Proc. Natl. Acad. Sci. USA
Vol. 77, No. 4, pp. 2265–2269, April 1980
Microbiology

Two distinct loci confer resistance to acycloguanosine in herpes simplex virus type 1

(thymidine kinase/DNA polymerase/recombination/complementation)

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Communicated by Bernard Roizman, November 26, 1979

ABSTRACT **Two distinct loci that confer resistance to acycloguanosine (acyclo-Guo) in herpes simplex virus type 1 have been identified. The first locus is the gene for the virus-specific thymidine kinase (TK). Mutations that decrease TK activity also render the virus resistant to acyclo-Guo, and the level of resistance corresponds to the decrease in TK activity. acyclo-Guo resistance due to defective TK expression is recessive to the wild-type phenotype, acyclo-Guo-sensitive (ACG^s). We term this locus *ACG^r-TK*. The second locus is defined by the properties of a mutant, PAA^r5, which is resistant to acyclo-Guo and to phosphonoacetic acid (PAA) yet exhibits wild-type TK activity. The acyclo-Guo-resistant locus in PAA^r5 is separable from *ACG^r-TK* mutations by recombination. Moreover, PAA^r5 and ACG^r-TK mutants can complement each other, producing drug-sensitive gene products which result in growth inhibition in the presence of acyclo-Guo. The acyclo-Guo resistance conferred by PAA^r5 behaves as though it were codominant with the wild-type phenotype. This second acyclo-Guo-resistance locus is closely linked to the mutation specifying resistance to PAA. Resistance to PAA is thought to result from mutations in the gene for viral DNA polymerase. Thus, the close linkage of the *ACG^r* and *PAA^r* loci suggests that resistance to both drugs is specified by a mutant DNA polymerase. We term this second locus *ACG^r-PAA*.**

9-(2-Hydroxyethoxymethyl)guanine [acycloguanosine (acyclo-Guo); generic name, acyclovir] is a promising new antiviral drug. It has been shown to inhibit the replication of herpes simplex virus (HSV) types 1 and 2 *in vitro* and *in vivo* (1–3) and of varicella zoster (3) and Epstein–Barr viruses (B. M. Colby, J. E. Shaw, G. B. Elion, and J. S. Pagano, personal communication) *in vitro*. Tests in laboratory animals have demonstrated its effectiveness in the treatment of herpes encephalitis in mice, herpes keratitis in rabbits, and cutaneous herpes infections in guinea pigs (1). acyclo-Guo does not appear to be toxic to laboratory animals or to primate cells *in vitro* at effective antiviral doses (1, 3).

The HSV-specified thymidine kinase (TK) appears to be essential for the antiviral effect of acyclo-Guo. Phosphorylation of acyclo-Guo to its mono-, di-, and triphosphate derivatives is associated with antiviral activity (2). The HSV TK and the enzyme that phosphorylates acyclo-Guo copurify; and an HSV mutant, tsA1, which lacks TK activity, is also deficient in acyclo-Guo phosphorylating activity (4). Similarly, we have found that 14 mutants independently isolated for their resistance to acyclo-Guo exhibit decreased HSV TK activity. These mutants and the TK^- mutations in tsA1 and other temperature-sensitive mutants behave in genetic studies as if they were mutated in a single locus—presumably the gene for TK (unpublished data). We have termed this locus *ACG^r-TK*.

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Elion *et al.* (2) have reported that the HSV-specified DNA polymerase is sensitive to a derivative of acyclo-Guo, acycloguanosine triphosphate (acyclo-GTP). Thus, mutations in the HSV DNA polymerase gene might be expected to confer resistance to acyclo-Guo. In this report, we present genetic and biochemical data demonstrating that a second locus is able to confer resistance to acyclo-Guo.

