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Resistance of herpes simplex virus to acycloguanosine: Role of viral thymidine kinase and DNA polymerase loci

(antiviral chemotherapy/drug-resistant mutants/selection of resistant virus)

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ABSTRACT **Acycloguanosine [9-(2-hydroxyethoxymethyl)guanine; acyclo-Guo] is a potent inhibitor of herpes simplex viruses (HSV); it is selectively phosphorylated in virus-infected cells. In order to define those viral functions that may mediate resistance to acyclo-Guo, the drug sensitivities of temperature-sensitive (*ts*) and phosphonoacetic acetic acid (PAA)-resistant mutants of HSV-1 and HSV-2 have been determined. Two distinct viral genetic loci are independently associated with acyclo-Guo resistance. Mutations resulting in diminished thymidine kinase activity are associated with resistance to inhibition by acyclo-Guo. Several PAA-resistant viruses that express wild-type levels of thymidine kinase activity are also resistant to acyclo-Guo. This suggests the importance of the viral DNA polymerase region in mediating acyclo-Guo resistance and is consistent with a close relationship between the *PAA*^{r} mutation site and the *ACG*^{r} locus. When wild-type HSV-1 is serially propagated under the selective pressure of acyclo-Guo, rapid emergence of resistant virus occurs, accompanied by the simultaneous appearance of thymidine kinase-deficient progeny.**

The nucleoside analogue 9-(2-hydroxyethoxymethyl)guanine [acycloguanosine (acyclo-Guo); Acyclovir] is a potent inhibitor of herpes simplex virus (HSV) replication (1–3). It is effective in the treatment of herpes infections in experimental animals (2) and has a high therapeutic ratio (2). Plaque formation by HSV is very sensitive to inhibition by the drug. Isolates from human infections are inhibited by acyclo-Guo at a mean 50% inhibitory dose (ID_{50}) of 0.15 μM for HSV type 1 (HSV-1) and 1.62 μM for HSV type 2 (HSV-2) in a plaque reduction assay (3).

acyclo-Guo must be phosphorylated in order for it to be an effective inhibitor of HSV (1). In the infected cell, viral thymidine kinase (TK) converts it to acyclo-GMP which is further phosphorylated to acyclo-GTP, an inhibitor of viral DNA synthesis (1). A temperature-sensitive (*ts*) mutant that fails to induce viral TK (*ts A1*) is relatively resistant to inhibition by acyclo-Guo (1), as is human cytomegalovirus, which does not induce a specific viral TK (3, 4). To identify other viral functions that might play a role in resistance to acyclo-Guo, mutants of HSV-1 containing defects in viral functions have been tested for sensitivity to acyclo-Guo.

This report presents evidence supporting the presence of two independent genetic loci in HSV-1 that mediate resistance of the virus to acyclo-Guo. These are the viral TK region and the viral DNA polymerase region of the genome that is associated with resistance to phosphonoacetic acid (PAA).

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MATERIALS AND METHODS

Cells and Viruses. The Vero line of African green monkey kidney cells was obtained from Microbiological Associates (Walkerville, MD) and maintained in medium 199 containing 2% fetal calf serum and antibiotics (penicillin, 250 units/ml; streptomycin, 250 μg/ml). Cell lines were free of mycoplasma when tested in Hayflick's medium.

HSV-1 strain KOS and *ts* mutants derived from this strain were a generous gift from Priscilla Schaffer. The mutants *ts* A1 and *ts* B2 fail to induce viral TK at the nonpermissive temperature (39°C) (5, 6). The *ts* C4, *ts* C7, and *ts* D9 mutants all possess thermolabile viral DNA polymerase activity and fail to synthesize viral DNA at the nonpermissive temperature (7). Several other mutants of HSV-1 KOS with known phenotypes were also studied (*ts* M19, *ts* K13, *ts* 022, and *ts* J12). All the viruses were grown in Vero cells by low-multiplicity inoculation (10^{-2}) and incubation at the permissive temperature (34°C). Pools were assayed by plaque formation in Vero cells, and all possessed a high titer [2×10^7 to 8×10^8 plaque-forming units (PFU)/ml]. A pyrimidine deoxyribonucleoside kinase mutant of HSV-1 (strain 17) $dPyK^{-7}$ deficient in viral TK and deoxycytidine kinase activities (isolated by the late A. T. Jamieson, Glasgow, Scotland) was also tested (8). Three independently derived *PAA*^{r} mutants were tested for acyclo-Guo sensitivity: HSV-1 *PAA*^{r}-5 (KOS) from Priscilla Schaffer (9), HSV-2 *PAA*^{r}-B1 (strain HG52) from Glasgow, Scotland, and *PAA*^{r}-K isolated in this laboratory from the KOS strain by plaque purification of virus grown in PAA at 100 μg/ml (10).

