JOURNAL OF VIROLOGY, Feb. 1985, p. 477–488
0022-538X/85/020477-12$02.00/0

Vol. 53, No. 2

# Sensitivity of Arabinosyladenine-Resistant Mutants of Herpes Simplex Virus to Other Antiviral Drugs and Mapping of Drug Hypersensitivity Mutations to the DNA Polymerase Locus

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Received 27 August 1984/Accepted 10 October 1984

**Seven herpes simplex virus mutants which have been previously shown to be resistant to arabinosyladenine were examined for their sensitivities to four types of antiviral drugs. These drugs were a pyrophosphate analog, four nucleoside analogs altered in their sugar moieties, two nucleoside analogs altered in their base moieties, and one altered in both. The seven mutants exhibited five distinct phenotypes based on their sensitivities to the drugs relative to wild-type strain KOS. All mutants exhibited resistance to acyclovir and arabinosylthymine, as well as marginal resistance to iododeoxyuridine, whereas all but one exhibited resistance to phosphonoformic acid. The mutants exhibited either sensitivity or hypersensitivity to the other drugs tested—2′-nor-deoxyguanosine, 5-methyl-2′-fluoroarauracil, 5-iodo-2′-fluoroarauracil, and bromovinyldeoxyuridine—some of which differed only slightly from drugs to which the mutants were resistant. These results suggest ways to detect and treat arabinosyladenine-resistant isolates in the clinic. Antiviral hypersensitivity was a common phenotype. Mutations conferring hypersensitivity to 2′-nor-deoxyguanosine in mutant PAA$^r$5 and to bromovinyldeoxyridine in mutant tsD9 were mapped to nonoverlapping regions of 1.1 and 0.8 kilobase pairs, respectively, within the herpes simplex virus DNA polymerase locus. Thus, viral DNA polymerase mediates sensitivity to these two drugs. However, we could not confirm reports of mutations in the DNA polymerase locus conferring resistance to these two drugs. All of the mutants exhibited altered sensitivity to two or more types of drugs, suggesting that single mutations affect recognition of the base, sugar, and triphosphate moieties of nucleoside triphosphates by viral polymerase.**

Considerable progress has been made in the last 20 years in developing antiviral drugs which are active against herpes simplex virus (HSV). The availability of such drugs has not only allowed treatment of HSV infections in the clinic (34), but has also aided the study of HSV in the laboratory—particularly through the analysis of mutants of HSV which are resistant to antiviral drugs. Such mutants can be valuable in several ways. First, measurement of the frequency of isolation of resistant mutants in the laboratory may help predict the frequency with which they arise in clinical settings. Second, knowledge of the properties of laboratory-isolated resistant mutants may allow one to anticipate the features of resistant isolates which are found in the clinic, e.g., their sensitivities to other antiviral drugs. Such studies could promote solutions to clinical resistance.

Third, studies of drug-resistant mutants can aid the elucidation of the mechanisms of action of antiviral drugs. The identification of mutants resistant to a particular antiviral drug implies that the drug acts selectively on the virus, not simply by rendering the host incapable of supporting virus replication; the identification of a gene which mutates to confer drug resistance implies that the product of the gene normally contributes to the selectivity of the drug. On this basis, it has been concluded that arabinosyladenine (araA) is a selective anti-HSV agent and that the HSV DNA polymerase is a target of araA (9, 24).

Fourth, investigations of drug-resistant mutants should abet the functional subdivision of viral genes. Mutations conferring drug-resistance are likely to affect sites on drug targets which interact with the drugs. Dissection of the function of the HSV DNA polymerase by analysis of such mutations has begun. For example, intratypic marker rescue and transfer experiments have mapped mutations in two mutants of strain KOS, PAA$^r$5 and tsD9, which confer differing degrees of resistance to the pyrophosphate analog, phosphonoacetic acid (PAA), and to acyclovir (ACG), a nucleoside analog altered in its sugar moiety, to different regions in the HSV DNA polymerase (*pol*) locus (7). These mutations were separable from certain temperature-sensitive (*ts*) *pol* mutations (7). Interestingly, PAA$^r$5, but not tsD9, contains a mutation conferring resistance to araA, also a nucleoside analog altered in its sugar moiety, mapping with the PAA$^r$ marker (7, 9). Other *pol* mutations conferring drug resistance have also been shown to be separable from various *ts pol* mutations (4, 5, 11, 13, 36).

Recently, mutants which are drug hypersensitive have been identified. These include mutants which are hypersensitive to the α-polymerase inhibitor aphidicolin (3, 8, 36) and to PAA (7). In the case of aphidicolin, hypersensitivity mutations have been mapped to various locations in the *pol* locus by marker rescue (8; unpublished data) and by genetic recombination (36). As with resistant mutants, hypersensitive mutants can aid in the analysis of drug action; identification of a gene which can mutate to confer drug hypersensitivity identifies the product of that gene as a mediator of drug sensitivity. Additionally, mutations which confer drug hypersensitivity should alter the functional sites which interact with the drugs, much as drug resistance mutations do. One might expect, then, that both resistance and hypersensitivity mutations could alter the pyrophosphate exchange-release site, the aphidicolin-binding site, the nucleoside triphosphate-binding site, or some combination of these sites with which the various drugs are likely to interact. Moreover, one would expect that mutations which alter sensitiv-

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ity to nucleoside analogs altered in particular moieties would alter the recognition site for that moiety of the natural nucleoside triphosphate. Such mutations could affect recognition directly by changing an amino acid in the recognition site or indirectly by altering the tertiary structure of polymerase.

In this report, we examine seven HSV mutants known or likely to be *pol* mutants which were previously shown to be resistant to araA and PAA (9, 24) and hypersensitive to aphidicolin (8) for their sensitivities to eight other antiviral drugs. These antiviral drugs are analogs of pyrophosphate or of nucleosides altered in their sugar moiety, base moiety, or both. Mutants can be distinguished from each other on the basis of their sensitivities to these drugs. For two mutants, we mapped mutations conferring hypersensitivity to either 2'-nor-deoxyguanosine (2'NDG) or bromovinyldeoxyuridine (BVdU) to small regions within the *pol* locus. Additionally, we reexamine two mutants reported to contain mutations mapping to the *pol* locus conferring resistance to BVdU and 2'NDG, respectively (12, 13). Our results have implications for the detection and possible treatment of clinically resistant isolates, for the mechanisms of action of these drugs, and for the functional dissection of the *pol* locus. (Portions of this study were presented at the International Herpesvirus Workshop, Oxford, England, August 1983, and at the Burroughs Wellcome Co.-UCLA Herpesvirus Symposium, Keystone, Colo., April 1984.)

## MATERIALS AND METHODS

**Cells and viruses.** African green monkey kidney cells (Vero) were propagated and maintained as described (48). Stocks of viruses were prepared by infecting Vero cells at a multiplicity of infection between 0.01 and 0.1 PFU per cell. Infected cells were incubated at 34°C until generalized cytopathic effects were obtained, scraped into the medium, dispensed into 50-ml conical tubes, and frozen at −80°C. Cells were disrupted either by three rounds of freezing and thawing or by one round of freezing and thawing and sonication in a cup horn sonic oscillator (Heat Systems Ultrasonics, Farmingdale, N.Y.) at full power for 45 s. Cell debris was removed by centrifugation, and supernatants were dispensed into small portions and frozen at −80°C. When possible, virus stocks were never more than a few passages removed from the original stock derived from plaque-purified virus. Relevant properties of the viruses used in this study are presented in Table 1.

TABLE 1. Mutants and recombinant viruses used in this study

| Wild-type strain | Mutant or recombinant | Phenotype[a] | | | | References |
|---|---|---|---|---|---|---|
| | | ts | araA | PAA | Aph | |
| KOS | PAA$^r$5 | + | R | R | HS | 7–10, 24, 31, 37 |
| | AraA$^r$6 | + | R | R | HS | 8, 9, 24 |
| | AraA$^r$7 | + | R | R | HS | 8, 9, 24 |
| | AraA$^r$8 | + | R | R | HS | 8, 9, 24 |
| | AraA$^r$9 | + | R | R/S | HS/S | 8, 9, 24, 31 |
| | AraA$^r$12 | + | R | R | HS | 8, 9, 24 |
| | AraA$^r$13 | + | R | R | HS | 8, 9, 24, 31 |
| | tsC4 | ts | S | HS | HS | 2, 7–9 |
| | tsC7 | ts | S | S | S | 2, 7–9 |
| | tsD9 | ts | S | R | HS | 2, 7–9, 40 |
| | ts022 | ts | S | S | S | 2, 8, 9, 48 |
| | P5P$^r$Ba8[b] | + | R | R | HS | 7, 8, 24, 31 |
| | P5P$^r$Kp3[c] | + | R | R | HS | 7, 8, 24, 31 |
| | P5Aph$^+$K2[d] | + | S | S | S | 8, 24; unpublished data |
| | P5Aph$^+$S1[d] | + | S | S | S | 8, 24; unpublished data |
| | D9$^+$E/K4[e] | + | ND | S | ND | 7 |
| | D9$^+$E/K2[e] | + | ND | S | ND | Unpublished data |
| 17 | paa$^r$-1[f] | + | R | R | ND | 5, 13, 32 |
| HG52 | ts6[f] | ts | ND | S | ND | 5, 13 |

[a] ts, Temperature sensitive; Aph, aphidicolin; R, resistant; S, sensitive; HS, hypersensitive; R/S, marginally resistant; HS/S marginally hypersensitive; ND, not determined. Definitions of the phenotypes are provided in the references and in this report.