MATERIALS AND METHODS

Cells and Viruses. African green monkey kidney (Vero) cells were routinely passaged as described (5). Stocks of wild-type HSV-1, strain KOS (6) and mutants of strain KOS were prepared in Vero cells as described by Parris *et al.* (7). acyclo-Guo-resistant mutants (ACG^r4, 8, 18, 33, and 35) were derived by plating KOS on Vero cells under methylcellulose containing acyclo-Guo. PAA^r5 was derived by passage of KOS in the presence of phosphonoacetic acid (PAA) (8).

Drugs. acyclo-Guo was kindly supplied by Gertrude Elion (Burroughs Wellcome, Research Triangle Park, NC). The drug was dissolved in equimolar NaOH, filter sterilized, and stored at −20°C. Disodium PAA was the generous gift of Abbott Laboratories (North Chicago, IL).

Virus Assays. Virus assays were performed on Vero cell monolayers by a plaque method utilizing a 2% methylcellulose overlay (9). For assaying resistance to acyclo-Guo or PAA, viruses were plated under methylcellulose containing the concentrations of acyclo-Guo or PAA indicated.

Recombination analysis was performed as described (10) except that Vero cells were used instead of HEL cells and recombination was performed at 37°C. Recombination frequencies were calculated as described in Table 2.

Single-Cycle Growth Experiments. Vero cells were inoculated at the multiplicities of infection indicated in Table 3 as described (10). After absorption, 2 ml of medium either with or without 5 μM acyclo-Guo was added to duplicate cultures (although 5 μM acyclo-Guo inhibited the plating efficiency of KOS by less than 2 orders of magnitude, it inhibited KOS growth in a single cycle by 4 orders while permitting substantially greater growth of partially resistant viruses). At 18 hr after infection (incubation at 37°C), virus was harvested as described (10) and assayed on Vero cell monolayers.

TK Assays. Vero cells were mock-infected or infected at 5 plaque-forming units (PFU) per cell as described (10). After 8.5 hr at 37°C, floating cells were pelleted by low-speed centrifugation, and the monolayers and pelleted cells were washed once with Tris/saline and then frozen at −80°C.

Just before assay, pellets were thawed, resuspended in 0.5 ml

Abbreviations: HSV, herpes simplex virus; acyclo-Guo, acycloguanosine; acyclo-GTP, acycloguanosine triphosphate; PFU, plaque-forming unit; TK, thymidine kinase; PAA, phosphonoacetic acid.

of 10 mM sodium phosphate buffer at pH 6.0, and sonicated at 10 kHz for 60 sec with a sonic oscillator (Heat Systems Ultrasonics, Plainview, NY). Extracts were centrifuged in a Brinkmann Microfuge (12,000 × *g*) for 1 min and the supernatant fluids were used for TK assays as described by Preston (11) with the addition to assay mixtures of bovine serum albumin at 0.1 mg/ml and 40 μM TTP. At this concentration of TTP, KOS-induced TK activity was unaffected and mock-infected activity was reduced by 93%. TK activities were calculated by using time points during which the rate of thymidine phosphorylation was linear, and normalized to the amount of protein present as determined by the method of Lowry *et al.* (12).

RESULTS

Resistance to acyclo-Guo. Fig. 1 illustrates the relative resistance to acyclo-Guo of KOS (the wild-type virus) and six mutants derived from KOS. It should be noted that plaque sizes generally did not remain constant as drug concentration increased and plating efficiencies decreased. Smaller plaques were usually observed at plating efficiencies of 50% or even 80%.

