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Herpes Simplex Virus Variants Resistant to High Concentrations of Acyclovir Exist in Clinical Isolates

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Acyclovir (ACV) has been shown to inhibit the replication of herpes simplex virus (HSV) in vitro. We examined a wide variety of HSV clinical isolates for the presence of naturally occurring ACV-resistant (ACV^r) variants. Although the ACV doses that inhibited 50% of these isolates were within the range of doses inhibiting 50% of the ACV-susceptible wild-type strains, we successfully isolated variants resistant to high ACV concentrations (25 to 75 μM) from each virion population even in the absence of prior drug exposure. Furthermore, we demonstrated, by fluctuation analysis of two encephalitis strains, that the ACV^r variants were clonally distributed in the virus populations before exposure to ACV and did not result from rapid adaptation to ACV. All variants isolated after a single exposure to a high dose of ACV were true ACV^r variants, as demonstrated by their high plating efficiencies in the presence of ACV. We found that 36 and 50% of the ACV^r variants of the two strains examined in detail displayed plating efficiencies in phosphonoacetic acid of >0.1, possibly indicating that many of the ACV^r variants contained alterations in the DNA polymerase gene locus. Because the distribution of ACV^r variants in natural populations is relatively high (10^{-4}), these results suggest that selection of ACV^r strains during ACV therapy is possible.

The drug 9-[(2-hydroxyethoxy)methyl]guanine (acycloguanosine; generic name, acyclovir [ACV]) has been shown to possess strong antiviral activity against a variety of herpesviruses in vitro and to have little toxicity to uninfected cells in culture (4, 6, 18). The specificity of action of ACV is based on the ability of the herpesvirus-specified thymidine kinase (TK) to phosphorylate the drug, which can then be converted to its active form, acycloguanosine triphosphate. Cellular TK, however, can phosphorylate ACV only inefficiently (6, 12). The acycloguanosine triphosphate form inhibits viral DNA synthesis at the level of the viral DNA polymerase, which allows the drug to become incorporated into growing DNA chains and thereby effect chain termination (6, 11).

Resistance to ACV can arise because of alterations in the herpes simplex virus (HSV) genes encoding either TK or DNA polymerase (1, 19). Viral TK gene mutations which result in resistance to ACV may be due to the loss of production of an active TK (1, 8, 19) or to the production of a TK with altered substrate specificity (7). Because the viral DNA polymerase gene is essential (14), alterations in its locus most likely lead to the production of a DNA polymerase with altered substrate or allosteric effector specificity. This is most evident by the fact that some ACV-resistant (ACV^r) mutants which are TK^+ are also resistant to phosphonoacetic acid (PAA), a competitive inhibitor of viral DNA polymerase (15), although the actual loci for ACV and PAA resistances may be separable (2, 10).

In the laboratory, it is possible to derive ACV^r mutants by growing HSV in the presence of ACV. In most instances, relatively low ACV concentrations (<10 μM) have been used to maintain selective pressure, resulting in the generation of mutants with alterations in the expression of the HSV TK phenotype (ACV^r-TK mutants [1, 19]). It has been demonstrated that many clinical isolates and laboratory strains of HSV are capable of plaque formation in 10 μM ACV without prior drug exposure (21). However, the investigators did not conclusively determine whether the virus within the plaques represented true, naturally occurring, ACV-resistant HSV mutants. In this study, we examined a variety of clinical HSV isolates that were passaged minimally in the laboratory without selective pressure and were isolated from patients with herpes simplex encephalitis or oral or genital HSV lesions. Although the 50% inhibitory doses (ID_{50}) for the wild-type populations did not vary substantially from those for ACV-susceptible laboratory strains, we demonstrated

that in these laboratory strain populations, variants existed which were resistant to up to 100 μM ACV without prior exposure to the drug. Some of these variants most likely contained mutations in the viral DNA polymerase gene (the locus of genes defining the ACV^r-PAA phenotype), as demonstrated by the altered susceptibility of virus growth to PAA.

(A preliminary report of these results was presented at the Fifth Cold Spring Harbor Workshop on Herpesviruses, 26 to 31 August 1981.)

MATERIALS AND METHODS

Growth of cells and virus. Cell lines were propagated at 37°C in minimum essential medium containing 5% (for CV-1 and Vero cells) or 10% (for human diploid fibroblasts) fetal bovine serum, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 0.075% sodium bicarbonate. Cultures grown in open vessels contained 0.225% sodium bicarbonate and were incubated in a humidified CO_2 incubator.

