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Herpes Simplex Virus Mutants Resistant to Arabinosyladenine in the Presence of Deoxycoformycin

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Herpes simplex virus mutants PAA^{r}5, AraA^{r}6, AraA^{r}7, AraA^{r}9, and AraA^{r}13, which previously had been shown to be resistant to arabinosyladenine (araA) alone, were found to be resistant to araA in the presence of high concentrations of the adenosine deaminase inhibitor, deoxycoformycin. The marker conferring resistance to araA and deoxycoformycin in mutant PAA^{r}5 was mapped finely to an 0.8-kilobase-pair region in the herpes simplex virus DNA polymerase locus. These results indicate that the mutants are resistant to araA itself rather than to its deamination product and confirm the importance of the viral polymerase in the antiviral action of araA.

Arabinosyladenine (9-β-D-arabinofuranosyladenine, vidarabine, adenine arabinoside, ViraA [araA]) was the first antiviral agent licensed in the United States for systemic use against herpes infections. Despite its proven clinical efficacy (21, 39), the details of its mechanism of action remain sparse. Although many of the biochemical studies addressing the mechanism of action of araA have advanced the notion that the viral DNA polymerase is an ultimate target of the drug (3, 13, 25, 28, 30, 33), conceivably, other enzymes could also serve as targets (for review, see references 14 and 27).

A definite role for the viral DNA polymerase in mediating susceptibility to araA has been posited on the basis of a study of herpes simplex virus (HSV) mutants resistant to araA (8). Several mutants were isolated for resistance to araA; selection for araA resistance also selected for resistance to phosphonoacetic acid (PAA), an established marker for the HSV DNA polymerase (*pol*) locus (4–6, 12, 16, 20, 23, 24, 29). Additionally, a mutant previously isolated for PAA resistance, PAA^{r}5 (24), was found to be resistant to araA. The araA resistance and PAA resistance markers of this mutant were shown to be closely linked; moreover, the mutant specified HSV DNA polymerase activity which exhibited a higher K_i for araATP (8). These findings have been extended in a study mapping the araA and PAA resistance markers of PAA^{r}5 to a 1.1-kilobase-pair (kbp) fragment in the *pol* locus (6) and in a report mapping an araA resistance marker from another mutant to a 2.6-kbp fragment in the *pol* locus (11). These studies led to the conclusions that araA acts selectively on HSV and that the DNA polymerase is a target of araA action (8).

Recently, several mutants isolated for resistance to phosphonoformic acid (PFA) (12), a congener of PAA, were found to be resistant to araA (2). However, when an inhibitor of adenosine deaminase, deoxycoformycin (pentostatin, covidarabine [DCF]) (40), was included with the araA, it was reported that resistance was abolished (2). These findings, along with kinetic analyses of the mutant polymerases, were used both to support the conclusion that the araA resistance observed for previously described mutants (8, 31) was probably due to the omission of an adenosine deaminase inhibitor and to suggest that what had been observed was not resistance to araA but resistance to the deamination product of araA, arabinosylhypoxanthine (araHx). On this basis, it was suggested that determinants other than the HSV DNA polymerase are probably important for the antiviral action of araA (2).

To address these questions, we have reexamined the araA-resistant mutants upon which previous conclusions implicating the HSV DNA polymerase as a target for this drug were based (6, 8). We find that these mutants retain their resistance to araA in the presence of DCF. Moreover, this resistance can be mapped finely to an 0.8-kbp region in the HSV DNA polymerase locus, confirming that the HSV DNA polymerase is important for the antiviral action of araA.

MATERIALS AND METHODS

Cells and viruses. African green monkey kidney cells (Vero cells) were propagated and maintained as described previously (38). Viruses used included HSV type 1 wild-type strain KOS, mutants PAA^{r}5 (6–8, 19, 24), AraA^{r}6, AraA^{r}7, AraA^{r}9, and AraA^{r}13 (7, 8, 19), and recombinant viruses P5P^{r}Ba8, P5P^{r}Kp3 (referred to as Ba8 and Kp3 in references 7 and 19), P5Aph$^+$K2, and P5Aph$^+$S1. The last two recombinant viruses were isolated after marker rescue experiments, using infectious PAA^{r}5 DNA and fragments of wild-type strain KOS DNA and selection for restoration of wild-type levels of aphidicolin susceptibility (7) (see Fig. 2 for the map locations of the fragments used to construct the recombinant viruses).