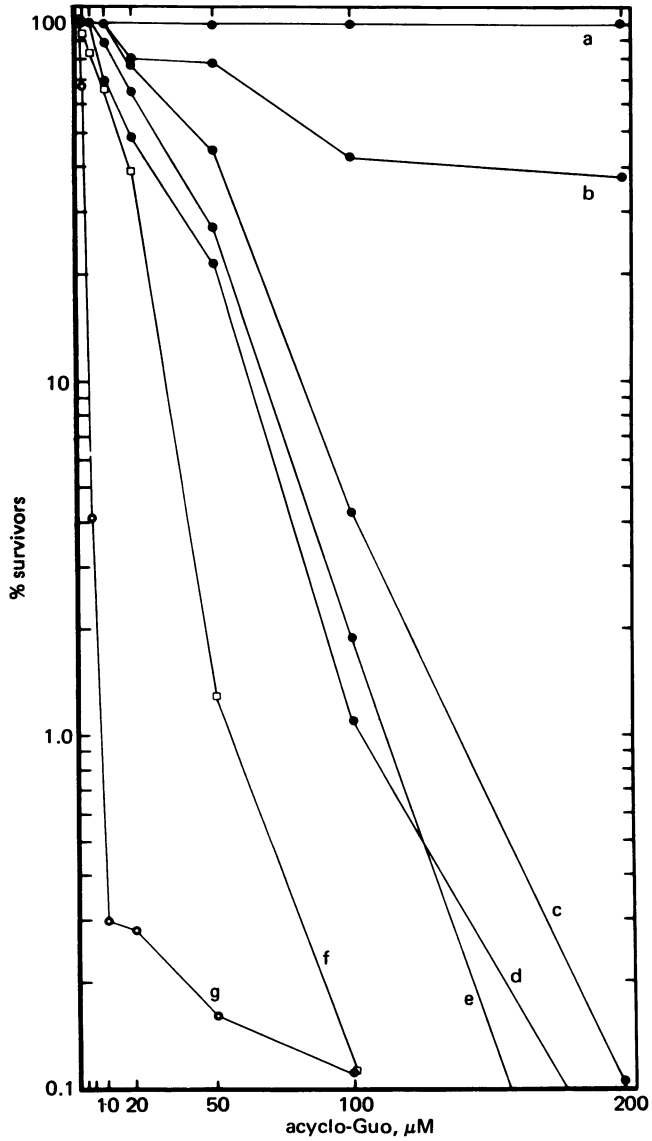

FIG. 1. Effect of varying concentrations of acyclo-Guo on the plating efficiency of KOS and six mutants derived from KOS. Viruses were assayed by a plaque method (9) under methylcellulose containing the concentrations of acyclo-Guo indicated. The number of plaques produced by each virus under methylcellulose without acyclo-Guo was considered to be 100%. Curves: a, ACG^{r}4; b, ACG^{r}8; c, ACG^{r}18; d, ACG^{r}33; e, ACG^{r}35; f, PAA^{r}5; g, KOS.

The wild-type virus was highly sensitive to acyclo-Guo; the plating efficiency was 0.3% at 20 μM. PAA^{r}5, which is resistant to PAA (8), proved to be significantly resistant to acyclo-Guo as well. At 20 μM acyclo-Guo, the plating efficiency of PAA^{r}5 was 39%. Mutants ACG^{r}18, ACG^{r}33, and ACG^{r}35 exhibited plating efficiencies similar to the efficiency of PAA^{r}5 at 20 μM but were significantly more resistant to the drug at higher concentrations. We have classified these three mutants and PAA^{r}5 as partially resistant to acyclo-Guo. ACG^{r}8 was more resistant than the partially resistant mutants, with a plating efficiency of 37% at 200 μM. In contrast, ACG^{r}4 was highly resistant to acyclo-Guo showing no difference in plating efficiency or plaque size when plated in the presence or absence of 200 μM acyclo-Guo. The relative plating efficiencies of these viruses in 20 μM acyclo-Guo are summarized in Table 1.

Resistance to PAA. Because PAA^{r}5 was resistant to both PAA and acyclo-Guo, the relative plating efficiencies of KOS and six ACG^r mutants in PAA at 100 μg/ml were compared (Table 1). acyclo-Guo-resistant mutants were not necessarily resistant to PAA; however, PAA^{r}5 and the highly acyclo-Guo-resistant mutant ACG^{r}4 were resistant to both drugs.