[b] Referred to as Ba8 in references 7, 8, and 31.

[c] Referred to as Kp3 in references 7, 8, and 31.

[d] Recombinants isolated after marker rescue of PAA$^r$5, selecting for restoration of wild-type levels of aphidicolin sensitivity (8). See Fig. 11 for the map locations of the fragments used to construct these recombinants.

[e] D9$^+$E/K4 and D9$^+$E/K2 are similar recombinants derived by marker rescue of tsD9. See Fig. 11 for the map location of the fragment used to construct them.

[f] J. H. Subak-Sharpe and H. Marsden and, independently, C. Crumpacker and L. Schnipper graciously provided stocks of these mutants and their corresponding wild-type strains.

**Assays of drug sensitivity.** Virus titrations were performed by the procedure of Dreesman and Benyesh-Melnick (18) 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 mulitiplicity of infection remains below 0.0005. The use of the lower dilution allows counting of statistically meaningful numbers of plaques [≥30 in duplicate dishes] at drug concentrations which substantially reduce plaque formation.) Diluted 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 cultures 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 incubated at 37°C (unless otherwise indicated) in a humidified $CO_2$ incubator. Generally, plaques were visualized by neutral red staining at 3 to 4 days postinfection and were counted on the following day by illuminating the dishes from below. Only plaques visible to the naked eye were counted. On certain occasions, however, plaques were counted microscopically or, after staining with crystal violet, under a dissecting microscope (14). Similar results were obtained by all three procedures. The 50% effective dose ($ED_{50}$) values were derived by interpolation. Data presented are either the average of two or more assays, each performed in duplicate, or from a single representative assay. We stress that the behavior of the mutants relative to their wild-type parent strain was quite reproducible, unless otherwise noted.

**Antiviral drugs.** Figure 1 shows the structures of 10 anti-HSV drugs. PAA and phosphonoformic acid (foscarnet sodium; PFA) are pyrophosphate analogs which bind at the pyrophosphate exchange-release site of HSV DNA polymerase (17). ACG and 2'NDG (also known as BIOLF 62, DHPG, BW759, and BWB759) are guanosine analogs lacking the 2' and 3' or the 2' moieties, respectively. Thus, ACG and 2'NDG differ only at the 3' position. AraA and arabinosylthymine (araT) are adenosine and thymidine analogs,

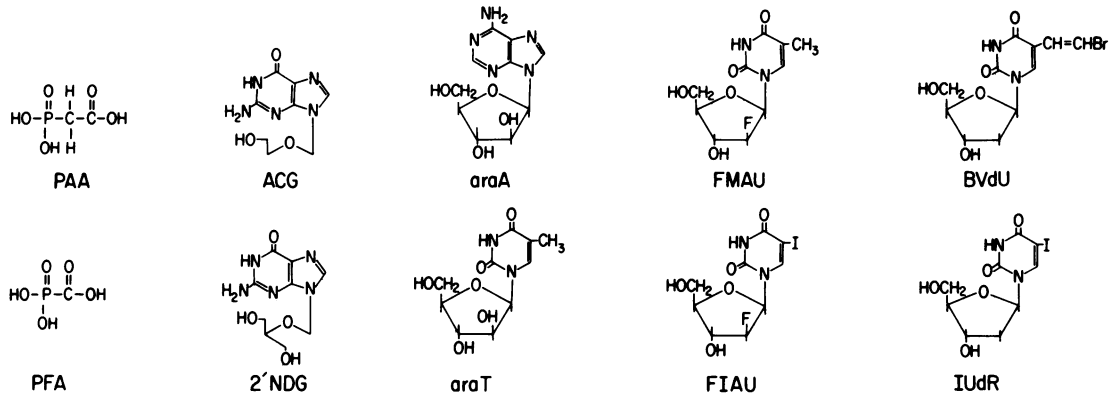


FIG. 1. Structures of antiviral compounds. The structures of each compound are given above a common abbreviation for each one. See the text for further description.

respectively, with the arabinosyl conformation at the 2' position. 2'-Fluoro-5-methylarauracil (FMAU) is a thymidine analog which differs from araT only at the 2' position by the presence of a fluorine atom rather than a hydroxyl group. ACG, 2'NDG, araA, araT, and FMAU, then, are all nucleoside analogs altered in their sugar moieties at the 2' or 3' positions, or both. 2'-Fluoro-5-iodoarauracil (FIAU), BVdU, and iododeoxyuridine (IUdR) are all thymidine analogs which replace the 5-methyl group of thymidine with iodine in the case of FIAU and IUdR or with a bromovinyl group in the case of BVdU. FIAU is additionally altered in its sugar moiety by the fluorine at the 2' position, similar to FMAU. Thus, it differs from IUdR only slightly. The triphosphates of these nucleoside analogs are competitive inhibitors or substrates, or both, for HSV DNA polymerase (1, 3, 9, 15, 23, 26, 29, 30, 39, 41, 42, 47) and can be presumed to bind at the nucleoside triphosphate-binding site of polymerase.

PFA was kindly provided by B. Erikkson, Astra Läkemedel AB, and was prepared as previously described for PAA (10). ACG and 2'NDG were graciously supplied by G. Elion and D. Barry, Burroughs Wellcome Co., and were prepared as previously described for ACG (10). AraT was purchased from Calbiochem-Behring and was prepared as previously described for araA (9). FMAU and FIAU were generously provided by C. McLaren, Bristol Meyers, BVdU was kindly supplied by E. De Clercq, Rega Institute, and IUdR was purchased from Sigma Chemical Co. These latter four drugs were each dissolved in distilled water and filter sterilized to prepare concentrated stock solutions. These stocks were stored frozen at −20°C. In the case of IUdR, the stock was kept shielded from light.

## RESULTS

**Sensitivity of araA-resistant mutants to pyrophosphate analogs. (i) Resistance to PAA.** A previous publication (9) described 13 mutants of HSV-1 strain KOS which were isolated for resistance to araA. Five mutants, AraA$^r$1 through 5, were isolated after extensive passage of KOS in increasing concentrations of araA. Eight, AraA$^r$6 through 13, were isolated by single-step selection for resistance to araA. Since AraA$^r$1 through 5 seemed likely to have suffered multiple mutations during their selection, we chose to concentrate our studies on the remaining mutants. Two of these mutants, AraA$^r$10 and 11, exhibited only very slight resistance to araA and have not been studied further. Thus, we report here on phenotypic properties of mutants AraA$^r$6 through 9, AraA$^r$12, and AraA$^r$13. Each of these mutants was plaque purified an additional two times in the absence of drug and expanded into a high-titer stock.

An additional mutant of strain KOS, PAA$^r$5, which was isolated for resistance to the pyrophosphate analog PAA (37) is resistant to araA (9, 13, 24). Its araA resistance marker has been mapped to within an 0.8-kilobase pair (kbp) region of the HSV DNA polymerase (*pol*) locus (7, 24).

All seven mutants (AraA$^r$6 through 9, AraA$^r$12, AraA$^r$13, and PAA$^r$5) have been shown to exhibit at least some resistance to PAA (9, 10, 27, 37). The resistance to PAA suggests that they contain mutations in the *pol* locus, since all PAA-resistant mutants which have been studied to date are so mutated (4, 5, 7, 36; unpublished data). In the case of AraA$^r$9, 12, and 13, this resistance was most evident at relatively high concentrations of PAA (e.g., 490 μM), in which plating efficiencies at least 200-fold greater than that of KOS were observed (Fig. 1B in reference 9). However, for these three mutants, this resistance was more subtle when considered in terms of the concentration of PAA required to reduce plaque formation 50% ($ED_{50}$), which was no more than 1.6-fold greater than that of KOS (Fig. 1B in reference 9; unpublished data). AraA$^r$6 and 8 exhibited $ED_{50}$s ca. threefold greater and AraA$^r$7 and PAA$^r$5 exhibited $ED_{50}$s more than threefold greater than that of KOS (13, 27; Fig. 1B in reference 9; unpublished data).

