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Susceptibility of Phosphonoformic Acid-Resistant Herpes Simplex Virus Variants to Arabinosylnucleosides and Aphidicolin

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A plaque-reduction assay was used to examine the susceptibility of five phosphonoformic acid-resistant variants of herpes simplex virus type 1 to arabinosylnucleosides and aphidicolin. These viruses were cross-resistant to arabinosylhypoxanthine and to arabinosyladenine when tested in the absence of deoxycoformycin, a deaminase inhibitor. In the presence of deoxycoformycin, no cross-resistance between arabinosyladenine and phosphonoformic acid was observed. The two variants tested were cross-resistant to arabinosylthymine, and all five variants were collaterally susceptible to aphidicolin inhibition.

In recent years, several anti-herpes simplex virus (HSV) agents have been discovered which depend on HSV-specified enzymes for activity. They can be divided into two general categories: those metabolized by HSV deoxythymidine kinase (HSV-TK) and those which are not (4). The activity of many of these agents can be changed through alterations in the biochemical properties of virus enzymes, and variants with different drug susceptibilities have been isolated and characterized by many laboratories (5–7, 10–15). A recent study claimed that mutation in the HSV DNA polymerase gene conferred marked resistance to arabinosyladenine (araA) (6), whereas other investigators have reported difficulty in isolating variants highly resistant to this agent (14, 21). Several of the araA-resistant variants described were cross-resistant to another anti-HSV agent, phosphonoacetic acid, which also exerts its action on HSV DNA polymerase (9, 13). Studies which describe araA-resistant HSV variants have not included an adenosine deaminase inhibitor (6, 19) which could prevent the conversion of araA to arabinosylhypoxanthine (araHx), a less active antivirus compound (16, 20, 22). Therefore, it is conceivable that the araA resistance observed by others could actually reflect decreased susceptibility of HSV to araHx. This communication describes a reexamination of araA resistance, using a group of five variants which were independently selected in 1 mM phosphonoformic acid (PFA), a congener of phosphonoacetic acid. The mechanism of PFA resistance is the induction of altered HSV DNA polymerase in cells infected with these viruses (8). Also, we report a preliminary study with aphidicolin, an agent which is a specific inhibitor of replicative DNA polymerase, including HSV DNA polymerase (2, 18). Since the HSV variants used in this study induce altered DNA polymerase, we examined these viruses for altered susceptibility to aphidicolin.

MATERIALS AND METHODS

Drug susceptibility was tested by a plaque-reduction assay. Confluent monolayers of Vero cells in 7-cm^2 plastic wells (Linbro) were inoculated with 150 or 1,500 PFU of virus in 0.2 ml of RPMI 1640 medium supplemented with 1% calf serum (GIBCO Laboratories). After 30 min of adsorption at 37°C, the inoculum was replaced with 1 ml of medium supplemented with carboxymethylcellulose to 1% and drugs at various concentrations. Each treatment was done in duplicate. The cells were fixed and stained after 2 days with 0.8% crystal violet in 50% ethanol, and the wells were examined microscopically for plaques. Determinations of drug susceptibility were repeated at least twice on separate occasions, and reproducible results were obtained. Stock solutions of drugs were dissolved in water, filter sterilized, and stored at −20°C, except for aphidicolin, which was made 30 mM in dimethyl sulfoxide.

RESULTS AND DISCUSSION

The dose responses of variants PFA^r-1 to PFA^r-5 and a fresh plaque-purified isolate of the parent strain (KOS) tested for inhibition by araA and an adenosine deaminase inhibitor, deoxycoformycin (dCF), by araA alone, and by araHx are shown in Fig. 1A, B, and C, respectively. The concentration of dCF used (3.5 μM) had no antiviral activity. A comparison among drugs for activity against the KOS strain shows that araHx was less potent than araA when tested alone (Fig. 1C compared with Fig. 1B) or in