TK Activity of ACG^r Mutants: PAA^{r}5 Induces Wild-Type Levels of TK. TK activities induced by the viruses that exhibited resistance to acyclo-Guo were measured. ACG^{r}4, ACG^{r}8, ACG^{r}18, ACG^{r}33, and ACG^{r}35 showed substantially decreased TK activity compared to that of the wild-type virus (Table 1). For these five mutants, the lower the TK activity, the higher the degree of acyclo-Guo resistance. In recombination tests these mutants behaved as alleles of a single locus which we term *ACG^r-TK* (Table 2; unpublished data). Surprisingly, however, PAA^{r}5 induced wild-type levels of TK activity (Table 1). Two hypotheses could explain this finding: (*i*) PAA^{r}5 could possess a mutation in its *TK* gene such that it could phosphorylate thymidine efficiently *in vitro* but not acyclo-Guo efficiently *in vivo* or (*ii*) PAA^{r}5 could possess a mutation in a second locus, other than the *TK* gene, conferring resistance to acyclo-Guo.

Recombination Between *ACG^r* Loci: Linkage of the Second *ACG^r* Locus to *PAA^r*. If the second hypothesis posited above were correct, one might expect that the putative second locus for acyclo-Guo resistance would map sufficiently far from the *ACG^r-TK* locus to recombine with it. We tested this hypothesis by performing crosses between PAA^{r}5 and ACG^{r}35. Our ability to measure recombination between these two viruses

Table 1. Drug-resistance phenotypes and TK activities

Virus	Resistance* 20 μM acyclo-Guo	Resistance* PAA at 100 μg/ml	% TK activity†
KOS	0.3	0.2	100
ACG^{r}4	100	87	0.5
ACG^{r}8	80	0.02	0.5
ACG^{r}18	77	0.08	5
ACG^{r}35	66	0.06	14
ACG^{r}33	50	0.04	16
PAA^{r}5	39	100	117

* Shown as percentage survivors, calculated by [(PFU/ml with drug)/(PFU/ml without drug)] × 100.

† The activity induced by each virus was normalized to that induced by KOS. Mock-infection activity was 0.7% of KOS-induced activity.

Table 2. Recombination between ACG^r loci

Virus(es)	PFU/ml: No drug	PFU/ml: In 100 μM acyclo-Guo	EOP,* acyclo-Guo	RF† between ACG^r loci, %	PFU/ml in acyclo-Guo (100 μM) and PAA (100 μg/ml)	EOP,* ACG and PAA	RF† between ACG^r-TK and PAA^r loci, %
PAA^r5	1.9×10^7	1.0×10^4	1.0×10^{-3}	—	2.0×10^3	1×10^{-4}	—
ACG^r8	2.1×10^7	1.4×10^7	6.7×10^{-1}	—	1.0×10^3	5×10^{-5}	—
ACG^r35	2.4×10^7	2.5×10^3	1.0×10^{-4}	—	1.0×10^1	$<4 \times 10^{-7}$	—
ACG^r33	1.7×10^7	2.9×10^4	2.0×10^{-3}	—	$<1.0 \times 10^1$	$<5 \times 10^{-7}$	—
$PAA^r5 \times ACG^r8$	1.8×10^7	1.2×10^7	6.7×10^{-1}	—	8.0×10^5	4.4×10^{-2}	8.8
$PAA^r5 \times ACG^r35$	1.5×10^7	6.2×10^5	4.1×10^{-2}	8.2	6.6×10^5	4.4×10^{-2}	8.8
$ACG^r33 \times ACG^r35$	3.0×10^7	2.2×10^4	7.0×10^{-4}	0.14	$<1.0 \times 10^2$	$<3 \times 10^{-7}$	6×10^{-5}

* Efficiency of plating (EOP) = (PFU/ml in presence of drug)/(PFU/ml in absence of drug).
† Recombination frequency (RF) between drug-resistance loci (RF) = [(PFU/ml in presence of drug)/(PFU/ml in absence of drug)] × 2 × 100%.