**(ii) Resistance and sensitivity to PFA.** Figure 2 depicts the results of plaque reduction experiments to test the sensitivity of the seven araA-resistant mutants and strain KOS to the pyrophosphate analog PFA (22). KOS typically exhibited an $ED_{50}$ for PFA of ca. 350 μM. One mutant, AraA$^r$9, appeared no more resistant or sensitive to PFA than wild-type strain KOS. Four mutants, AraA$^r$6, 8, 12, and 13 exhibited modest resistance to PFA, with $ED_{50}$s ca. fourfold higher than that of KOS, whereas two mutants, AraA$^r$7 and PAA$^r$5, exhibited substantial resistance, with $ED_{50}$s nearly sevenfold higher than that of KOS. Thus, for the two pyrophosphate analogs, the seven mutants exhibited similar patterns of resistance in that AraA$^r$9 is sensitive to PFA and marginally resistant to PAA, AraA$^r$7 and PAA$^r$5 are substantially resistant to both, and the other mutants are intermediate. The differences observed presumably reflect different alterations in pyrophosphate exchange-release sites of these mutants.

**Sensitivity of araA-resistant mutants to acyclic guanosine analogs. (i) Resistance to ACG.** The potent antiherpesvirus compounds ACG and 2'NDG (6, 19, 21, 45–47) are both guanosine analogs with acyclic sugar moieties (Fig. 1). Figure 3 depicts the results of experiments to test the

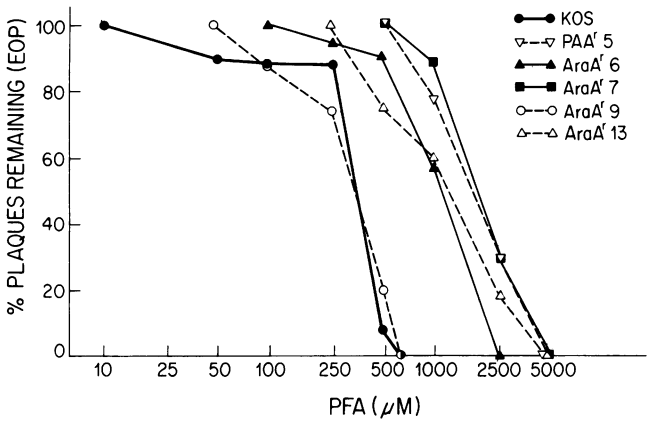


FIG. 2. Effect of PFA on plaque formation by strain KOS and mutant viruses. The dose-response curves for the various viruses are indicated. Results for mutant AraA$^r$8 were virtually identical to those shown for AraA$^r$6; results for mutant AraA$^r$12 were virtually identical those shown for AraA$^r$13.

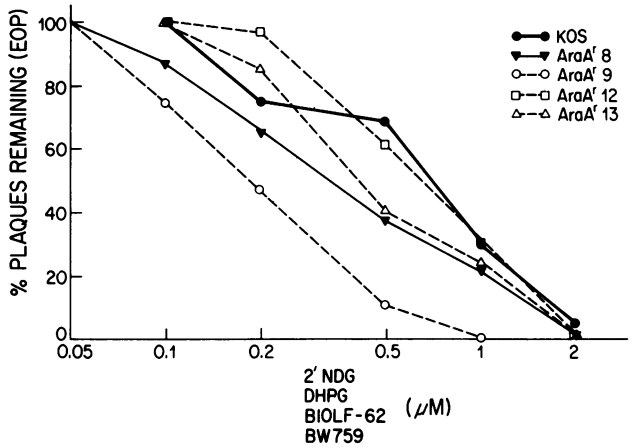


FIG. 4. Effect of 2'NDG on plaque formation by strain KOS and mutant viruses. The dose-response curves for the various viruses are indicated. Results for mutant AraA$^r$6 were virtually identical to those shown for AraA$^r$8; results for mutant AraA$^r$7 were virtually identical to those shown for mutant AraA$^r$13.

sensitivity of araA-resistant mutants to ACG relative to wild-type strain KOS. All seven mutants exhibited appreciable resistance to ACG. AraA$^r$6, 8, and 9 exhibited $ED_{50}$s ca. fourfold greater than that of KOS, whereas AraA$^r$7 and 12 exhibited $ED_{50}$s roughly 15-fold greater than that of KOS. The $ED_{50}$ of AraA$^r$13 appeared intermediate between these two groups, but at higher concentrations of drug it was indistinguishable from that of AraA$^r$12. PAA$^r$5 has previously been shown to exhibit an $ED_{50}$ for ACG ca. 20-fold greater than that of KOS (10, 27, 44) due to a *pol* mutation (7, 10, 27).

**(ii) Sensitivity and hypersensitivity to 2'NDG.** The six mutants were tested for their sensitivities to the close relative of ACG, 2'NDG (Fig. 4). Strain KOS exhibited an $ED_{50}$ for this drug that varied from experiment to experiment from ca. 0.5 to 1.5 μM (note the differences between Fig. 4 and 5). These values are similar to those reported for this virus (0.2 to 1 μM) by others (12, 46, 47). In contrast to the results with ACG, all of the mutants were either sensitive or hypersensitive to 2'NDG. AraA$^r$9 was the most hypersensitive of the mutants to 2'NDG, exhibiting an $ED_{50}$ ca. fivefold lower than that of strain KOS. Thus, the inclusion of the 3' carbon moiety in 2'NDG which is absent in ACG makes the difference between sensitivity and resistance to these drugs, respectively, for these mutants. This finding is similar to that found by others with other known or likely *pol* mutants (6, 45–47).

Three other groups have reported that PAA$^r$5 is similar to wild-type strain KOS in terms of its sensitivity to 2'NDG (12, 46, 47). In contrast, we found that this mutant was hypersensitive to 2'NDG, exhibiting an $ED_{50}$ ca. threefold lower than that of strain KOS (Fig. 5). In more than 10 assays, PAA$^r$5 always exhibited hypersensitivity to this drug, regardless of the variations in absolute $ED_{50}$ values for strain KOS mentioned above.

**(iii) The 2'NDG hypersensitivity marker of PAA$^r$5 maps to the *pol* locus.** We next asked whether the hypersensitivity to 2'NDG in PAA$^r$5 resulted from mutation in the *pol* locus. This analysis was made possible by the previous construction of recombinant viruses containing regions of the *pol* locus derived either from PAA$^r$5 of KOS (7, 8). P5P$^r$Ba8 and P5P$^r$Kp3 are recombinant viruses of the former type derived by transfer of the PAA$^r$ marker of PAA$^r$5 into a KOS

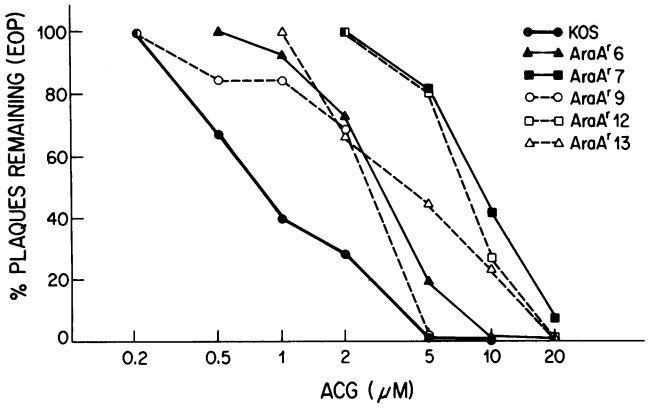


FIG. 3. Effect of ACG on plaque formation by strain KOS and mutant viruses. The dose-response curves for the various viruses are indicated. Results for mutant AraA$^r$8 were virtually identical to those shown for AraA$^r$6.

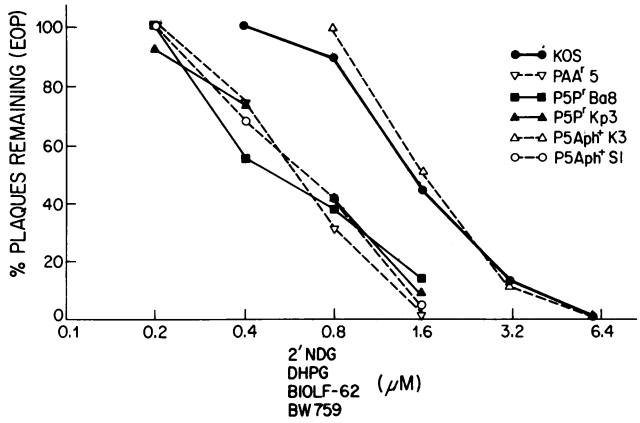


FIG. 5. Effect of 2'NDG on plaque formation by strain KOS, PAA$^r$5, and recombinant viruses. The dose-response curves for the various viruses are indicated. The construction of the recombinant viruses is described in the text and Fig. 11.