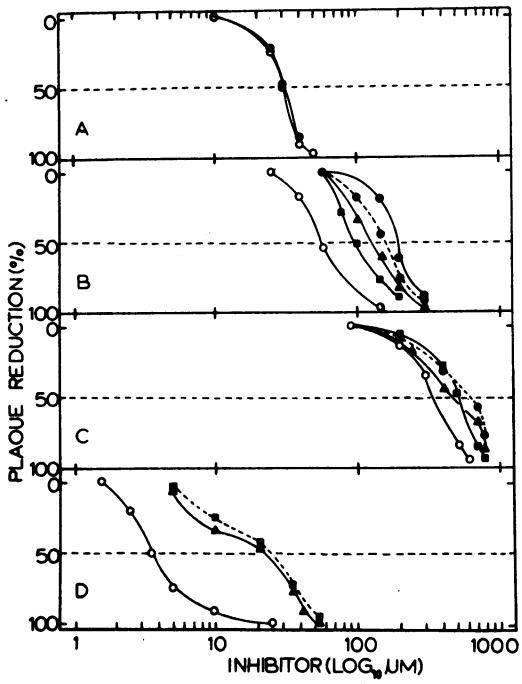

FIG. 1. Susceptibilities of viruses to arabinosylnucleotides, using a plaque-reduction assay. Inhibition of plaque formation by araA and 3.5 μM dCF (A), araA alone (B), araHx (C), and araT (D). Results are expressed as plaque number in the presence of drug given as a percentage of plaques in control, untreated cultures. Symbols: ○, KOS; ●——●, PFA^r-1; ■——■, PFA^r-2; ▲, PFA^r-3; ■------■, PFA^r-4; ●------●, PFA^r-5. For clarity, the data in (A) shows only the result for the parent virus and for PFA^r-1; however, the other variants had the same susceptibility to araA. When araA was tested alone (B), PFA^r-4 was indistinguishable from PFA^r-2. For araHx (C), PFA^r-1 and PFA^r-4 were indistinguishable from PFA^r-2.

combination with dCF (Fig. 1C compared with Fig. 1A). dCF increased the potency of araA (Fig. 1A compared with Fig. 1B), suggesting that cellular adenosine deaminase played an important role in determining the activity of araA. These results are consistent with previous reports (16, 20, 22). A comparison among viruses for each drug shows that all viruses were equally susceptible to araA tested in the presence of dCF (Fig. 1A). However, the variants were less susceptible to araA alone since plaque formation by these viruses was unaffected by 55 μM araA, which was the 50% effective dose for KOS (Fig. 1B). A similar result was obtained with araHx (Fig. 1C). In this case, the degree of resistance did not appear as marked, but this could partly reflect a limitation of the plaque-reduction assay which has been discussed elsewhere (19), since the higher concentrations of araHx used were close to levels which made the scoring of plaques difficult after 2 days of incubation. The results from a yield-reduction assay confirmed that variants were resistant to araHx because growth of these viruses at 18 h postinfection was unaffected by 160 μM araHx, which was the 50% effective dose for KOS. The simplest interpretation of these results is that PFA resistance confers decreased susceptibility to araHx, but the data in Fig. 1B and C are not consistent with this proposal, since araA without dCF was much more active against the variants than araHx. This discrepancy could be explained if there was some interaction between the metabolites of araA and araHx on biochemical targets. HSV DNA polymerase may be involved because the arabinosylnucleotide triphosphates, araATP and araHxTP, are known to compete with different deoxynucleotide triphosphates for DNA polymerization (8, 9). Other biochemical targets could also be involved since HSV DNA polymerase is probably not the sole determinant of the antiviral action of araA (10; this communication).

Two of the variants (PFA^r-3 and PFA^r-4) were tested for susceptibility to arabinosylthymine (araT), and both were found to be less susceptible than KOS (Fig. 1D). HSV-TK is one determinant of araT sensitivity (1); however, both PFA^r-3 and PFA^r-4 induced activities of HSV-TK similar to those found in the KOS-infected cells (data not shown). This suggests that differences in the activity of HSV-TK were not responsible for the araT resistance we observed. Another determinant of araT sensitivity is HSV DNA polymerase (1), and it has also been suggested that susceptibility of HSV to araA corre-

TABLE 1. Sensitivity of purified HSV DNA polymerase to arabinosylnucleotide triphosphates in synthetic reactions

Analog	Competing substrate	K_i (μM) of HSV DNA polymerase from:[a]					
		KOS	PFA^r-1	PFA^r-2	PFA^r-3	PFA^r-4	PFA^r-5
araATP	dATP	0.29	0.78	1.50	1.60	1.30	0.48
araHxTP	dGTP	2.6	22	31	28	28	12
araTTP	dTTP	0.14	0.52	1.14	1.10	1.01	0.24

[a] Competitive inhibition analysis and the determination of apparent K_i values have been described previously (8). The apparent K_i's for araHxTP were given in the same report and are included here for comparison.