Drugs. AraA was purchased from Calbiochem-Behring, LaJolla, Calif., and was prepared as described previously (8). DCF (pentostatin, covidarabine), in the form of a powder also containing mannitol and phosphate buffer, as supplied by the National Cancer Institute, was a generous gift from D. Kufe, Dana-Farber Cancer Institute, Boston, Mass. It was dissolved in water to yield a 10 mM stock solution and was stored at −20°C. Under these conditions, its activity remained stable for months.

Assays of virus drug susceptibility. Virus titrations were performed by the procedure of Dreesman and Benyesh-Melnick (15) as modified in this laboratory and the laboratory of P. A. Schaffer. Briefly, confluent monolayers of Vero cells in 35-mm^2 dishes were infected with two dilutions of the viruses such that dishes received between 15 and 50 or 150 and 500 PFU. (It should be noted that even at the lower dilution, the multiplicity of infection remains below 0.0005. The use of the lower dilution allows the counting of statistically meaningful numbers of plaques [≥30] at drug concentrations which substantially reduce plaque formation.) Dilut-

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ed virus was absorbed to cells in a volume of 0.1 ml of Dulbecco modified minimal essential medium containing 2% or more newborn calf serum for 1 h at 37°C in a humidified CO_2 incubator, with gentle shaking every 15 min. After absorption, duplicate dishes of cells infected at each dilution were overlaid with 2% methylcellulose in Dulbecco modified minimal essential medium plus 2% serum containing the concentrations of the drugs indicated in the figures and text and then incubated at 37°C in a humidifed CO_2 incubator. Plaques were visualized by neutral red staining 3 to 4 days postinfection and counted on the next day by illuminating the dishes from below. Only plaques visible to the naked eye were counted.

RESULTS

PAA^{r}5 is resistant to araA and DCF. We reexamined the resistance to araA of PAA^{r}5, a mutant derived from wild-type HSV type 1 strain KOS (24). Previously, we analyzed araA susceptibility by plotting the log of the percent plaques remaining versus drug concentration, since differences between sensitive and resistant viruses could more easily be recognized in such a plot (8). In this way, PAA^{r}5 was first shown to be resistant to araA (8). We have plotted the data in this report as percent plaques remaining on the ordinate versus the log of araA concentration on the abscissa to make our results directly comparable to those of Bastow et al. (2), who reported that resistance of certain mutants to araA was abolished when an adenosine deaminase inhibitor, DCF, was included.

Figure 1A shows the results of a plaque reduction assay of KOS and PAA^{r}5 at different concentrations of araA in the absence of DCF. Examination of the curves indicates a 50% effective dose (ED_{50}; dose required to reduce plaque formation by 50%) of ca. 60 μM for strain KOS (similar to that reported by Bastow et al. [2]) and an ED_{50} about threefold higher for PAA^{r}5. These ED_{50} values are different from those reported by another group for these two viruses (11) or from those values which can be abstracted from Fig. 2 of reference 8; however, the relative degree of resistance of PAA^{r}5 is similar. Substantial variation from experiment to experiment in levels of araA susceptibility is common (6, 8; W. O'Brien, personal communication), which may explain the discrepancy.

When the relative susceptibilities of KOS and PAA^{r}5 to araA were examined in the presence of 3.5 μM DCF (the dose used by Bastow et al. [2]), we found that although DCF potentiated the antiviral effect of araA two- to threefold for both viruses, PAA^{r}5 remained markedly resistant to araA in its presence (ED_{50}, three- to fourfold greater than that of strain KOS) (Fig. 1B). Importantly, virtually identical plaque reduction curves were obtained in the presence of either two- or fourfold greater concentrations of DCF. As has been previously reported (10, 26), these concentrations of DCF displayed no detectable antiviral effect in the absence of araA (data not shown). The degree of the potentiation by DCF of the antiviral effect of araA for strain KOS which we observed was very similar to that observed by Bastow et al. (2).