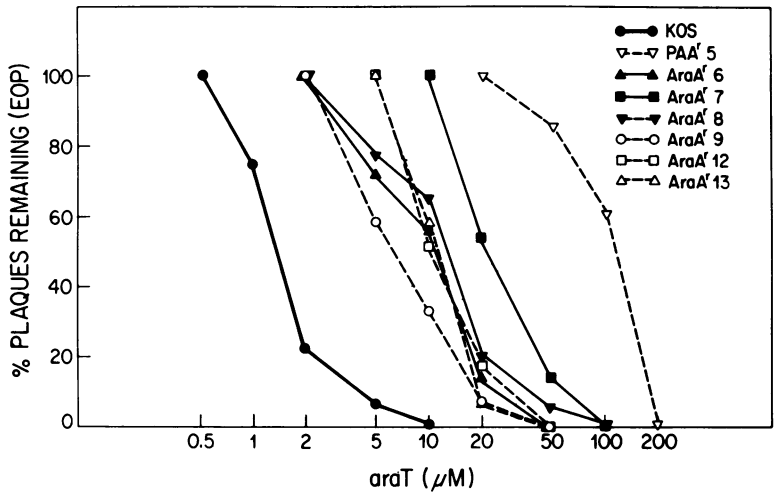


FIG. 6. Effect of araT on strain KOS and mutant viruses. The dose-response curves for the various viruses are indicated.

background. They are indistinguishable from PAA$^r$5 in terms of PAA, ACG, and araA resistance and aphidicolin hypersensitivity (7, 8, 24; unpublished data). Both of these recombinant viruses were similarly indistinguishable from PAA$^r$5 in terms of hypersensitivity to 2'NDG (Fig. 5). These observations permit the mapping of the 2'NDG hypersensitivity marker of PAA$^r$5 within the 1.1-kbp region (map coordinates 0.427 to 0.434) which the PAA$^r$5 DNA fragments used to construct these recombinants overlap (7) (for summary, see Fig. 11).

P5Aph$^+$K3 and P5Aph$^+$S1 are recombinant viruses derived by restoration of wild-type levels of aphidicolin sensitivity by recombination of KOS DNA fragments into a PAA$^r$5 background (8). These recombinants exhibit wild-type levels of PAA, araA, and aphidicolin sensitivity (8, 24; unpublished data). P5Aph$^+$K3, as expected, exhibited wild-type levels of sensitivity to 2'NDG, confirming that this marker maps in the *pol* locus. Surprisingly, however, P5Aph$^+$S1 exhibited hypersensitivity to 2'NDG similar to that of PAA$^r$5. Aphidicolin$^+$ recombinants derived from independent transfections with the same wild-type DNA fragment used to generate P5Aph$^+$S1 also exhibited hypersensitivity to 2'NDG. Two possible explanations for this result seem most likely. First, the 2'NDG hypersensitivity marker of PAA$^r$5 is separable from the other *pol* markers of this mutant. Alternatively, the cloned wild-type DNA sequence used to construct P5Aph$^+$S1 differs from most wild-type KOS sequences such that it confers 2'NDG hypersensitivity.

**(iv) Reexamination of a mutant reported to be 2'NDG resistant.** Mutant ts6 was derived from HSV-2 strain HG52 in the 1970s (5); at that time it was not examined for sensitivity to 2'NDG. Recently, ts6 has been reported to be substantially resistant to 2'NDG, exhibiting an $ED_{50}$ nearly 10-fold higher than that of its parental strain (12). Recombinants derived in the late 1970s by marker rescue of the *ts* mutation of ts6 with fragments of HSV-1 strain 17 mutant paa$^r$-1 DNA by Chartrand et al. (5) were quite sensitive to 2'NDG (12). On this basis, it was stated that a 2'NDG$^r$ mutation mapped within the same region of the *pol* locus as the *ts* mutation of ts6 (12).

Because none of the known or likely *pol* mutants which we had examined were resistant to 2'NDG, we reexamined the sensitivity of ts6 to this drug. We used samples of early passage stocks of mutant ts6 and strain HG52 kindly provided to us by H. Marsden and J. H. Subak-Sharpe. In our hands, this stock of ts6 was no more resistant to 2'NDG than its parental strain, HG52 ($ED_{50}$s, 3.2 and 3.8 μM, respectively).

In an effort to resolve the discrepancy between our results and those recently reported (12), we tested ts6 and HG52 stocks graciously provided by L. Schnipper. In our hands, this ts6 stock was indeed substantially resistant to 2'NDG ($ED_{50}$ of >25 μM), as had been reported (12), thus differing from the early passage ts6 stock. It thus appears that mutant ts6 exists in two forms—one, 2'NDG resistant (12), which is altered relative to the original 2'NDG-sensitive ts6 whose *ts pol* mutation was mapped by Chartrand et al. (5). Further study is required to determine whether the 2'NDG resistance exhibited by one form of ts6 is due to a *pol* mutation.

**Sensitivity of araA-resistant mutants to arabinosyl analogs. (i) Resistance to araA.** In the original description of the araA-resistant mutants (9), the data demonstrating the resistance of the mutants to araA was presented to emphasize the 10-fold or greater differences in plating efficiency of the mutants relative to strain KOS at high araA concentrations (e.g., 400 μM; Fig. 1A in reference 9). When these data are restated in terms of $ED_{50}$ values, as with PAA, the differences are more subtle, with values ranging from ca. two-fold to four-fold greater than that of strain KOS (9, 24; unpublished data). AraA$^r$9 was the least resistant, AraA$^r$6, 7, 8, 12, and 13 were more resistant, and PAA$^r$5 was the most resistant.

**(ii) Resistance to araT.** We examined the relative sensitivities of the araA-resistant mutants to the related drug, araT. All of the mutants exhibited substantial resistance to this drug (Fig. 6). Wild-type strain KOS generally exhibited an $ED_{50}$ for araT of ca. 1.2 μM. This value is slightly lower than that reported by another group (3). AraA$^r$9 exhibited an $ED_{50}$ for araT ca. fivefold greater than that of strain KOS, whereas four mutants, AraA$^r$6, 8, 12, and 13, exhibited $ED_{50}$s ca. 10-fold greater than that of strain KOS. AraA$^r$7 was more resistant still, exhibiting an $ED_{50}$ ca. 15-fold greater than that of KOS, whereas PAA$^r$5 was highly resistant to araT, exhibiting an $ED_{50}$ of ca. 120 μM.

**(iii) Sensitivity and hypersensitivity to FMAU.** FMAU, a compound with potent HSV activity (25), differs only slightly from araT in that the arabinosyl hydroxyl group of araT is

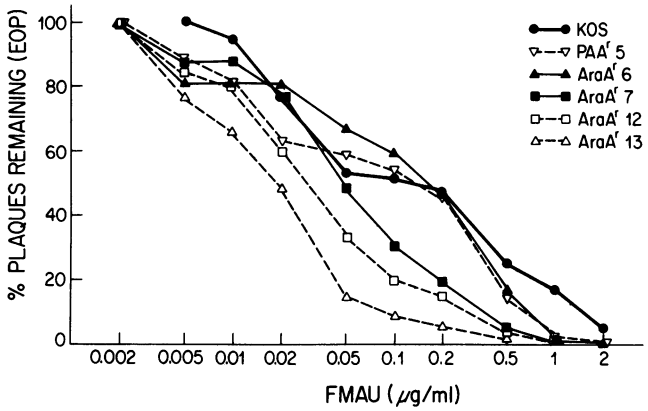


FIG. 7. Effect of FMAU on strain KOS and mutant viruses. The dose-response curves for the various viruses are indicated. Results for mutants AraA$^r$8 and AraA$^r$9 were virtually identical to those shown for mutant AraA$^r$6.

replaced with a fluorine atom (Fig. 1). This substitution had a major effect on the sensitivities of the araA-resistant mutants relative to that of KOS (Fig. 7). Wild-type strain KOS typically exhibited an $ED_{50}$ for FMAU of ca. 0.1 μg/ml (0.4 μM). Similar values have been obtained by C. McLaren (personal communication). Mutants AraA$^r$6, 8, and 9 and PAA$^r$5 appeared as sensitive to FMAU as strain KOS, whereas AraA$^r$7, 12, and 13 appeared modestly hypersensitive, exhibiting $ED_{50}$s two- to fivefold lower than that of KOS. Additionally, these latter three mutants plated much less efficiently than strain KOS at concentrations of FMAU between 0.2 and 0.5 μg/ml.