Drugs. acylo-Guo was supplied by Burroughs Wellcome (Research Triangle Park, NC) with the cooperation of the Antiviral Substances Program of the National Institute of Allergy and Infectious Diseases. A 10 mM stock solution of acyclo-Guo was prepared in distilled water and stored at −20°C. PAA (disodium salt) was the generous gift of Abbott Laboratories.

Determination of Antiviral Activity. Resistance of HSV to acyclo-Guo was determined by described methods (3). Briefly, Vero cell monolayers were prepared in six-well (35 mm) plastic tissue culture plates (Linbro, Flow Laboratories Inc., McLean, VA). When monolayers were confluent, they were inoculated with 100 PFU in 0.2 ml of medium. After 1 hr at 37°C, the inoculum was removed and 3 ml of 1% methycellulose containing 2% serum and appropriate acyclo-Guo concentrations was added. After 5 days in a 5% CO_2 incubator, monolayers were fixed and stained, and the plaques were counted. The ID_{50} for acyclo-Guo was based on the number of plaques present in

Abbreviations: acyclo-Guo, acycloguanosine; PAA, phosphonoacetic acid; HSV, herpes simplex virus; TK, thymidine kinase; *ts*, temperature sensitive; ID_{50}, 50% inhibitory dose; PFU, plaque-forming units.

the control wells without drug and was calculated directly from the semilogarithmic plot of surviving plaques in the presence of increasing concentrations of acyclo-Guo. The plot of percentage survivors against logarithm of acyclo-Guo concentration usually is linear; when necessary, a linear plot was obtained by the method of a least-squares fit.

Resistance to PAA was determined by performing a plaque assay on Vero cells in the presence and absence of PAA at 100 μg/ml. The efficiency of plating is the ratio of virus titers in the presence and absence of drug (+PAA/−PAA). It was 0.8 for *PAA*^{r}-5, 0.7 for *PAA*^{r}-B1, 0.65 for *PAA*^{r}-K, 0.004 for HSV-2 (strain HG-52), and 0.001 for HSV-1 (KOS).

Selection of *ACG*^{r} Virus by Passage in acyclo-Guo. HSV-1 resistant to acyclo-Guo (*ACG*^{r}) was selected by serial passage in the presence of the antiviral drug. Confluent Vero cell monolayers were infected with a cloned clinical isolate of HSV-1, at 1 PFU per cell, and incubated at 37°C in a 5% CO_2 atmosphere in the presence of 10 μM acyclo-Guo. At maximal cytopathic effect, the cultures were harvested and clarified, and the titer was determined. Five serial passages were made at 1 PFU per cell in 10 μM acyclo-Guo, and the sensitivities (ID_{50}) of the progeny were determined after each passage.

Viral TK Assays. The method of Jamieson *et al.* (11) was used. Vero cells were grown to confluence in 60-mm^2 petri dishes and infected with the appropriate HSV at 5 PFU per cell. After a 1-hr adsorption period, the monolayers were washed with phosphate-buffered saline (pH 7.4), and harvested by scraping at 14 hr after infection. The cell pellet was washed twice in buffered saline, once in 10 mM Tris/5 mM 2-mercaptoethanol/5 μM thymidine, pH 7.5, sonicated at 10 kHz for 60 sec with a Branson Sonifier, and centrifuged at 11,000 rpm in a Sorvall RC-15 centrifuge, and the supernatant fluid was used for the TK assay. The assay mixture had a total volume of 0.1 ml and contained 50 μl of the enzyme sample, 10 mM ATP, 10 mM $MgCl_2$, 0.2 M phosphate buffer (pH 6.0), and 10 μM [^{3}H]thymidine (50 Ci/mmol; New England Nuclear). The assay mixture was incubated for 15 min at 37°C, and the reaction was stopped by immersion in boiling water. After cooling in an ice bath, duplicate 10 μl samples were plated onto Whatman DE81 paper discs. The discs were air dried and then washed in 4 mM ammonium formate, pH 4.0/10 μM thymidine for 7 min at 37°C. The discs were then washed in 4 mM ammonium formate at 37°C, washed in distilled water, dried, and assayed for radioactivity in a Beckman L200 liquid scintillation counter. TK activity was calculated and normalized for the amount of protein present in the control sample as determined by the method of Lowry *et al.* (12) with bovine serum albumin for the standard curve.