was dependent upon the fact that both PAA^r5 and ACG^r35 are only partially resistant to acyclo-Guo. Thus, neither virus plated efficiently at 100 μM acyclo-Guo, producing, at best, minute plaques that could readily be distinguished from truly resistant plaques (Fig. 1; and Table 2, lines 1 and 3). However, when cells were infected with both viruses in the absence of acyclo-Guo and the progeny virus was assayed, 4.1% of the progeny was resistant to 100 μM acyclo-Guo (Table 2, line 6). Thus, the two loci recombined with a frequency of 8.2%. We then asked whether the acyclo-Guo-resistance locus of PAA^r5 was linked to the PAA^r locus of this virus. If the two drug-resistance loci were *not* closely linked, one would expect a number of the recombinant progeny to be resistant to one drug but not the other, the number being proportional to the distance between the two loci. In fact, all recombinants that were resistant to acyclo-Guo were also resistant to PAA (Table 2, line 6). Thus, the locus in PAA^r5 that confers resistance to acyclo-Guo is closely linked to the mutation that confers resistance to PAA. Moreover, a recombination frequency (8.8%) similar to that obtained from crosses between the two ACG^r loci was obtained in crosses between the PAA^r locus of PAA^r5 and the ACG^r-TK locus of the more resistant mutant ACG^r8 (Table 2, line 5).

Similar results were obtained in crosses between PAA^r5 and the partially acyclo-Guo-resistant mutants ACG^r18 and ACG^r33 (not shown). In contrast, crosses between ACG^r33 and ACG^r35 yielded fewer highly acyclo-Guo-resistant progeny than did the parental ACG^r33 mutant alone (Table 2, lines 4 and 7). These data thus define a second acyclo-Guo-resistance locus which we term ACG^r-PAA, which maps approximately 8.5 units from the ACG^r-TK locus. This map distance is much greater than any between mutants within the same complementation group which map in unique sequences of the genome (unpublished data).

Dominance and Recessiveness of acyclo-Guo Resistance. When KOS was grown for one cycle in 5 μM acyclo-Guo, virus yield was decreased by 4 orders of magnitude compared to growth in the absence of the drug (Table 3, experiment I, line 1). In contrast, PAA^r5 and the ACG^r-TK mutant ACG^r8 grew efficiently in the presence of acyclo-Guo (Table 3, experiment I, samples 2 and 3). When cells were subjected to mixed infection with acyclo-Guo-resistant mutants ACG^r8 or PAA^r5 and KOS in acyclo-Guo, however, virus growth was inhibited (Table 3, experiment I, samples 5–12). The degree of inhibition can be expressed as the reduction in titer in mixed infections relative to the titer in infections with an acyclo-Guo-resistant virus alone (at the same multiplicity) in the presence of acyclo-Guo. The growth of ACG^r8 was decreased to $1/450$th at equal multiplicities of the two viruses (experiment I, sample 5 and control sample 2). Even when there was a 3- to 4-fold excess of input ACG^r8, growth was inhibited to $1/40$th (sample 7 and control sample 2). Thus, the ACG^r phenotype of this mutant was recessive to the wild-type phenotype, ACG^s. Nevertheless, the dominance of the ACG^s phenotype was not absolute because the virus yield from mixed infections (experiment I, samples 5 and 6) was greater than that from an infection with KOS alone (experiment I, sample 1).

When cells were subjected to mixed infection with the ACG^r-PAA mutant PAA^r5 and KOS at varying input multiplicities, growth was inhibited (Table 3, experiment I, samples 8 and 10–12). However, the degree of inhibition observed was not as great as that seen in mixed infections with KOS and ACG^r8. In fact the actual virus yield was greater even though ACG^r8 by itself was more resistant (compare experiment I, samples 5 and 8). Moreover, when cells were infected with a greater ratio of PAA^r5 relative to KOS, growth inhibition was even less. At ratios of PAA^r5 to KOS of 5, growth was inhibited only to about ½ (Table 3, experiment I, sample 12 and control sample 4). Thus, the wild-type and ACG^r-PAA alleles behave not as if one were simply recessive and the other dominant but more as if they were codominant.