An unusual feature of plaque reduction assays with FMAU was noted. The interaction of most antiviral drugs with HSV produces sigmoid plaque reduction curves in which virtually all plaque reduction occurs between concentrations of drug which differ by 10- to 20-fold (e.g., reduction of plating efficiency of KOS is first observed between 0.2 and 0.5 μM ACG and is nearly 100% complete by 5 μM [Fig. 3]). In contrast, FMAU produced relatively flat plaque reduction curves, spanning concentrations of the drug which differed by several hundred-fold. Similar flat dose-response curves have been observed by S. Sacks (personal communication) with dye uptake assays of FMAU sensitivity. This suggests competing processes affecting the antiviral action of FMAU.

**(iv) Sensitivity and hypersensitivity to FIAU.** FIAU differs from FMAU by substitution of an iodine residue for the 5-methyl group of FMAU. It thus differs from the normal nucleoside, thymidine, in both its base and sugar moieties. Like FMAU, it possesses potent antiviral activity (25), typically yielding in our hands an $ED_{50}$ for KOS of ca. 0.05 μg/ml (0.1 μM) (Fig. 8). Slightly higher $ED_{50}$ values have been obtained by C. McLaren (personal communication). All of the araA-resistant mutants were sensitive or modestly hypersensitive to this drug, with AraA$^r$7, 12, and 13 being the most hypersensitive, having $ED_{50}$s two- to threefold lower than that of KOS. Thus, the pattern of sensitivity to this drug mimicked that to FMAU; however, FIAU did not produce flat plaque reduction curves like FMAU. Interestingly, all the mutants appeared somewhat hypersensitive to 0.1 μg of FIAU per ml compared with strain KOS.

Taken together, the data in Fig. 3 through 8 indicate that *pol* mutations can confer resistance or hypersensitivity to nucleoside analogs altered in their sugar moieties. The observed drug sensitivities depend exquisitely on the alteration in the sugar. These results presumably reflect the sugar recognition properties of the mutant polymerases.

**Sensitivity of araA-resistant mutants to thymidine analogs with an altered base. (i) Slight resistance to IUdR.** IUdR, which is licensed in the United States for topical treatment of HSV ophthalmic infections, is a thymidine analog with iodine substituting for the 5-methyl group. We examined the relative sensitivities of the araA-resistant mutants to IUdR (Fig. 9). In our hands, strain KOS generally exhibited an $ED_{50}$ of 7 μg/ml (20 μM). All the mutants exhibited slight resistance to IUdR, with $ED_{50}$s ca. 1.4-fold greater than that of KOS. The resistance was particularly evident at 20 μg of IUdR per ml, at which the mutants exhibited plating efficiencies ranging from 5- to 15-fold greater than that of KOS. Thus, restoration of the hydrogen atom at the 2′ position in IUdR compared with FIAU converted modestly hypersensitive mutants like AraA$^r$13 to slightly resistant ones.

**(ii) Sensitivity and hypersensitivity to BVdU.** We next examined the sensitivity of the araA-resistant mutants to BVdU (16), a thymidine analog in which a bromovinyl moiety substitutes for the 5-methyl group (Fig. 1). All the mutants were either sensitive or hypersensitive to this drug (Fig. 10, bottom). KOS exhibited an $ED_{50}$ of ca. 0.03 μg/ml (0.09 μM), which is identical to the value reported by another group (13). AraA$^r$6 and 8 exhibited sensitivities to BVdU indistinguishable from that of KOS, whereas mutants AraA$^r$7 and 9 exhibited $ED_{50}$s ca. 2.5-fold lower than that of

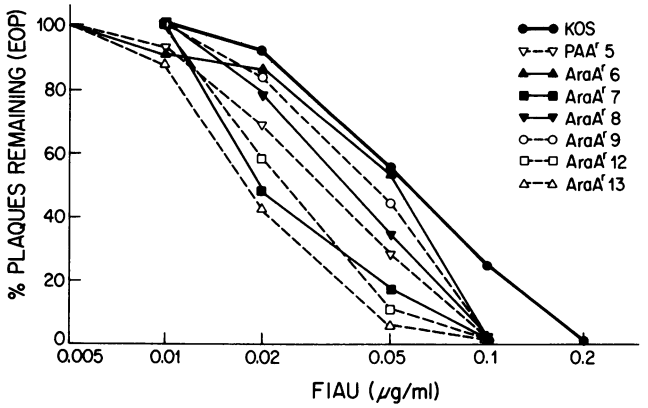


FIG. 8. Effect of FIAU on strain KOS and mutant viruses. The dose-response curves for the various viruses are indicated.

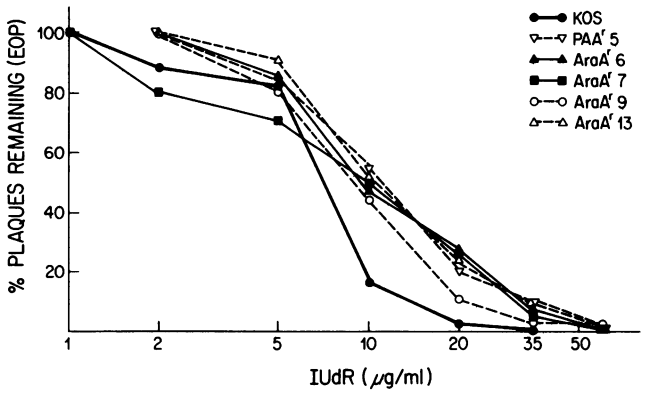


FIG. 9. Effect of IUdR on strain KOS and mutant viruses. The dose-response curves for the various viruses are indicated. Results for mutants AraA$^r$8 and AraA$^r$12 were similar to those shown for PAA$^r$5.

strain KOS. Mutants AraA$^r$12 and 13 were even more hypersensitive, exhibiting $ED_{50}$s ca. fourfold lower than that of strain KOS (data for AraA$^r$12 not shown). Mutant PAA$^r$5 has previously been reported to be sensitive to BVdU (13). We found on several occasions that PAA$^r$5 was slightly hypersensitive to BVdU, exhibiting $ED_{50}$s ca. twofold lower than that of KOS, but that this hypersensitivity was not always reproducible (unpublished data). We would therefore classify this mutant as, at most, marginally hypersensitive.

**(iii) Mapping a BVdU hypersensitivity marker to the HSV *pol* locus.** Because these araA-resistant mutants are known or likely to be mutated in the *pol* locus and were all sensitive or hypersensitive to BVdU, we examined known HSV *pol* mutants of another class—temperature sensitive—for their sensitivity to BVdU. Mutants tsC7, tsC4, tsD9, and tsO22 (2, 7, 37, 40) were tested at the permissive temperature, 34°C. None of the mutants exhibited either marked resistance or hypersensitivity to BVdU. As shown in Fig. 10, top, this was true even for tsD9, which exhibited an $ED_{50}$ less than twofold greater than that of KOS, even though this mutant specifies substantially less thymidine kinase (TK) activity than the wild type (11, 44; unpublished data). TK-deficient mutants are usually substantially resistant to BVdU (13, 15, 22). This result suggested that mutant tsD9 might contain a *pol* BVdU hypersensitivity mutation which counteracted the TK deficiency, leading to only slight BVdU resistance. This suggestion was confirmed by examination of two recombinant viruses, D9$^+$E/K2 and D9$^+$E/K4, which were constructed by rescue of the *ts* marker of tsD9 by an 0.8-kbp wild-type DNA fragment (7). These recombinants are *ts*$^+$ and PAA sensitive (7; unpublished data). They were actually more resistant to BVdU than tsD9 itself, with $ED_{50}$s ca. twofold higher (Fig. 10, top). This maps a BVdU hypersensitivity marker in tsD9 to the 0.8-kbp region (map coordinates 0.422 to 0.427) where the tsD9 *ts*, PAA$^r$, and ACG$^r$ markers are located (7) (summarized in Fig. 11).

The data in Fig. 8, 9, and 10 taken together indicate that *pol* mutations can confer altered sensitivity to nucleoside analogs altered in their base moieties, suggesting that the base recognition properties of the polymerase are changed in these mutants.

**(iv) Reexamination of a mutant reported to be BVdU resistant.** Mutant paa$^r$-1, derived from HSV-1 strain 17 (32), has been reported to be highly resistant to BVdU, with an $ED_{50}$ of 10.4 μg/ml (13), ca. 300-fold greater than that of KOS (13; Fig. 10) or wild-type strain 17 (13; unpublished data). This resistance was attributed to a mutation mapping in the *pol* locus (13). Since none of the *pol* mutants we examined exhibited anything approaching this degree of resistance to BVdU, we reexamined the sensitivity of paa$^r$-1 to BVdU.