RESULTS

acyclo-Guo Sensitivity of TS Mutants of HSV-1. Plaque reduction tests were performed on several previously characterized DNA$^-$, DNA$^\pm$, and DNA$^+$ *ts* mutants of HSV-1 (6), with increasing concentrations of acyclo-Guo in the overlying medium. Table 1 shows the relative sensitivities of each virus to acyclo-Guo as well as their viral DNA synthesis, TK, and DNA polymerase phenotypes. Mutants *ts* A1 and *ts* B2 were DNA$^-$ at 39°C, deficient in TK production at permissive and nonpermissive temperatures, and resistant to acyclo-Guo at 34°C. The kinetics of inhibition of HSV-1 *ts* A1 and *ts* B2 in relation to KOS are shown in Fig. 1. Another mutant of HSV-1, dPyK^{-7}, isolated by Jamieson (8) was TK$^-$ and acyclo-Guo resistant as well. The ID_{50}s for *ts* A1, *ts* B2, and dPyK^{-7} were 52, 28.5, and 27 μM, respectively, compared to 0.72 μM for the KOS wild type. Because these mutants induce viral DNA polymerase at the permissive temperature (7), the importance of viral TK activity to the antiviral effect of acyclo-Guo is emphasized. *ts* mutants belonging to complementation groups M, K, O, and J were sensitive to acyclo-Guo and their ID_{50}s ranged between 0.36 and 2.3 μM (Table 1).

Table 1. Effects of acyclo-Guo on mutants of HSV-1

Virus	ID_{50}, μM	DNA phenotype*	TK activity, %†	DP phenotype‡
HSV-1 (KOS)	0.72	+	100	+
ts A1	52.0	−	7.6	+
ts B2	28.5	−	8	±
dPyK^{-7}§	21.0	+	0	+
ts C4	6.0	−	51	−
ts C7	1.26	−	109	−
ts D9	18.0	−	20	−
ts M19	0.36	±	130	ND
ts K13	2.25	±	50	ND
ts O22	2.25	±	92	±
ts J12	2.3	+	66	+

* Viral DNA phenotypes of *ts* mutants at 39°C (6, 7).
† The activity of each mutant is expressed as a % of the wild-type virus activity after subtraction of mock-infected cell activity [8% of HSV-1 (KOS)]. TK activity was normalized to the amount of protein present in the wild-type control sample.
‡ Viral DNA polymerase activity at 39°C (6, 7). ND, not done.
§ ID_{50} was determined in Glasgow, Scotland, on BHK C13 cells. TK activity was determined in (TK$^-$/dCK$^-$) cells (8).

Two mutants in the C complementation group had different sensitivities to acyclo-Guo in that *ts* C7 was inhibited by the antiviral agent at a lower concentration than *ts* C4 (Table 1; Fig. 1). Both viruses were defective in induction of the viral DNA polymerase, and they induced levels of TK activity equivalent to or greater than those induced by drug-sensitive *ts* mutants belonging to complementation groups M, K, O, and J (Table 1). The moderate acyclo-Guo resistance of *ts* C4 may therefore be related to inefficiency of its TK at phosphorylating the drug or to the presence of the mutation rendering it deficient in DNA polymerase activity. Mutant *ts* D9 was defective in TK and DNA polymerase expression (7) and had an acyclo-Guo ID_{50} of 18 μM (Table 1). Due to the presence of two defects, the contribution of each mutation to the acyclo-Guo-resistant phenotype of *ts* D9 cannot be evaluated.