Complementation of acyclo-Guo-Resistant Mutants. In order to determine whether ACG^r-TK and ACG^r-PAA mutations affected different gene products, complementation tests were performed. Unlike standard yield-of-progeny virus tests, complementation was measured by an increase in drug sensitivity (i.e., a decreased yield) rather than an increase in virus yield. In mixed infections with the ACG^r-PAA mutant, PAA^r5, and either of the ACG^r-TK mutants ACG^r8 or ACG^r35 in acyclo-Guo, the virus yield was lower than with either of the two mutants alone (Table 3, experiment I, samples 13–15 and control samples 2 and 3; experiment II, samples 5 and 6 and control samples 1–3). Although the reductions in titer (relative to the growth of the *less* resistant of the pair) were small, varying from 1.9-fold to 4.8-fold, they were reproducible with both ACG^r8 and ACG^r35 in a total of six experiments. Moreover, only small reductions in titer were expected, based on results of mixed infections with PAA^r5 and KOS. These reductions in titer were drug-specific and were not due to other interference phenomena because, when co-infection was performed without drug, normal virus yields were obtained (experiment I, samples 13–15, and unpublished data). In contrast, with mixed infection with two ACG^r-TK mutants in acyclo-Guo, the yields were at least as high as those from cells infected with the less resistant of the two viruses grown alone (experiment II, samples 8–10 and control samples 2–4). These data indicate that PAA^r5 and ACG^r-TK viruses can complement each other for growth *inhibition* in the sense that PAA^r5 encodes a wild-type drug-sensitive TK, whereas the ACG^r-TK mutants encode the wild-type drug-sensitive counterpart of the acyclo-Guo-resistant gene product of PAA^r5. Thus, in acyclo-Guo, the replication of both viruses was inhibited.

By these criteria, the highly acyclo-Guo-resistant mutant

Table 3. Dominance, recessiveness, and complementation

Sample	Infecting virus(es)	Multiplicity of infection (ratio)	PFU/ml: No acyclo-Guo*	PFU/ml: 5 μM acyclo-Guo	Reduction†
		Experiment I			
1	KOS	25	2.1×10^7	1.9×10^3	—
2	ACG^{r}8	25	1.7×10^7	1.8×10^7	—
3	PAA^{r}5	15	1.5×10^7	1.2×10^6	—
4	PAA^{r}5	25	ND	2.3×10^6	—
5	KOS + ACG^{r}8	25, 25 (1:1)	2.0×10^7	4.0×10^4	450
6	KOS + ACG^{r}8	25, 7 (3.6:1)	2.2×10^7	5.2×10^3	—
7	KOS + ACG^{r}8	7, 25 (1:3.6)	1.6×10^7	4.5×10^5	40
8	KOS + PAA^{r}5	25, 15 (1.7:1)	2.0×10^7	6.9×10^4	17.4
9	KOS + PAA^{r}5	25, 5 (5:1)	3.7×10^7	1.2×10^4	—
10	KOS + PAA^{r}5	7, 15 (1:2.1)	2.0×10^7	3.6×10^5	3.3
11	KOS + PAA^{r}5	25, 25 (1:1)	ND	5.2×10^5	4.4
12	KOS + PAA^{r}5	5, 25 (1:5)	ND	1.1×10^6	2.1
13	PAA^{r}5 + ACG^{r}8	15, 25 (1:1.7)	2.2×10^7	2.5×10^5	4.8
14	PAA^{r}5 + ACG^{r}8	15, 7 (2.1:1)	1.4×10^7	5.3×10^5	—
15	PAA^{r}5 + ACG^{r}8	5, 25 (1:5)	2.0×10^7	3.9×10^5	3.5
		Experiment II			
1	PAA^{r}5	5	ND	3.6×10^6	—
2	ACG^{r}8	5	ND	1.2×10^7	—
3	ACG^{r}35	5	ND	6.8×10^6	—
4	ACG^{r}4	5	ND	3.2×10^7	—
5	PAA^{r}5 + ACG^{r}8	5, 5 (1:1)	ND	1.9×10^6	1.9
6	PAA^{r}5 + ACG^{r}35	5, 5 (1:1)	ND	1.1×10^6	3.3
7	PAA^{r}5 + ACG^{r}4	5, 5 (1:1)	ND	4.1×10^6	0.88
8	ACG^{r}8 + ACG^{r}35	5, 5 (1:1)	ND	1.3×10^7	0.52
9	ACG^{r}8 + ACG^{r}4	5, 5 (1:1)	ND	1.2×10^7	1
10	ACG^{r}4 + ACG^{r}35	5, 5 (1:1)	ND	3.2×10^7	0.21