Mutant paa$^r$-1 was kindly provided by C. Crumpacker and independently by H. Marsden and J. H. Subak-Sharpe. In our hands, both stocks of this mutant exhibited sensitivity to BVdU which was indistinguishable from that of wild-type strains 17 and KOS ($ED_{50}$ of ca. 0.03 μg/ml). Our results and those reported previously (13) were obtained by using similar procedures in Vero cells. We obtained the same $ED_{50}$ values for strains 17 and KOS as reported previously (13) and were able to observe substantial BVdU resistance exhibited by TK-deficient mutants (unpublished data). Although different Vero cell lines have yielded degrees of drug resistance which vary 5- to 10-fold with the same mutant, they also yielded differences in $ED_{50}$ values for wild-type viruses (D. Coen and P. Furman, unpublished data). Thus, it is difficult to attribute the 300-fold difference between our results and those published (13) to such effects. For now, at least, we cannot confirm reports of mutants which exhibit marked resistance to BVdU due to a *pol* mutation (13).

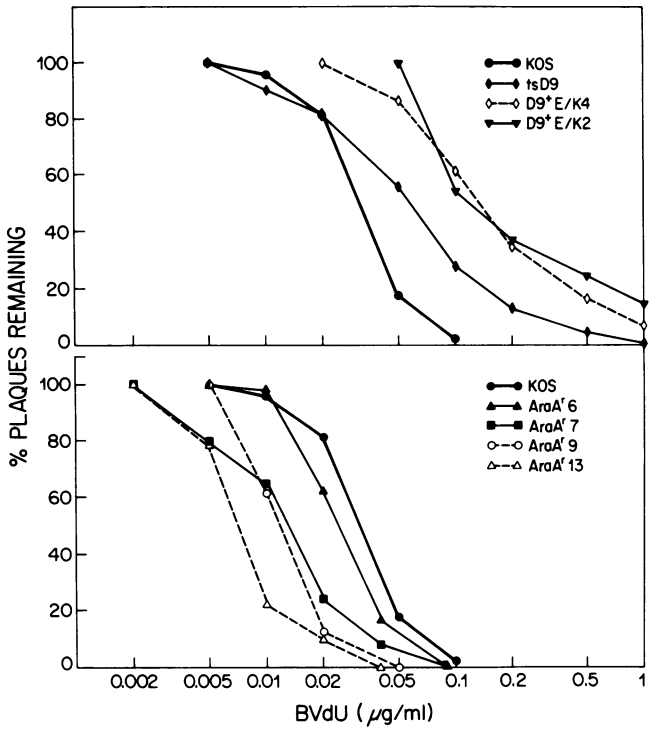


FIG. 10. Effect of BVdU on strain KOS and mutant and recombinant viruses. Top panel, Dose-response curves for strain KOS, tsD9, and the recombinant viruses are indicated. Dose-response curves for tsC4, tsC7, tsO22, paa$^r$-1, and strain 17 were similar to that of strain KOS. Bottom panel, Dose-response curves for strain KOS and AraA$^r$ mutants are indicated. Results for mutant AraA$^r$8 were virtually identical to those shown for mutant AraA$^r$6; results for mutant AraA$^r$12 were virtually identical to those shown for mutant AraA$^r$13.

## DISCUSSION

**Definitions of resistance, sensitivity, and hypersensitivity.** Table 2 summarizes drug sensitivity data for seven araA-resistant mutants, AraA$^r$6 through 9, AraA$^r$12, AraA$^r$13, and PAA$^r$5. For each drug, we designated each mutant as resistant, marginally resistant, sensitive, marginally hypersensitive, or hypersensitive. We based these designations on two criteria: first, the $ED_{50}$ of each mutant relative to that of strain KOS, and second, plating efficiency differences at the various drug concentrations tested. Mutants were designated as sensitive to a given drug when we could not distinguish their behavior in the presence of that drug from that of KOS. When the $ED_{50}$ for a mutant was more than twofold greater or more than twofold less than the $ED_{50}$ for KOS, we designated that mutant as resistant or hypersensitive, respectively. When the $ED_{50}$ value for a mutant was less than twofold greater or less than twofold smaller than the $ED_{50}$ for strain KOS, yet the mutant exhibited plating efficiencies twofold or greater different than that of strain KOS at certain drug concentrations, we designated that mutant as marginally resistant or marginally hypersensitive, respectively. For some mutants, the two criteria were in conflict. For example, AraA$^r$8 exhibited an $ED_{50}$ twofold lower than that of strain KOS in 2′NDG, yet it did not exhibit a plating efficiency twofold lower than that of KOS at

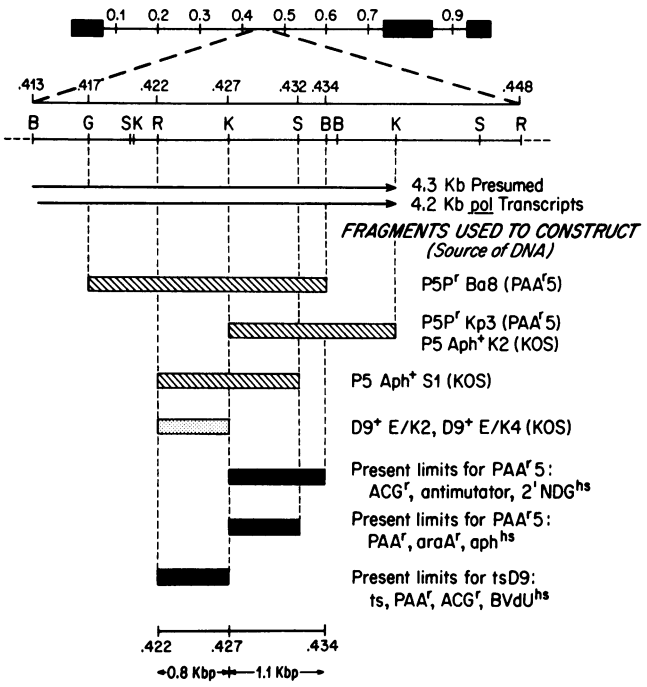


FIG. 11. Physical map locations of the drug hypersensitivity markers of mutants PAA$^r$5 and tsD9. The top line is a schematic representation of the HSV DNA molecule, with physical map coordinates provided. The second and third lines show an expanded view of the region between coordinates 0.413 and 0.448 which contain the *pol* locus (7), with coordinates and restriction endonuclease recognition sites for enzymes *Bam*HI (B), *Bgl*II (G), *Sal*I (S), *Kpn*I (K), and *Eco*RI (R) indicated. Coordinates and location of restriction enzyme sites are as previously described (7, 8, 48). Below are two lines with arrowheads depicting the approximate location of two transcripts described by Holland et al. (35), which span finely mapped *pol* mutations (7) and are thus good candidates to encode polymerase. The arrowheads denote the 3' ends of the transcripts. The map locations of the fragments used to construct the recombinant viruses used in the experiments depicted in Fig. 5 and 10 are shown as hatched or stippled boxes. The names of the recombinant viruses are indicated to the right of the boxes and the viruses from which the DNA fragments used to construct the recombinants were derived are within parentheses (7, 8). Below these are three black boxes; the upper box depicts the map limits for the ACG resistance (7), antimutator (31), and 2'NDG hypersensitivity markers (Fig. 5) or PAA$^r$5. The middle box depicts the map limits for the PAA and araA resistance and aphidicolin hypersensitivity markers of PAA$^r$5 (8, 24; unpublished data). The bottom box depicts the map limits for the temperature sensitivity PAA and ACG resistance, and BVdU hypersensitivity markers of tsD9 (7; Fig. 10). At the bottom of the figure are the map coordinates and sizes of the regions containing the aforementioned markers. Abbreviations: r, resistant; hs, hypersensitive.

any drug concentration tested (Fig. 4). We classified this mutant and others like it as sensitive to 2'NDG. On the other hand, certain mutants exhibited $ED_{50}$s for PAA less than twofold greater than that of strain KOS, yet at certain PAA concentrations (e.g., ≥490 μM) exhibited high plating efficiencies, whereas KOS exhibited a plating efficiency of less than 1% (9; unpublished data). These mutants were classified as resistant. We would stress the importance of examining the entirety of a plaque reduction curve in evaluating whether a given mutant is resistant, sensitive, or hypersensitive.

**Five distinct araA-resistant mutants.** As summarized in Table 2, the seven araA-resistant mutants exhibit five distinct phenotypes based on their sensitivities to the 10 antiviral drugs shown in Fig. 1 and to aphidicolin (8). These phenotypes are distinct, both qualitatively and quantitatively (e.g., AraA$^r$7 and PAA$^r$5 are somewhat similar qualitatively, but PAA$^r$5 is considerably more resistant to araT, etc.). We grouped AraA$^r$6 and 8 together and AraA$^r$12 and 13 together. In both cases, the mutants grouped together have the same qualitative sensitivity classification (resistant, sensitive, etc.). In plaque reduction assays with ACG, 2'NDG, and FIAU, the two mutants in each of these pairs behaved slightly differently from each other (Fig. 3, 4, and 8); however, we did not judge these differences to be significant. For example, AraA$^r$13 exhibited a lower $ED_{50}$ for ACG than AraA$^r$12; however, their plating efficiencies were less than twofold different from each other at all drug concentrations tested and nearly identical at higher concentrations (Fig. 3). Each mutant pair arose from the same selection procedure (9) and could well be clonally related. Clearly, the five distinct araA-resistant mutants arose from different mutational events, presumably in the *pol* locus.

