rows A through D and rows E through H, Fig. 7A). The presence of 10 μM ara-A had little effect on the amount of virus-specific DNA made for either parent virus (column 4, rows A through H, Fig. 7A) or ACV-11 (column 12, rows E through H, Fig. 7A). Surprisingly, there appeared to be no difference in the amount of viral DNA made in the presence of 50 μg of PAA per ml between ACV-11 (a PAA-resistant virus; column 11, rows E through H, Fig. 7A) and the parent viruses (PAA-susceptible viruses; column 3, rows A through H, Fig. 7A). In contrast, no virus-specific DNA was detected when cells infected with ACV-1 were cultured in the presence of 50 μg of PAA per ml (column 11, rows A through D, Fig. 7A), and very little DNA was detected in the presence of ara-A (column 12, rows A through D, Fig. 7A). To control for possible variations in the DNA extractions, a second dot blot experiment run in parallel with the first one was hybridized with a mouse DNA-specific probe. There was little, if any, variation in the amount of mouse DNA among the samples for each virus. MCMV titers from the samples used for hybridization are presented in Table 7.

DISCUSSION

The isolation of MCMV mutants resistant to ACV strongly suggests that this analog of guanosine has antiviral activity against MCMV rather than being a secondary inhibitor of virus as a result of toxic effects on the host cell. Because these mutants were isolated independently of each other, each isolate presumably represents an unrelated mutational event. Unlike HSV, in which two distinct loci (TK and DNA polymerase genes) can confer resistance to ACV, complementation data presented here demonstrate a single complementation group involved in resistance of MCMV to ACV. The fact that the PAA-derived resistant mutant also failed to complement any of the ACV-derived mutants suggests that all the resistant mutants are mutated at or very close to the gene that codes for DNA polymerase. The failure to show recombination between any of the mutants parallels the complementation data. The negative recombination results necessitated the use of the temperature-sensitive mutant to establish a positive control for the recombination experimental system. In classical recombination experiments, the numerator in the RF equation should represent only recombined progeny virus. Because the ACV-resistant mutants grew well at 40°C, it was not possible to obtain a true RF by positive selection. Therefore, the alternative methods described above were used to detect, indirectly, ACV-resist-

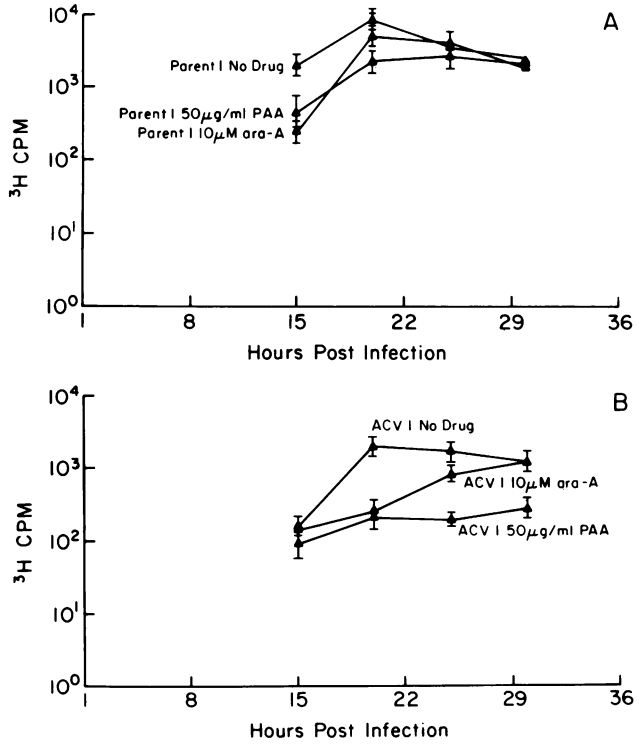

FIG. 6. Effect of PAA and ara-A on [^{3}H]thymidine incorporation in MEF cells infected with parent 1 or ACV-1.