* ND, not determined.

† Fold reduction = (PFU/ml, single infection in acyclo-Guo)/(PFU/ml, mixed infection in acyclo-Guo). This value represents the reduction in titer in mixed infections relative to that in infections with a single acyclo-Guo-resistant mutant in the presence of acyclo-Guo (e.g., compare experiment I, samples 2 and 5: the yield of virus in the presence of acyclo-Guo in sample 5 is $1/450$th that with ACG^{r}8 alone in sample 2). In mixed infections in which both mutants were acyclo-Guo-resistant (e.g., experiment I, sample 13), the reduction was calculated by using the *less-resistant* mutant of the pair (sample 13 is thus compared with sample 3).

ACG^{r}4, which is also resistant to PAA (Table 1), did not appear to complement PAA^{r}5, ACG^{r}8, or ACG^{r}35 (Table 3, experiment II, samples 7, 9, 10 and control samples 1–4).

DISCUSSION

The data presented here demonstrate the existence of two distinct loci conferring resistance to acyclo-Guo in HSV-1. The first of these loci is the HSV *TK* gene; mutations that significantly decrease TK activity render the virus acyclo-Guo resistant (4) (Fig. 1; Table 1). Recombination analysis indicates that such mutations map to one locus which corresponds to the known physical location of the *TK* gene (unpublished data). We term this locus *ACG^r-TK*.

The second locus is defined by the properties of the mutant PAA^{r}5. This virus is resistant to acyclo-Guo, yet it exhibits wild-type levels of TK activity. It recombines efficiently with partially resistant *ACG^r-TK* viruses, yielding more highly resistant recombinant progeny. Furthermore, in the presence of acyclo-Guo, PAA^{r}5 complements *ACG^r-TK* mutants, producing wild-type, drug-sensitive gene products that inhibit viral replication. Thus, the *ACG^r* locus in PAA^{r}5, which we term *ACG^r-PAA*, appears to be a structural gene other than *TK*. These data imply therefore that the wild-type virus encodes a gene product—other than TK—that renders the virus sensitive to acyclo-Guo.

The most likely candidate for the *ACG^r-PAA* locus is the gene for the HSV-specified DNA polymerase. The *ACG^r-PAA* locus in PAA^{r}5 is closely linked to the *PAA^r* locus. Resistance to PAA is a recognized marker for the gene for HSV DNA polymerase (8, 13–15). Although our measurement of linkage is not yet sufficiently fine to place the *ACG^r-PAA* locus within the same gene as the *PAA^r* locus, the simplest interpretation of the data is that resistance to both drugs is specified by a mutant DNA polymerase and that a single mutation gave rise to both resistances. Buttressing this interpretation are results of studies by Schnipper and Crumpacker (16) who have described two other *PAA^r* mutants that are acyclo-Guo-resistant yet induce wild-type levels of TK. This interpretation is consistent with the findings of Elion *et al.* (2) that acyclo-GTP inhibits HSV DNA polymerase 10–30 times more effectively than cellular DNA polymerase.