***In Vitro* Selection of Viruses Resistant to acyclo-Guo.** Earlier studies documented the uniform sensitivity of clinical isolates of HSV-1 to this nucleoside analogue (3). To determine the rapidity with which acyclo-Guo resistance develops in a population of HSV, a cloned clinical isolate of HSV-1 sensitive to the drug (ID_{50} = 0.16 μM) was used to infect Vero cells at a

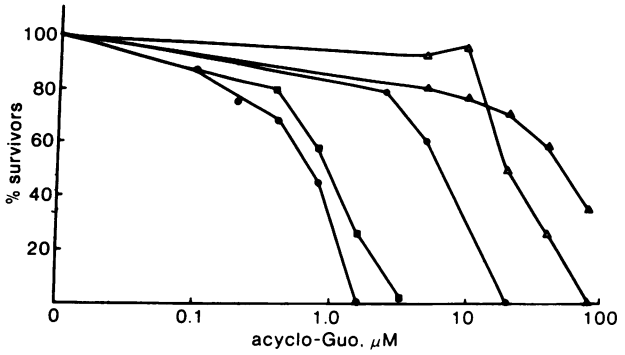

FIG. 1. Inhibition by acyclo-Guo of plaque formation by wild-type HSV-1 or selected *ts* mutants. ●, HSV-1 (KOS); △, *ts* B2; ▲, *ts* A1; ○, *ts* C4; ■ *ts* C7.

multiplicity of infection of 1 PFU per cell, in the presence of 10 μM acyclo-Guo. At maximal cytopathic effect the progeny were harvested and quantitated by plaque titration, and four subsequent serial passages were carried out in the same concentration of this agent. The observed titer of the HSV-1 from passages 1 through 5 were 6.8×10^5, 3.3×10^6, 3.1×10^7, 3.5×10^6, and 1.25×10^7 PFU/ml, respectively. In this series, growth in acyclo-Guo resulted in rapid emergence of resistant HSV-1 (Fig. 2). A population of HSV-1 that was 30-fold more resistant than the parent virus emerged after the initial exposure to acyclo-Guo. Subsequently, the resistance to acyclo-Guo increased, reaching a plateau by passage 3. The earlier observations from this and other laboratories that TK-deficient HSV are resistant to acyclo-Guo (1) suggest the likelihood of selecting for such particles with high concentrations of this nucleoside analogue. Accompanying the prompt selection of drug-resistant viruses was a parallel reduction in the expression of viral-specific TK activity. From passages 2 through 5, the TK activity ranged between 3.5 and 7.6% of the control. Of interest is the increase in ID_{50} between passages 2 and 5, from 48 to 82 μM, with little or no decrease in the level of TK activity within these populations.

Effect of acyclo-Guo on PAA-Resistant Mutants of HSV-1. PAA-resistant HSV-1 was derived by propagating the HSV-1 strain KOS in PAA at 100 μg/ml, harvesting progeny from the resulting plaques, and passing the virus in the absence of the drug (10). After two serial passages, one of the PAA mutants selected, *PAA*^{r}-K, demonstrated an efficiency of plating in PAA (100 μg/ml) of 0.65, compared with 0.004 for parent virus. In addition to *PAA*^{r}-K, the PAA-resistant HSV-1 mutant *PAA*^{r}-5 (9) and the HSV-2 mutant *PAA*^{r}-B1, were studied. The three