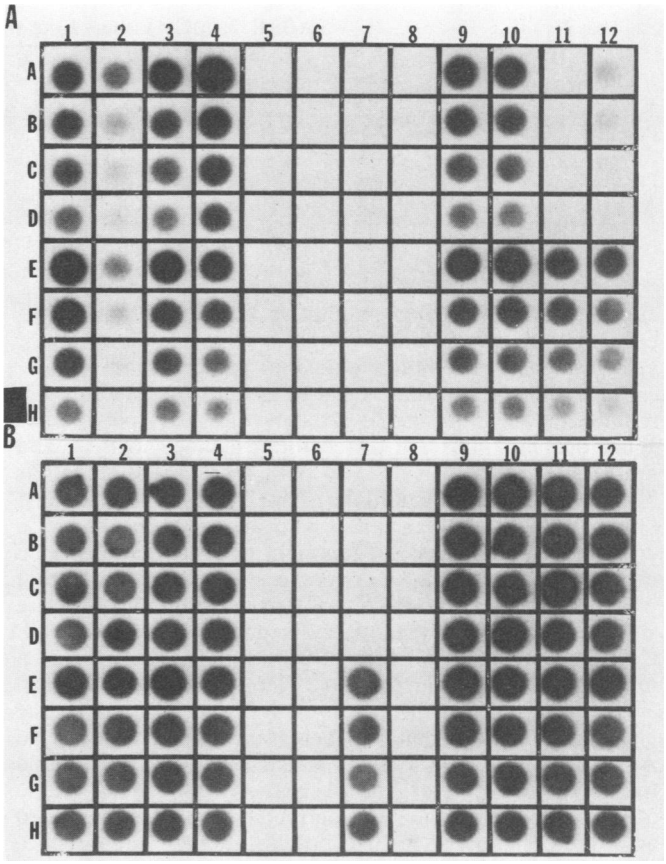

FIG. 7. Dot blot hybridization. (A) Hybridization with a ^{32}P-labeled MCMV probe. (B) Hybridization with a ^{32}P-labeled mouse DNA probe. For rows A through D, the columns contain the following: 1, parent 1 virus, no drug; 2, parent 1 virus, 5 μM ACV; 3, parent 1 virus, 50 μg of PAA per ml; 4, parent 1 virus, 10 μM ara-A; 9, ACV-1 virus, no drug; 10, ACV-1 virus, 5 μM ACV; 11, ACV-1 virus, 50 μg of PAA per ml; 12, ACV-1 virus, 10 μM ara-A. For rows E through H, the columns contain the following: 1, parent 11 virus, no drug; 2, parent 11 virus, 5 μM ACV; 3, parent 11 virus, 50 μg of PAA per ml; 4, parent 11 virus, 10 μM ara-A; 7, no virus, no drug; 9, ACV-11 virus, no drug; 10, ACV-11 virus, 5 μM ACV; 11, ACV-11 virus, 50 μg of PAA per ml; 12, ACV-11 virus, 10 μM ara-A.

TABLE 7. MCMV titers from samples used in dot blot hybridization

Virus	Titer (PFU/ml) (percent survivors[a]) at the indicated concn of the following drugs:			
	No drug	5 μM ACV	50 μg of PAA per ml	10 μM ara-A
Parent 1	1.7×10^6	5.3×10^3 (0.3)	5.5×10^4 (3.2)	6.2×10^5 (36)
ACV-1	2.4×10^6	1.1×10^6 (46)	1.0×10^2 (0.004)	1.1×10^3 (0.05)
Parent 11	1.3×10^6	8.5×10^3 (0.7)	5.2×10^4 (4)	1.8×10^5 (14)
ACV-11	2.3×10^6	7.9×10^5 (34)	1.1×10^6 (48)	5.0×10^5 (22)

[a] Percent survivors was calculated by (PFU per milliliter with drug)/(PFU per milliliter without drug) × 100.

ant-temperature-sensitive mutant recombinants. The existence of these recombinants suggests that conditions were appropriate for recombination events to occur.

Since the genetic data suggest that all the mutants are mutated in the same gene, possibly the DNA polymerase gene, the mutants were tested for resistance to PAA and ara-A. Increased resistance to both these compounds has been correlated with mutations in the DNA polymerase gene (3, 5, 16, 25). Although little or no increased resistance to ara-A was found, seven of the eight mutants demonstrated increased resistance to PAA (Table 5). The one mutant that was not coresistant to PAA (ACV-1) was found to be more susceptible to PAA than was the parent virus (Table 5). Coen et al. (4) and Honess et al. (15) have shown that a mutation leading to an increase in resistance to PAA and ara-A usually results in hypersensitivity to Aph. When the eight ACV-derived mutants were tested for sensitivity to Aph, the same seven mutants resistant to PAA were found to be hypersensitive to Aph whereas ACV-1 was not (Table 5). That all eight of the ACV-derived mutants demonstrated an altered phenotype in susceptibility to PAA supports the genetic data, which suggest that a single gene, the gene coding for the DNA polymerase, is responsible for the susceptibility of MCMV to ACV.

If only one MCMV gene product is required for susceptibility to ACV, then cellular enzymes must be necessary for the phosphorylation of this antiviral compound. St. Clair et al. (27) have shown that ACV is phosphorylated to low levels in mouse cells, and their data further suggest that uninfected murine cells may phosphorylate ACV to a greater extent that uninfected human cells. We found that MCMV infection of MEF cells results in increased phosphorylation of ACV (unpublished data). It may be that the wild-type MCMV DNA polymerase is exquisitely sensitive to phosphorylated ACV, and even the low level of acyclo-GTP found in murine cells is sufficient to inhibit the polymerase and block replication.