**Drug hypersensitivity as a common phenotype.** The data summarized in Table 2 make it clear that mutants which are hypersensitive to antiviral drugs are common, and the results of our mapping experiments (Fig. 5, 10, and 11) establish that antiviral-drug hypersensitivity mutations can be ascribed to particular loci, in these cases, the *pol* locus. There are numerous examples in the literature of data which might indicate drug hypersensitivity, perhaps due to altered DNA polymerase, but which were described as indicative of wild-type drug sensitivity (6, 9, 12, 15, 27). In some cases this is difficult to judge in the absence of complete plaque reduction curves. Additionally, our studies with mutant tsD9 indicate that drug hypersensitivity mutations at one locus can counteract resistance mutations at other loci, leading to intermediate degrees of resistance.

**Implications for detection of araA resistance.** Despite the proven clinical efficacy of AraA (34, 49), there have been numerous cases in which araA treatment of HSV infections has proven unsuccessful. In certain of these cases, araA-resistant HSV strains have been reported (see reference 43 for selected references); however, in many cases it has been difficult to document either that a given strain is more resistant to araA than most wild-type strains or that post-treatment isolates are any more resistant than pretreatment isolates. The level of araA resistance of the most araA-resistant mutant we studied (PAA$^r$5) is relatively slight. As summarized in Table 2, all five distinct araA-resistant mutants are resistant to two anti-HSV agents which are more selective than araA—araT and ACG. In every case, the araA-resistant mutants exhibited more resistance to these two drugs than they did to araA. We would suggest therefore that those attempting to detect araA-resistant mutants in clinical isolates screen their isolates for the more readily detectable resistance to araT or ACG, or both.

**Implications for treatment of araA-resistant infections.** There are currently three drugs licensed for treatment of HSV infections in the United States: IUdR, araA, and ACG. Numerous other drugs, including several examined in this study, are in various phases of development. The availability of several antiviral drugs may make it possible to combat infections resistant to any one antiviral drug. Our results indicate that five distinct araA-resistant mutants are at least slightly resistant to the two other licensed antivirals, IUdR and ACG, and that all but one are at least modestly resistant to PFA. This might suggest that these three antiviral drugs would be less successful in treating araA-resistant clinical

TABLE 2. Drug sensitivity phenotypes of araA-resistant mutants[a]

| Mutant(s) | $PP_i$ | | Acyclic G | | Arabinosyl | | | | T altered at the 5-position | | Aph |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | PAA | PFA | ACG | 2'NDG | araA | araT | FMAU | FIAU[b] | IUdR | BVdU | |
| AraA$^r$9 | R/S | S | R | HS | R | R | S | HS/S | R/S | HS | HS/S |
| | 1.3 | 1 | 4 | 5 | 2 | 5 | 1 | 1.3 | 1.4 | 3 | 1.3 |
| AraA$^r$6 and 8 | R | R | R | S | R | R | S | HS/S | R/S | S | HS |
| | 3 | 4 | 4 | 1–2 | 3 | 10 | 1 | 1–1.5 | 1.4 | 1 | 1.6 |
| AraA$^r$12 and 13 | R | R | R | S | R | R | HS | HS | R/S | HS | HS |
| | 1.6 | 4 | 5–15 | 1–2 | 3 | 10 | 4 | 3 | 1.4 | 4 | >3 |
| AraA$^r$7 | R | R | R | S | R | R | HS/S | HS | R/S | HS | HS |
| | >3 | 7 | 15 | 1.8 | 3 | 15 | 2 | 3 | 1.4 | 3 | >3 |
| PAA$^r$5 | R | R | R | HS | R | R | S | HS | R/S | HS/S | HS |
| | >10 | 7 | 20 | 3 | 4 | 100 | 1 | 2 | 1.4 | 1–2 | 10 |

[a] Phenotypes are described as resistant (R), marginally resistant (R/S), sensitive (S), marginally hypersensitive (HS/S), or hypersensitive (HS), as defined in the text. The values below the descriptive abbreviations are the fold differences in $ED_{50}$ between the mutant and wild-type strain KOS. Data are from this report, with the exception of the data for PAA, araA, and aphidicolin, which are from references 7–9 and 27. Classes of drugs: PPI, pyrophosphate analogs; acyclic G, acyclic guanosine analogs; T, thymidine analogs; Aph, aphidicolin.

[b] FIAU is also classified as a thymidine analog altered at the 5-position.

infections than the other antiviral drugs. Nevertheless, the levels of resistance to IUdR, ACG, and PFA which we have detected may not be sufficient to vitiate the clinical use of these antiviral drugs against araA-resistant infections. Moreover, the use of antiviral drugs in combination might lessen problems with resistant strains (43).

**Implications for mechanisms of drug action. (i) Genetic criteria for polymerase contributing to antiviral selectivity.** The antiviral drugs examined in this study are all selective against HSV infections, as indicated by pharmacological studies and by the existence of viral mutants resistant to them. The identification of drug resistance mutations in a particular gene implies that the wild-type form of the product of that gene contributes to the selectivity of the drug. For example, one could hypothesize that the selectivity of ACG was due entirely to its activation by HSV TK, leading to a poisoning of numerous processes, thereby preventing viral replication. However, the identification of ACG-resistant *pol* mutations demonstrates that particular properties of the HSV polymerase contribute to the selectivity of ACG. Firm identification of the *pol* gene as a site for drug resistance mutation requires genetic studies linking the mutation to the *pol* locus. Nevertheless, the initial identification and mapping of the *pol* locus also rested upon biochemical analyses of mutant polymerases. However, with our increased understanding of the *pol* locus from fine mapping (7, 8, 24; unpublished data) and from analyses of transcripts and DNA sequences (35; unpublished data), we would argue that properly executed physical mapping studies can identify a *pol* mutation, even in the absence of biochemical studies associating the mutation with an appropriately altered DNA polymerase.

**(ii) Polymerase mediating sensitivity to 2'NDG and BVdU.** The demonstration of a drug hypersensitivity mutation within a particular gene does not imply that the product of that gene contributes to the selectivity of the drug; nevertheless, it does show that the product of that gene *mediates* drug sensitivity. Most of the araA-resistant mutants exhibit hypersensitivity to 2'NDG or BVdU, or both. Moreover, a 2'NDG hypersensitivity marker of PAA$^r$5 was shown to lie within a 1.1-kbp region of the *pol* locus, and a BVdU hypersensitivity marker of tsD9 was shown to be located in an adjacent 0.8-kbp fragment in the *pol* locus (Fig. 5, 10, and 11). Thus, *pol* mutations can confer hypersensitivity to these two drugs.

The 2'NDG hypersensitivity of PAA$^r$5 may be explained by the biochemical data of St. Clair et al. (47), who showed that this mutant specifies a polymerase with an apparent $K_i$ for the triphosphate of 2'NDG identical to that of the wild-type (KOS) enzyme, but with an apparent $K_m$ for the competing natural nucleotide, dGTP, that is three- to fourfold higher. Thus, the ratio of $K_i$ to $K_m$ is three to fourfold lower for PAA$^r$5 compared with KOS, a value similar to the degree of hypersensitivity we observe. One would expect that the importance of $K_i/K_m$ ratios in determining relative levels of sensitivity might depend on the intracellular concentrations of nucleoside triphosphates relative to the nucleoside triphosphate-binding constants of the polymerase. Furman et al. (28) observed different levels of triphosphates in different Vero cell lines, which might account for why we observed the 2'NDG hypersensitive phenotype of PAA$^r$5, whereas others (12, 46, 47) have not.

The triphosphates of 2'NDG and BVdU have been shown to serve as substrates for HSV DNA polymerase and to inhibit HSV DNA polymerase with lower $K_i$ values than cellular DNA polymerase α (1, 15, 26, 30, 42, 47). This suggests that preferential affinity for HSV DNA polymerase contributes to the selective antiviral effect of these drugs. The identification of *pol* mutations conferring resistance to these drugs would demonstrate that this is so. Although reports of mutations conferring resistance to these two drugs mapping in the *pol* locus have appeared (12, 13), we have been unable to confirm them. Clearly, HSV DNA polymerase mediates sensitivity to 2'NDG and BVdU; it has not yet been demonstrated that the properties of this enzyme contribute substantially to the antiviral selectivity of these drugs.