Other workers have also observed that PAA^{r}5 is resistant to araA plus DCF (L. Schnipper and W. O'Brien, personal communications).

The marker conferring resistance to araA and DCF in PAA^{r}5 maps to an 0.8-kbp sequence in the HSV *pol* locus. The marker conferring araA resistance in PAA^{r}5 had previously been mapped to a 1.1-kbp DNA fragment in the HSV *pol* locus (6). We wished to determine whether the mutation(s)

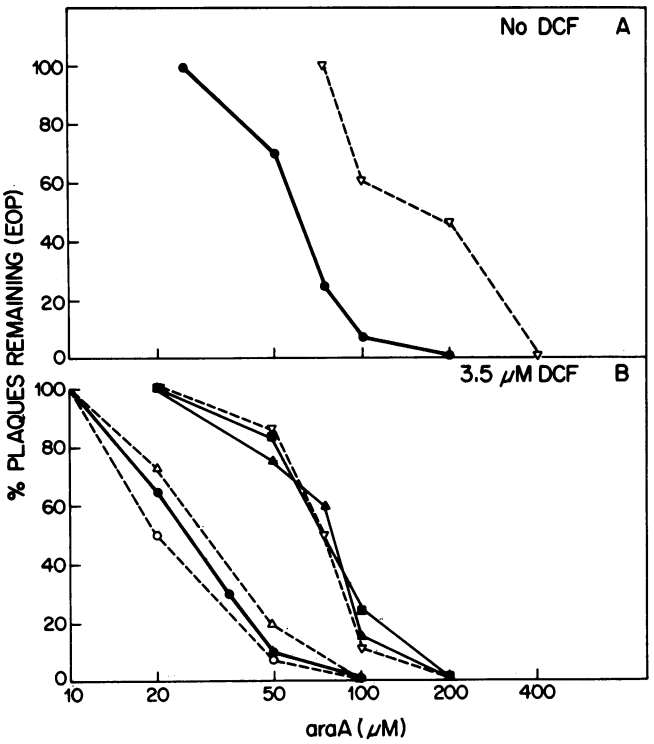

FIG. 1. Effect of araA, with or without DCF, on plaque formation by KOS, PAA^{r}5, and recombinant viruses. (A) Results of plaque reduction assays performed as described in the text with the indicated concentrations of araA and no DCF. The dose-response curves for KOS (●) and PAA^{r}5 (▽) are indicated. (B) Results of plaque reduction assays performed with the indicated concentrations of araA and 3.5 μM DCF. The dose-response curves for KOS (●), PAA^{r}5 (▽), and recombinant viruses P5P^{r}Ba8 (■), P5P^{r}Kp3 (▲), P5Aph$^+$K2 (△), and P5Aph$^+$S1 (○) are indicated. The derivation of the recombinant viruses is explained in the text and Fig. 2. Virtually superimposable dose-response curves for KOS and PAA^{r}5 were obtained in the presence of either 7 or 14 μM DCF. Each of the curves in both panels represents the average of two or more plaque reduction assays.

responsible for the resistance to araA plus DCF mapped to the same sequences. Recombinant viruses constructed by marker transfer of DNA fragments from a recombinant DNA plasmid containing the PAA^{r}5 drug resistance markers into a KOS background (6) were used. The map locations of the fragments used to construct these recombinant viruses, P5P^{r}Ba8 and P5P^{r}Kp3, are shown in Fig. 2. These fragments overlap by 1.1 kbp. P5P^{r}Ba8 and P5P^{r}Kp3 have previously been shown to be resistant to araA alone (6). These viruses were as resistant to araA and 3.5 μM DCF as was PAA^{r}5 (Fig. 1B). These results map the marker for resistance to araA and DCF in PAA^{r}5 to the 1.1-kbp fragment (Fig. 2).