The DNA synthesis experiments reported here demonstrate that ACV inhibits MCMV at the level of DNA replication and supports the probability that it is the polymerase that is the ultimate site of action of ACV for MCMV. Acyclo-GTP has been shown to competitively inhibit dGMP incorporation into HSV DNA (11). If the mechanism of inhibition of MCMV DNA by acyclo-GTP is similar to the proposed mechanism of action in the HSV system, then partial blocking of [^{3}H]thymidine incorporation in cells infected with parent virus in the presence of ACV was to be expected. The exact mechanism of action is still not known. It has been demonstrated that acyclo-GMP (derived from acyclo-GTP) is incorporated into activated calf thymus DNA more efficiently with HSV DNA polymerase compared with Vero DNA polymerase alpha (13). The incorporation by HSV DNA polymerase was very rapid for the first 2 min but virtually ceased after 15 min, probably due to chain termination. This seemed to be a logical hypothesis because the incorporated acyclo-GMP does not have the 3′-hydroxyl group that is necessary for the attachment to the alpha phosphate moiety of the entering deoxyribonucleoside triphosphate, thereby preventing chain elongation. However, affinity of HSV DNA polymerase for incorporated acyclo-GMP may also play a role. By incubating HSV DNA polymerase with calf thymus DNA, of which 10% was terminated with acyclo-GMP, Derse et al. (9) were able to show that HSV DNA polymerase had a 50-fold higher affinity for acyclo-GMP-terminated DNA than for 3′-hydroxyl terminated DNA. These results suggest the mechanism by which acyclo-GTP competes with deoxyguanine triphosphate may be twofold. Preferential incorporation of acyclo-GMP over deoxyguanine monophosphate by HSV DNA polymerase leading to chain termination and inhibition of the viral polymerase via increased binding to the acyclo-GMP-terminated template. How the proposed mechanism of action of acyclo-GTP at the level of the DNA polymerase in the HSV system reflects the MCMV system remains to be determined.

The results obtained with the virus-specific DNA probe strongly suggest that the [^{3}H]thymidine incorporation experiments actually measured differences in viral-specific DNA synthesis. The specificity of the probe can be determined when Fig. 7A (column 7, rows E though H) is compared with Fig. 7B (column 7, rows E through H). No hybridization could be detected between the virus-specific DNA probe and uninfected MEF cells. For both parent viruses, the presence of 5 μM ACV during the one-step growth cycle resulted in a decrease in virus-specific DNA production, whereas the same amount of ACV had little effect on resistant virus DNA production. These results paralleled the titer data presented in Table 7 in that 5 μM ACV inhibited parent virus replication by more than 99%. At the concentrations of drug used, both parent viruses were almost as sensitive to PAA as to ACV (based on production of infectious virus as seen in Table 7); however, little or no difference was seen in the amount of virus-specific DNA made. This may reflect the different mechanisms of action of ACV and PAA at the level of DNA replication.

The increased susceptibility of ACV-1 to both PAA and ara-A (Table 2 and Fig. 3) is seen in the hybridization experiment (Fig. 7A and B). No viral DNA was detected when ACV-1 was grown in the presence of 50 μg of PAA per ml, whereas very small amounts were seen when grown in the presence of ara-A. These results were probably not due to inconsistencies in the extraction of DNA in that all the samples had approximately the same amount of mouse DNA (Fig. 7B).

The data presented illustrate that for all the ACV-derived mutants, except ACV-1, the mutation responsible for increased resistance to ACV was associated with an increase in resistance to PAA and hypersensitivity to Aph. In contrast, the mutation in ACV-1 leading to resistance to ACV resulted in increased sensitivity to PAA and ara-A with little

or no change in sensitivity to Aph. At least three possibilities could account for the properties of ACV-1. It could be that ACV-1 is mutated within the polymerase gene, affecting the same functional domains as in the other mutants but resulting in different phenotypic expression. It is also possible that ACV-1 represents a mutation at a different region of the polymerase gene, affecting a different functional domain while maintaining the ACV-resistant phenotype similar to that of the other mutants. A third possibility is that this mutant represents a double-mutated virus. Furman et al. (12) have described an HSV DNA polymerase mutant (BW^r) that is resistant to ACV but remains sensitive (and not hypersensitive) to PAA. Coen et al. (5) have suggested that this mutant may be more susceptible to ara-A than is the parent virus, and the polymerase mutation did not result in hypersensitivity to Aph (4). It may be that ACV-1 possesses a polymerase mutation that results in a phenotype similar to that of BW^r in conjunction with a second mutation at the pyrophosphate-binding site, resulting in increased susceptibility to PAA. It is also possible that a mutation occurs outside of the DNA polymerase gene. Chiou et al. (H. Chiou, E. Fleming, L. Leslie, M. Retondo, S. Weller, and D. Coen, Abstr. Ninth Int. Herpesvirus Workshop, p. 6, 1984) have reported that a mutation in the gene coding for the DNA binding protein (ICP-8) can result in increased susceptibility to PAA.

The phenotypic similarities between MCMV and HSV resulting from mutations in the DNA polymerase suggest that these enzymes may be similar in genetic organization and function and may predict similarities among other herpesviruses.

ACKNOWLEDGMENT

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