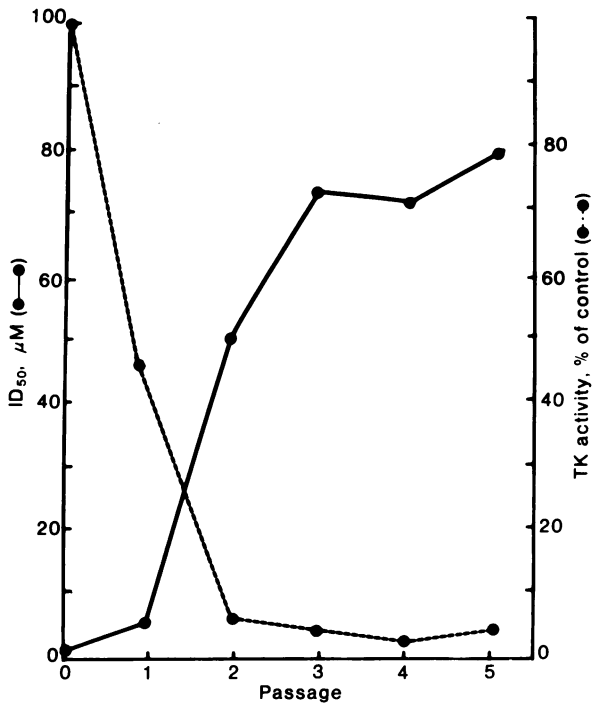

FIG. 2. Selection of acyclo-Guo-resistant HSV-1 during serial passage in the presence of the drug. Vero cells were infected with a cloned isolate of HSV-1 (1 PFU/cell) in the presence of 10 μM acyclo-Guo and harvested at maximal cytopathic effect; HSV-1 progeny were passaged under identical conditions. At each passage samples were assayed for sensitivity to acyclo-Guo and viral TK activity. Viral TK activity is expressed as a percent of the wild-type HSV-1 level. The TK activity of mock-infected Vero cells was 7.6% of that of wild-type HSV-1, and has been substracted from the total activity observed in virus-infected cells.

Table 2. Resistance of HSV *PAA*^{r} mutants to acyclo-Guo

Virus	ID_{50}, μM	TK activity,* %
HSV-1 KOS	0.72	100
PAA^r-5 (9)	23.0	105
PAA^r-K	8.2	90
HSV-2 (strain HG 52)	1.1	100
PAA^r-B1 (strain HG 52)	7.2	109

* Viral specific TK activities were determined as described in Table 1, by the method of Jamieson *et al.* (11).

PAA^r viruses were resistant to acyclo-Guo when compared to the parent HSV-1 or HSV-2 strains, and each expressed a level of TK activity comparable to that of the wild-type virus (Table 2). The finding that resistance to both PAA and acyclo-Guo is expressed in three independently isolated TK$^+$ viruses suggests that a mutation in both HSV-1 and HSV-2 conferring resistance to PAA is associated with acyclo-Guo resistance as well.

DISCUSSION

Mutants of HSV-1 defective in expression of viral TK, DNA polymerase, or other functions were evaluated for their sensitivity to acyclo-Guo. The results extend the findings of Elion *et al.* (1) that indicated that HSV TK activity is necessary for plaque inhibition by acyclo-Guo. Two of the mutants, HSV-1 *ts* A1 and HSV-1 *ts* B2, have a TK$^-$ phenotype at the permissive temperature (5) and induce low levels of a 44,000-dalton polypeptide (6). This polypeptide has the size of the HSV-induced TK and its presence correlates with viral TK activity (13). These mutants induce wild-type levels of viral DNA polymerase activity at the permissive temperature (7) and are 20 to 40-fold more resistant than wild-type HSV-1 (KOS) to acyclo-Guo. The dPyK^{-7} mutant, isolated in Glasgow (by A. T. Jamieson) by passaging HSV-1 (strain 17) in the presence of BrUrd, is deficient in TK and deoxycytidine kinase activities and is resistant to acyclo-Guo (8). The physical map location of the mutation dPyK^{-7} on the HSV genome has been defined by marker rescue (14). The inability of acyclo-Guo to inhibit three independently selected TK$^-$ viruses known to possess defects in viral TK activity indicates the essential role of the viral TK locus in determining resistance to acyclo-Guo.

The serial passage of a cloned isolate of HSV-1 in the presence of acyclo-Guo (10 μM) rapidly selects for acyclo-Guo-resistant progeny. The acyclo-Guo ID_{50} increases from 01.6 μM for the parent virus to >58 μM after three passages. There is a concomitant stepwise decrease in viral TK activity. Growth in acyclo-Guo appears to result in the selection of a population of HSV-1 that is deficient in TK expression. Beyond passage 3 there is nearly complete absence of measurable TK activity, but the level of resistance to acyclo-Guo continues to increase. Further studies are required to determine whether increasing resistance to acyclo-Guo in the presence of profound TK deficiency is related to a defect in the expression of another viral gene locus.