To map this marker more finely, we used recombinant viruses constructed by marker rescue of PAA^{r}5 from aphidicolin hypersensitivity to wild-type sensitivity by fragments of a recombinant DNA plasmid containing wild-type sequences (7). The map locations of the fragments used to construct these recombinant viruses, P5Aph$^+$K2 and P5Aph$^+$S1, are shown in Fig. 2. These recombinants display wild-type levels of susceptibility to PAA and aphidicolin (unpublished data). They also display wild-type levels of susceptibility to araA in the presence of 3.5 μM DCF (Fig. 1B). Thus, the marker for resistance to araA and DCF in

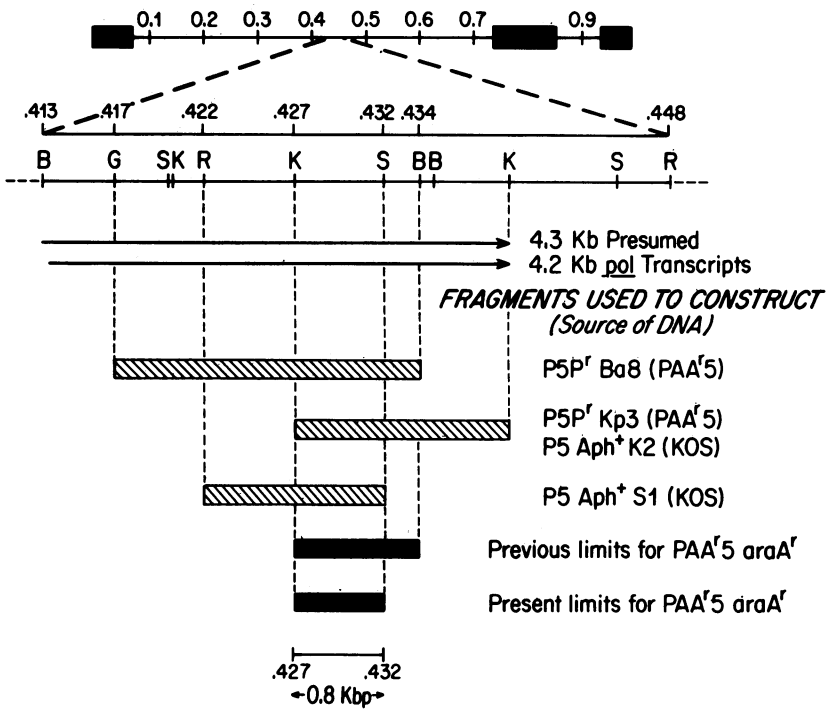

FIG. 2. Physical map location of the araA resistance marker of PAA^{r}5. The top line is a schematic representation of the HSV DNA molecule, with physical map coordinates provided. The second and third lines show an expanded view of the region between coordinates 0.413 and 0.448 which contains the *pol* locus (6) with coordinates and restriction endonuclease recognition sites for enzymes *Bam*HI (B), *Bgl*II (G), *Sal*I (S), *Kpn*I (K), and *Eco*RI (R) indicated. Coordinates and location of restriction enzyme sites are as previously described (6, 8, 39). Below are two lines with arrowheads depicting the approximate location of two transcripts described by Holland et al. (22), which span finely mapped *pol* mutations (6) and thus are good candidates to encode polymerase. The arrowheads denote the 3′ ends of the transcripts. The map locations of the fragments used to construct the recombinant viruses used in the experiment depicted in Fig. 1 are shown as hatched boxes. The names of the recombinant viruses are indicated to the right of the boxes, and the viruses from which the DNA fragments used to construct the recombinants were derived are within parentheses. At the bottom of the figure are two black boxes; the upper box depicts the previous map limits for the araA^r marker of PAA^{r}5 (6), and the bottom box indicates the map limits from the experiment depicted in Fig. 1. These map limits span coordinates 0.427 to 0.432, a distance of 0.8 kbp, as indicated.