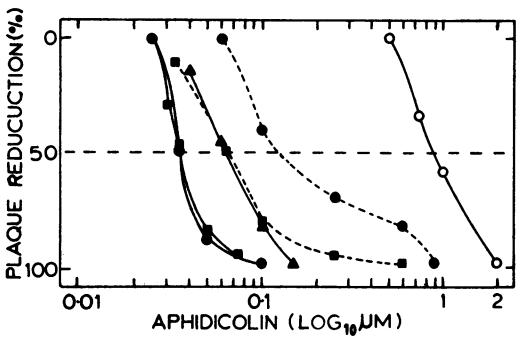

FIG. 2. Susceptibilities of viruses to aphidicolin, using a plaque-reduction assay. Results are expressed as plaque number in the presence of drug given as a percentage of plaques in control untreated cultures. Symbols: ○, KOS; ●——●, PFA^r-1; ■——■, PFA^r-2; ▲, PFA^r-3; ■------■, PFA^r-4; ●------●, PFA^r-5.

lated with the sensitivity of HSV DNA polymerase to araATP inhibition in vitro (6, 9). Therefore, the triphosphate derivatives of araA, araHx, and araT were tested against purified variant DNA polymerases to see if the biological and kinetic data were in agreement (Table 1). araATP, araHxTP, and araTTP all inhibited the incorporation of their competing substrates into DNA, but these triphosphates were less potent inhibitors of variant enzymes than the parent DNA polymerase. These results show that the changes in HSV DNA polymerase which were associated with PFA resistance (8) had also altered the binding behavior of arabinosylnucleotides, despite their base moiety. Furthermore, a general correlation between the kinetic and biological data did apply to araHx and araT, but not to araA. Kinetic alterations in HSV DNA polymerase which lower its affinity for araATP without affecting susceptibilities of variants to araA in culture have also been reported by others (10). These observations suggest that biochemical determinants other than the HSV DNA polymerase may be important for the antiviral action of araA. The data presented support the conclusion that the araA resistance described previously (6, 19) was probably due to omission of an adenosine deaminase inhibitor from experiments. However, our results clearly do not rule out kinetic alterations in HSV DNA polymerase as a mechanism for araA resistance in some cases. One possibility is that mutagenesis could increase the probability of such variants arising in a viral population.

A close examination of the apparent K_i values given in Table 1 and the biological data in Fig. 1 reveals a poor correlation between these results for some variants. The DNA polymerases induced by PFA^r-1 and PFA^r-5 had the highest affinity for araATP and araHxTP of the variant enzymes examined, but these two viruses were the least susceptible to araA and araHx in culture (Fig. 1B and C). This observation could be explained if these variants were capable of altering concentrations of adenosine deaminase in infected cells. There is a report which demonstrated that one strain of HSV had no effect on the levels of this enzyme in HeLa cells (3); however, this possibility has not been excluded for the variants described here.

Aphidicolin inhibition of virus plaque formation is shown in Fig. 2. The variants were more susceptible than KOS. Catalysis by DNA polymerases purified from variant-infected cells was also more sensitive to inhibition by aphidicolin than control reactions catalyzed by the parent enzyme (unpublished observation). The detailed information will be published elsewhere. Aphidicolin inhibition was not selective for the parent virus since the 50% inhibitory dose (0.3 μM) for Vero cell growth was similar to that concentration which reduced the yield of KOS by 50% (0.4 μM). In contrast, aphidicolin at 0.05 μM inhibited the yield of PFA^r-1 and PFA^r-2 by 50% and had a nominal effect on cell growth, which suggests that the drug may be acting selectively on these variants. These results could have some implication for antiviral chemotherapy, but it is not yet known whether polymerase variants will present a problem for effective treatment of disease associated with HSV infection. However, a recent study showed that variants resistant to 9-[2-hydroxyethoxy)methyl]guanine (acyclovir) were present in populations from clinical isolates and that some viruses possibly induced altered HSV DNA polymerase (17). The viruses used in this study behaved as other variants selected in PFA or phosphonoacetic acid (5, 10, 13, 15), and all five were cross-resistant to acyclovir (50% effective dose from plaque-reduction assay: KOS, 1.5 μM; PFA^r-1, 22 μM; PFA^r-2, 23 μM; PFA^r-3, 26 μM; PFA^r-4, 22 μM; PFA^r-5, 32 μM). If the increased susceptibility to aphidicolin is always associated with kinetic alterations in HSV DNA polymerase, then the use of aphidicolin in combination chemotherapy could be considered for treatment of HSV infections.

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