Three of the mutant viruses tested for acyclo-Guo sensitivity (*ts* C4, *ts* C7, and *ts* D9) are deficient in the induction of viral DNA polymerase activity at both permissive and nonpermissive temperatures (7). Although mutants *ts* C4 and *ts* C7 are in the same complementation group (15) and both induce viral TK activity, only mutant *ts* C4 is resistant to acyclo-Guo. A variation in the level of viral TK expression may not be the only way to explain the different sensitivities of *ts* C4 and *ts* C7 to acyclo-Guo because other *ts* mutants inducing TK activities comparable to those of *ts* C4 are inhibited by lower concentrations of drug. An altered proficiency of the viral TK at phosphorylating acyclo-Guo might explain this observation,

but there are no data to support this possibility. Another explanation for the resistance of mutant *ts* C4 to acyclo-Guo might be the mutation in the DNA polymerase locus of *ts* C4. The different acyclo-Guo sensitivities of *ts* C4 and *ts* C7 may be explained by different physical map positions of these mutations within the DNA polymerase locus as determined by marker rescue with intertypic recombinant viruses (16). The mutant *ts* D9 has a mutation in the viral DNA polymerase locus (7) and induces decreased amounts of viral TK activity. Therefore, the function(s) responsible for acyclo-Guo resistance in *ts* D9 is unclear.

Further evidence that the DNA polymerase locus is a second site mediating acyclo-Guo resistance is provided by the observations that PAA-resistant mutants of HSV-1 and HSV-2 are also resistant to acyclo-Guo (Table 2). Since these resistant viruses induce normal levels of viral TK activity, decreased TK activity cannot be invoked to explain the resistance to acyclo-Guo. Selective inability of the viral TK to recognize acyclo-Guo as a substrate is improbable because resistance is observed in three independently isolated TK^+/PAA^r HSV-1 mutants. The PAA^r phenotype presumably is the result of a mutation in the structural gene for viral DNA polymerase (9, 17) and the association of PAA^r and acyclo-Guo resistance suggests that a second locus for acyclo-Guo^r also resides in the DNA polymerase region of HSV.

Because the PAA^r mutation in HSV segregates as a single marker in genetic cross studies (18), and the mutations *ts* D9 and PAA^r appear to be the result of a single mutation (9, 17), it is likely that there is only one PAA^r mutation site in the HSV genome. Therefore it is probable that an ACG^r mutation is closely related to the PAA^r mutation in the DNA polymerase locus. These findings are in agreement with those of Coen and Schaffer (19) and confirm the importance of the PAA^r mutation in the DNA polymerase region to the resistance of HSV to acyclo-Guo.

The role of other genetic loci in determining acyclo-Guo resistance cannot be excluded. However, several other *ts* mutants were tested for sensitivity to acyclo-Guo (HSV-1 *ts* J12, *ts* M19, *ts* 022, and *ts* K13). These mutants induce the viral TK, differ in their ability to synthesize DNA at the nonpermissive temperature (5, 7), and are inhibited by low concentrations of acyclo-Guo (ID_{50}, 0.36–2.3 μM). The polypeptide profiles induced by these mutants differ from those of the wild-type KOS in many respects, but the nature of the mutations have not been functionally defined (6). In any case, these mutations do not appear to confer resistance to acyclo-Guo.

This report provides evidence for the importance of the virus-specific TK and DNA polymerase gene functions in determining sensitivity to acyclo-Guo. Mutations or alterations affecting expression of either of these enzyme activities can confer resistance to acyclo-Guo. The rapid emergence of resistance to acyclo-Guo after growth in the presence of the drug is associated with loss of TK activity and may have importance for the clinical use of the drug. There is no current evidence suggesting that growth of virus in the presence of acyclo-Guo results in selection of mutants expressing altered DNA polymerase activity. The nature of the relationship of PAA^r and ACG^r mutations in the DNA polymerase locus must await precise mapping studies, but our data suggest that they may be closely linked. Further definition of the role of the viral DNA polymerase in the development of acyclo-Guo resistance requires studies on the interaction of acyclo-GTP with the purified enzyme.

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