Elion *et al.* (2) suggest that acyclo-GTP not only is an inhibitor of HSV DNA polymerase but also may act as a substrate for this enzyme, causing chain termination upon insertion into replicating DNA. Recent studies by Furman *et al.* (17) indicate that, at least *in vitro*, acyclo-GTP can serve both as a competitive inhibitor and a substrate for HSV DNA polymerase. Some of our data may shed light on the role of acyclo-GTP.

The dominance or recessiveness of a drug-resistant phenotype often reflects the mechanism of action of a drug. Often when resistance is recessive, as in the case of *ACG^r-TK* mutants (Table 3), the wild-type gene product (in this case, TK) utilizes

the drug as a substrate (4). Alternatively, when resistance is at least partially dominant, the wild-type gene product frequently is inhibited by the drug without utilizing it. This, for example, is the case with PAA-resistant viral DNA polymerase (14, 18, 19). Our data (Table 3) indicate that the acyclo-Guo resistance conferred by PAA^{r}5 is at least partially dominant. This supports the notion that acyclo-GTP inhibits the HSV DNA polymerase without necessarily being utilized by the enzyme. However, our data are also consistent with the suggestion of Furman *et al.* (17) that utilization of acyclo-GTP causes inactivation of wild-type enzyme via nondissociative binding to template terminating with an acyclo-Guo moiety. Regardless, future studies on the mechanism of action of acyclo-Guo should take into account the partial dominance of the *ACG^r-PAA* allele.

Our results raise the question of whether all mutations that render a virus resistant to PAA also render it resistant to acyclo-Guo. In the present study, PAA^{r}5 and, apparently, ACG^{r}4 possess mutations that render them PAA resistant and at least partially acyclo-Guo resistant. In the case of ACG^{r}4, this resistance is manifested by its apparent failure to complement PAA^{r}5 for growth inhibition (Table 3) and by the virus being more resistant than other *ACG^r-TK* mutants (Fig. 1). This highly resistant character of ACG^{r}4 is mimicked by recombinant viruses produced by crosses between ACG^{r}8 [which, like ACG^{r}4, induces little or no TK activity (Table 1)] and PAA^{r}5 (unpublished results). In contrast, however, tsD9, a temperature-sensitive mutant of KOS which is PAA^r (8) and exhibits greatly reduced TK activity, is not highly resistant to acyclo-Guo (unpublished results) as is ACG^{r}4 or as are recombinant viruses which contain both types of ACG^r mutations (Table 2 and unpublished results). This suggests that two classes of PAA^r mutants exist: those that render the virus resistant to acyclo-Guo and those that do not.

The existence of two loci for acyclo-Guo resistance may have clinical significance. There is evidence that *ACG^r-TK* mutants are less pathogenic than their parents (20). Although the *ACG^r-PAA* locus in PAA^{r}5 confers only partial resistance and although *ACG^r-TK* mutants arise more frequently *in vitro* than do *ACG^r-PAA* mutants (unpublished results), *ACG^r-PAA* mutants may be more pathogenic *in vivo*. The frequency with which *ACG^r* mutants in either locus arise upon treatment with acyclo-Guo and the pathogenicity of such mutants remain to be determined.

Note Added in Proof. Because resistance to acyclo-Guo in *ACG^r-TK* mutants is recessive, henceforth we suggest the use of small letters to designate this locus: "*acg^r-tk*." Because the *ACG^r-PAA* mutant behaves as though it were codominant, we wish to retain the use of capital letters in designating this locus.

By using partially purified preparations, the DNA polymerase activities induced by both PAA^{r}5 and ACG^{r}4 have been shown to be more resistant to inhibition by acyclo-GTP than is wild-type DNA polymerase (P. A. Furman and M. H. St. Clair, personal communication).

The authors wish to acknowledge the excellent technical assistance of P. A. Temple and L. B. Sandner and manuscript preparation by J. G. Driscoll. This investigation was supported by Research Grant CA20260 from the National Cancer Institute. D.M.C. was a Fellow in Cancer Research supported by Grant DRG-257-F from the Damon Runyon–Walter Winchell Cancer Fund.

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