**(iii) Evidence for polymerase as target for PFA, araT, IUdR, FMAU, and FIAU.** The observation that known or likely *pol* mutants, such as the araA-resistant mutants, are resistant or hypersensitive to a particular drug does not prove that polymerase is a target for that drug, but it does provide evidence in support of that concept. Two or more araA-resistant mutants were resistant to PFA and araT,

marginally resistant to IUdR, or hypersensitive to FMAU and FIAU. Our data, then, supplement previous similar observations (3, 17, 20) to support the idea that *pol* mutations can confer resistance to two of these drugs, PFA and araT. There has previously been much less evidence, however, linking *pol* mutations with altered sensitivity to IUdR, FMAU, or FIAU. Indeed, certain known or likely *pol* mutants have been shown to be sensitive to IUdR (18). There have been reports of IUdR-resistant viruses isolated from ophthalmic HSV infections which were TK proficient or cross-resistant to araA, or both, but none have been shown to be resistant due to *pol* mutations (see reference 43 for selected references). On the other hand, a recent report by Darby et al. (15) has indicated that an IUdR resistance marker cosegregated with PAA resistance and altered polymerase in mutant $R_9C_2$. Our data further suggest that *pol* mutations can confer IUdR resistance. We are not aware of any previous published reports examining *pol* mutants for sensitivity to FMAU and FIAU. In summary, our studies tend to support the concepts developed from biochemical studies that HSV DNA polymerase is a target for PFA, araT, IUdR, FMAU, and FIAU (3, 17, 20, 23, 33, 39, 42).

**Implications for functional dissection of the pol locus. (i) Expanding the number of mutational sites.** Previous studies have identified five distinct mutants of HSV strain KOS as *pol* mutants; temperature-sensitive mutants tsC7, tsC4, tsD9, and tsO22 and mutant PAA$^r$5 (7). Mutations in all but tsO22 have been mapped to separate regions in the *pol* locus; the map limits of the tsO22 mutation overlap those of tsD9 and PAA$^r$5, but the three mutants can be distinguished by their drug and temperature sensitivity phenotypes (7, 8, 48). AraA$^r$6 through 9, AraA$^r$12, and AraA$^r$13 are likely to be *pol* mutants on the basis of their altered drug sensitivities (8, 9; this report) and antimutator phenotypes (31) and on the basis of genetic and biochemical studies (unpublished data; P. Furman and M. H. St. Clair, personal communication). Thus, four more distinct *pol* mutants can likely be added to the list, bringing the total to nine for this strain. Additionally, nine other likely *pol* mutants have been isolated from strain KOS (3, 10, 17, 27, 35). These nine mutants may well differ from each other and from the four temperature-sensitive and five distinct araA-resistant mutants, which would raise the number of distinct *pol* mutants of strain KOS to 18. Since such mutations can be cloned and mapped finely (7, 8, 24), it should be possible to determine the amino acid changes responsible for the different phenotypes of these mutants.

**(ii) Mutations affecting the pyrophosphate exchange-release site.** If we assume that the phenotypes of the araA-resistant mutants are due to *pol* mutations (as has been demonstrated for many of the phenotypes of PAA$^r$5), then their drug sensitivity phenotypes can shed light on the nature of the catalytic sites of HSV polymerase, particularly with regard to how they interact with analogs of natural substrates. Both PAA and PFA are pyrophosphate analogs thought to bind to at least a portion of the polymerase pyrophosphate exchange-release site (17, 38). This site most likely overlaps that portion of the nucleoside triphosphate-binding site which recognizes the β and γ phosphates. A previous study examining purified polymerases specified by five mutants isolated for PFA resistance indicated that for those mutants, resistance to PFA was always associated with a similar degree of resistance to PAA but not to β-phenyl-PAA (17). None of our mutants exhibit any drastic differences in resistance to PFA and PAA. Nevertheless, subtle differences in the degree of resistance to these two drugs can be observed. For example, AraA$^r$9 and PAA$^r$5 were more resistant to PAA than PFA, whereas AraA$^r$6 and AraA$^r$13 were more resistant to PFA than PAA. The two studies taken together suggest the existence of subtle changes in or near the pyrophosphate exchange-release site in the different mutant polymerases, which confer various degrees of discrimination against phosphono derivatives of different chain lengths.

**(iii) Mutations affecting sugar recognition at the nucleoside triphosphate-binding site.** ACG, 2′NDG, araA, araT, and FMAU are all nucleoside analogs with altered sugar moieties; *pol* mutations conferring altered sensitivity to them can be expected to affect sugar recognition at the nucleoside triphosphate-binding site of polymerase. In contrast to the case of the pyrophosphate analogs, drastic differences in resistance to different members of this class of analogs were seen depending on the alterations in the sugar moiety. Addition of the 3′ moiety to ACG, turning it into 2′NDG, converted resistance to sensitivity or hypersensitivity. An even subtler change, substituting the arabinosyl hydroxyl of araT with a fluorine, also converted resistance to sensitivity or hypersensitivity. In the case of PAA$^r$5, this represented a 100-fold change in $ED_{50}$ relative to the wild type. In both cases, the alterations leading to greater relative sensitivity can be considered to have changed the analog into one more closely resembling the natural substrate. 2′NDG clearly more closely resembles deoxyguanosine than ACG; it lacks only the 2′ moiety. FMAU resembles deoxythymidine more than araT in that the 2′ fluorine atom is closer in size to the normal hydrogen atom than the hydroxyl group of araT. It may also be, however, that the mutant polymerases recognize the fluoroarabinosyl derivatives better than the natural substrates, relative to the recognition properties of wild-type polymerase. In support of this hypothesis, all of the mutants exhibited some degree of hypersensitivity to FIAU, yet all were marginally resistant to IUdR.

**(iv) Mutations affecting base recognition at the nucleoside triphosphate-binding site.** IUdR and BVdU are thymidine analogs with alterations at the 5-position. *pol* mutations conferring altered sensitivity to these compounds can be expected to be altered in their recognition of this portion of thymidine triphosphate. The mutants studied here behaved differently depending upon the substitution for the normal methyl group at the 5-position: the iodo substitution led to slight resistance, and the bromovinyl substitution led to hypersensitivity in several cases. This may reflect amino acid changes in the polymerase which lead to enhanced recognition of a bromovinyl group and decreased recognition of an iodo group relative to recognition of the methyl group of the natural substrate.

**(v) Mutations affecting recognition at more than one site.** Although the number of analogs we tested does not constitute an exhaustive set, it is clear that different mutants display different alterations in recognition of the different classes of analogs. For example, AraA$^r$9 exhibits substantial alterations in sensitivity to ACG, 2′NDG, araT, and BVdU but minimal alterations in sensitivity to PAA and PFA. It may be altered in an amino acid in the nucleoside triphosphate-binding site, which recognizes both the sugar and the base but does not seem as involved in pyrophosphate recognition. AraA$^r$6 and PAA$^r$5, on the other hand, are substantially altered in sensitivity to PAA, PFA, ACG, 2′NDG, and araT, but are minimally altered in sensitivity to analogs with fraudulent base moieties. They may contain mutations which alter amino acids recognizing both sugar and pyrophosphate moieties but which are not as involved in base recognition. AraA$^r$7 and AraA$^r$13 are altered in sensitivities to all three classes of analogs, suggesting that they

contain mutations which alter amino acids involved in recognition at all three sites. Examination of models of araT-triphosphate and B-DNA indicates that a single amino acid residue could conceivably contact the 5-position, the 2′ position, and the pyrophosphate moiety of araT-triphosphate. Alternatively, mutations might change amino acids in only one or two sites or lie entirely outside the binding site and alter recognition of different portions of nucleoside triphosphates by indirect effects on tertiary structure. It will be of interest to determine whether the phenotypes of these mutants are due to single amino acid changes in the *pol* locus and to determine where the altered amino acids are located relative to the polymerase nucleotide-binding site.

## ACKNOWLEDGMENTS

We thank H. Chiou, A. Ellington, P. Furman, J. Gibbs, I. Goldberg, D. Knipe, and P. Schaffer for helpful discussions. L. Schnipper, C. Crumpacker, H. Marsden, and J. H. Subak-Sharpe graciously provided viral mutants and B. Eriksson of Astra, G. Elion and D. Barry of Burroughs Wellcome Co., C. McLaren of Bristol Meyers, and E. De Clercq of Rega Institute generously supplied us with PFA, ACG and BW759U, FMAU and FIAU, and BVdU, respectively. We thank M. H. St. Clair, P. Furman, C. McLaren, and S. Sacks for communicating unpublished data. Portions of some of these experiments were begun in the laboratory of P. Schaffer, whose help and encouragement are gratefully acknowledged. Assistance in manuscript preparation was provided by J. Spognardi and K. Cox.

This work was supported by a Public Health Service grant AI19838 from the National Institute of Allergy and Infectious Diseases and a PMA Foundation Research Starter Grant to D.M.C.

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