PAA^{r}5 maps to the 0.8-kbp DNA sequence, which the fragments used to construct the recombinants overlap (map coordinates, 0.427 to 0.432).

Resistance of mutants selected for araA resistance to araA and DCF. We tested several of the mutants which had been isolated for resistance to araA in the absence of DCF (8), AraA^{r}6, AraA^{r}7, AraA^{r}9, and AraA^{r}13, for their relative susceptibility to araA in the presence of 3.5 μM DCF. These mutants are distinct from one another on the basis of their relative susceptibilities to a variety of drugs (7, 8; D. M. Coen, H. E. Fleming, L. K. Leslie, and M. J. Retondo, manuscript in preparation). All mutants tested were resistant to the drug combination. Results from two of the mutants, AraA^{r}9, and AraA^{r}13, are shown in Fig. 3. The curves for the other two mutants fall between those of AraA^{r}9 and AraA^{r}13 (data not shown). These mutants, then, exhibit ED_{50}s two- to threefold greater than that of strain KOS. Thus, their selection for araA resistance was not due to the omission of an adenosine deaminase inhibitor.

DISCUSSION

Our results clearly demonstrate that HSV mutants can be isolated which are resistant to araA in the presence of 3.5 μM DCF, an inhibitor of adenosine deaminase. We believe that in our experiments adenosine deaminase was, in fact, inhibited for the following reasons: (i) 3.5 μM DCF has been shown to completely inhibit the deamination of araA in a monkey kidney cell line (9, 37); (ii) the K_i for DCF of

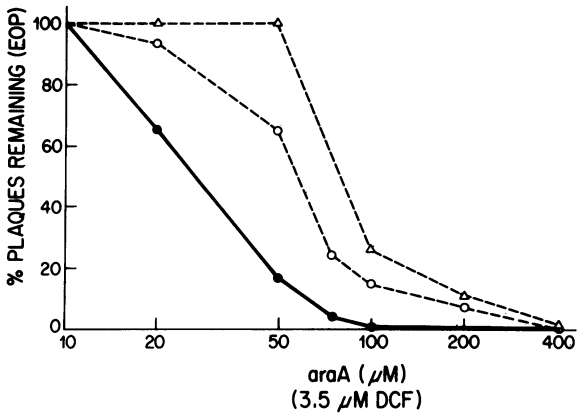

FIG. 3. Effect of araA with 3.5 μM DCF on plaque formation of KOS (●), AraA^{r}9 (○), and AraA^{r}13 (△). Plaque reduction assays were performed as described in the text, with the indicated concentrations of araA and 3.5 μM DCF. The dose-response curves for the three viruses are indicated in the figure.

adenosine deaminase has been reported to be at least five orders of magnitude lower than the concentration we used (1); (iii) the antiviral effect of araA was potentiated by DCF to the extent reported by others (2, 10); and (iv) increasing the DCF concentration fourfold did not increase the potentiating effect. Nevertheless, we still observed araA resistance in the mutants studied (Fig. 1 and 3). Thus, it is highly unlikely that the araA resistance which we observed reflects decreased susceptibility to araHx (2). Rather, it appears to be due to araA itself.

It should be noted that HSV strains which were more resistant than other strains to araA in the presence of DCF were described by Connor et al. in 1975 (10).

The results of our mapping experiments refine the map limits for the mutation(s) conferring araA resistance in mutant PAA^{r}5 to an 0.8-kbp region within the *pol* locus (Fig. 2). DNA sequencing studies are under way to determine the amino acid changes which result in the araA resistance phenotype. The data reported here combined with the previous report that PAA^{r}5 specifies DNA polymerase with a higher K_i for araATP than that of wild-type-specified polymerase (8) confirm that alterations in HSV DNA polymerase can result in resistance to araA. Thus, HSV polymerase is clearly a determinant of araA susceptibility.

Similarly, mutants which had been selected for resistance to araA retained resistance to araA in combination with DCF (Fig. 3). The phenotypes of these mutants seem likely to be due to mutations in the *pol* locus, based on their susceptibilities to other drugs (7, 8; Coen et al., manuscript in preparation), their antimutator phenotypes (19), physical mapping studies (H. Chiou and D. M. Coen, unpublished data), and their specification of altered polymerases (M. St. Clair and P. Furman, personal communication).