HSV type 1 (HSV-1) strain KOS, obtained from P. A. Schaffer, Sidney Farber Cancer Institute, Boston, Mass., was plaque purified and passaged once to obtain the stock virus used in these studies. HSV-2 strain HG-52 was obtained from J. Hay, Uniformed Services University of the Health Sciences, Bethesda, Md., and was passaged twice. Both strains were propagated in Vero cells as described previously (17).

Stocks of HSV encephalitis strains were obtained after only two passages in human diploid fibroblasts. Briefly, brain biopsy tissue was obtained from untreated patients suspected of having herpes simplex encephalitis. The tissue was collected in a small amount of 0.85% saline and homogenized in 2 to 3 ml of medium, and a 0.2-ml portion of the resulting mixture was used to inoculate each culture tube (16 by 125 mm) of cells for laboratory diagnosis. When characteristic HSV cytopathology was evident, the cultures were frozen at −70°C. Cultures were later thawed, sonicated, and clarified by centrifugation at 1,000 × g. The supernatant, diluted 1:100 (multiplicity of infection, 0.001 to 0.01 PFU per cell), was inoculated onto human diploid fibroblasts for the production of the virus stocks used in these studies. Stocks of HSV isolated from oral and genital lesions were prepared in a similar manner.

Typing of virus isolates. All isolates were typed on the basis of characteristic DNA cleavage patterns with *Hin*dIII and *Eco*RI (13, 20). Cultures of CV-1 cells in prescription bottles (8 oz [240 ml]) were inoculated with virus at a multiplicity of infection of 5 PFU per cell and incubated at 37°C for 18 h. The cells were scraped into medium, which was pelleted, washed once in TNE buffer (10 mM Tris-hydrochloride [pH 7.4], 100 mM NaCl, 1 mM EDTA) and pelleted again. The cell pellets were then stored at −70°C and processed later for DNA isolation. Thawed cell pellets were lysed by the addition of TNE containing 0.5% Sarkosyl and 0.2% sodium dodecyl sulfate. Pronase which had been autodigested was added to a final concentration of 50 μg/ml, and the lysate was incubated at 37°C for 2 h until it appeared clear. A portion was then added to CsCl in TNE buffer, and the density was adjusted to 1.685 g/ml. Ethidium bromide was added to a final concentration of 15 μg/ml. The material was centrifuged in a Beckman 75 Ti rotor at 125,000 × g for 42 h at 22°C. DNA bands were visualized with a short-wave UV lamp, and the viral DNA in the lower band was collected. The ethidium bromide was removed by extraction with isoamyl alcohol, and the DNA was dialyzed against 0.1× SSC (SSC = 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.4]) to remove the cesium salt. Sodium acetate was added to a concentration of 0.5 M, and the DNA was extracted twice with chloroform: and twice with isoamyl alcohol (24:1) and precipitated with ethanol at −20°C. The DNA pellets were resuspended in 0.1× SSC and digested at 37°C with 4 U of *Eco*RI or *Hin*dIII (Bethesda Research Laboratories, Gaithersburg, Md.) per μg of DNA for 4 to 5 h. The DNA was analyzed by electrophoresis on 0.4% agarose gels, stained with ethidium bromide, and visualized with UV light (302 nm).

Plaque reduction assay. Virus titrations were performed by plaque assay on CV-1 or Vero cells in culture dishes (35 mm; Lux Scientific Corp., Newbury Park, Calif.) with a 2% methylcellulose overlay. As indicated below, antiviral drugs were incorporated into the methylcellulose before the assay. ACV was a gift of the Burroughs Wellcome Co. (Research Triangle Park, N.C.), and PAA was obtained from Ron Duff at Abbott Laboratories (North Chicago, Ill.). ACV was dissolved in medium to yield a final concentration of 4 mM, and this solution was added to the methylcellulose overlay medium to yield desired concentrations. Cultures were incubated at 34°C in a humidified CO_2 incubator for 5 days, stained with neutral red, and enumerated. Virus titers were determined in duplicate cultures inoculated with dilutions of virus yielding 20 to 150 plaques per plate.

Isolation of ACV^r variants. Plaques which appeared in the presence of various concentrations of ACV (see below) were picked and inoculated into culture tubes (16 by 125 mm; Lux) containing 3×10^5 freshly trypsinized CV-1 cells in 1 ml of medium without ACV. Cultures were incubated at 34°C and observed daily for cytopathology. The medium was changed as required. When cultures exhibited 75 to 100% cytopathology, the cells were scraped into the medium, and the material was frozen at −70°C.