It is intriguing that mutants such as those described by Bastow et al. (2) could be resistant to araA alone and not to araA and DCF. This would tend to counter suggestions (14, 27, 34) that the antiviral effects of araHx are due to conversion of araHx-monophosphate to araAMP by sequential action of the enzymes adenylosuccinate synthetase and adenylosuccinate lyase (35, 36). This also suggests that resistance of *pol* mutants to nucleoside analogs that are altered in their sugar moiety can depend absolutely on the nature of the base moiety as well. As yet, we have observed quantitative differences in the degree of resistance of various mutants to analogs which differ in this fashion but no qualitative differences (Coen et al., manuscript in preparation). Nevertheless, there is considerable variation among HSV *pol* mutants with respect to araA resistance, even among those which are resistant to PAA or PFA or both. For example, PAA^{r}5 has been reported to be more resistant to araA than are other araA-resistant mutants (8, 11; this report), whereas other PAA^r mutants have been reported to be sensitive to araA (6, 8, 16, 17). Two PAA^r mutations have been mapped to separate locations in the *pol* locus (6).

The results of Bastow et al. also raise an interesting semantic question: if a virus is resistant to a metabolite of a drug (e.g., araHx) rather than to the drug itself (e.g., araA), how should its resistance be characterized? We would argue that the PFA^r mutants studied by Bastow et al. should still be characterized as araA resistant. First, araA was the drug added to the infected cultures in which resistance was observed. Second, it is not entirely clear that all of the araA resistance displayed by their mutants is due to resistance to araHx; synergistic effects of DCF with araA other than inhibition of adenosine deaminase might have led to the loss of resistance of these mutants. Third, examination of resistance to araA alone is more relevant clinically; araA is not generally administered to patients with herpesvirus infections with an adenosine deaminase inhibitor. Fourth, it can be argued that the active forms of many antiviral agents are, in fact, metabolites; for example, one can say that the active forms of iododeoxyuridine and acyclovir are their triphosphates. Thus, we prefer to retain the terminology "araA resistance."

The isolation of araA-resistant mutants (8, 31) implies that araA acts selectively on HSV, and the mapping of resistance mutations to the *pol* locus implies that at least some of this selectivity resides in the HSV DNA polymerase. One could hypothesize that the properties of the polymerase account for all of the selectivity. It is noteworthy that PAA^{r}5 exhibits an ED_{50} three- to fivefold higher than that of its parental strain, KOS (8, 11; this communication). This difference is similar to the reported difference between the inhibition by araA of viral DNA synthesis in infected cells and cellular DNA synthesis in uninfected cells (32, 33). Thus, the activity of araA against PAA^{r}5 may not be selective but due to effects on the host cell which decrease its ability to support HSV replication.

These effects of araA on cells rendering them less able to support HSV replication might also explain why the PFA^r mutants studied by Bastow et al. (2) were much more susceptible to araA alone than to araHx alone. AraHx is much less cytotoxic than araA (14, 27, 32, 33).

Marshalled against the hypothesis that all of the selectivity of araA against HSV resides in its interaction with viral polymerase are arguments based on araA-resistant mutants which specify DNA polymerase less resistant in vitro to araATP than one would expect (2, 16). These results, however, could have been due to subtle differences in the degree of purity of enzyme preparations; Furth and Cohen have noted substantial changes in the character of araA inhibition of mammalian DNA polymerase upon further purification (18). Moreover, resistance to araA could be mediated by DNA polymerase properties other than those measured by the K_i for araATP; for example, the ratio of the K_i to the K_m for dATP or the degree to which araATP is incorporated into and excised from viral DNA (13) could be altered in viral mutants. We would not, by any means, rule out the possibility that other viral gene products mediate araA susceptibility. However, a convincing case for such other mechanisms may require genetic as well as biochemical evidence.

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