Fluctuation analysis. For every strain analyzed, 20 tube cultures, each containing 2.4×10^5 Vero cells, were inoculated with 500 PFU of virus. We prepared corresponding bulk cultures by inoculating two petri dish (100 mm) cultures (3.5×10^6 cells per dish) with virus to yield the same input multiplicity. All cultures were incubated at 34°C and harvested 72 h later when 75 to 100% cytopathology was observed. We harvested the bulk cultures by scraping the cells into the medium and pooling the contents of the two plates. We harvested the tube cultures individually and froze them at −70°C. After freezing and thawing three times, the cultures were clarified by low-speed centrifugation, and the supernatant was retained for virus titration. Bulk cultures were divided into 10 portions and assayed at the same time as the individual cultures in the presence or absence of 25 μM ACV.

RESULTS

Susceptibility of clinical isolates to ACV. We were interested in determining whether variants

TABLE 1. Inhibition of HSV clinical isolates by ACV

Strain	HSV type[a]	Origin	ID_{50} (μM)[b]
KOS	1	Lab strain (oral)	0.40 (0.94)[c]
450	1	Face	0.14 (0.89)
1010	1	Throat	0.14 (0.87)
2021	1	Face	0.29 (0.86)
2526	1	Face	0.78 (0.92)
7-370	1	Adult brain	0.11 (0.95)
8-1973	1	Adult brain	0.06 (0.97)
0-1116	1	Adult brain	1.8 (0.97)
2053	2	Leg	0.89 (0.98)
HG-52	2	Lab strain (genital)	1.7 (0.86)
1482	2	Vagina	1.5 (0.98)
3325	2	Penis	1.2 (0.99)
Patient 1	2	Neonate brain	1.0 (0.94)
D-8575	2	Neonate brain	0.65 (0.98)
7-2667	2	Neonate brain	1.8 (0.93)

[a] Isolates were typed on the basis of the characteristic pattern produced after electrophoresis of *Hin*dIII- or *Eco*RI-cleaved DNA.

[b] Determined by plaque reduction assay in Vero cells. Values were calculated from the logarithmic curve ($y = a + b \ln x$) which best fit the data. A minimum of five ACV concentrations spanning the ID_{50} were used to derive the logarithmic function.

[c] The degree of fit of the points of data to the logarithmic curve is indicated by the coefficient of determination (r^2) shown in parentheses.

resistant to relatively high ACV concentrations were present in populations of uncloned, low-passaged, clinical HSV isolates. To determine a correlation between our ability to isolate such ACV^r variants and the overall susceptibility of each population to ACV, we measured the ID_{50} of ACV for the isolates in Vero cells. Thirteen isolates obtained from patients with a variety of clinical manifestations of HSV disease, including encephalitis, gingivostomatitis, genitalis, and systemic infections, were tested, and the ID_{50} values were compared with those determined under the same conditions for HSV-1 KOS and HSV-2 HG-52 laboratory strains. Each isolate was also typed on the basis of *Hin*dIII and *Eco*RI DNA restriction patterns obtained after electrophoresis on 0.4% agarose gels (see above). The results of these studies are summarized in Table 1.

The mean ID_{50} for the HSV-1 isolates was 0.47 ± 0.60 μM, and that for the HSV-2 isolates was 1.3 ± 0.39 μM. Although the ID_{50} of ACV for HSV-1 adult brain isolate 0-1116 was two standard deviations above the mean ID_{50} for HSV-1 isolates, it did fall within the range of the HSV-2 isolates. Thus, although a wide spectrum of susceptibilities to ACV was found among the populations studied, none of the strains displayed ID_{50} values comparable to those for resistant strains derived by repeated passage in the presence of ACV (1, 8, 19).

Isolation of ACV^r variants. Although the majority (>50%) of the virion population from each of the laboratory and clinical strains was susceptible to low concentrations of ACV, it was not apparent whether individual virions within the populations were resistant to much higher concentrations (25 to 100 μM). Because the existence of such ACV^r variants might lead to the development of general viral resistance during drug treatment, we chose to examine isolates from clinical encephalitis infections, for which effective drug therapy is essential. Stocks of these strains were titrated by plaque assay in the presence of increasing concentrations of ACV and in the absence of the drug.

Table 2 shows the plating efficiency of each encephalitis strain in the presence of ACV. Plating efficiencies of similar magnitudes were observed for the oral and genital isolates as well as the laboratory strains (data not shown). Although the plating efficiencies ranged from 5×10^{-3} to 5×10^{-5}, distinct plaques were detectable for all isolates in the presence of 25 to 75 μM ACV. Interestingly, the size of the plaques was not significantly smaller than that of plaques detected in the absence of drug, although most of the plaques detectable in the presence of 100 μM ACV were smaller. In contrast, for plaques detected in the presence of the lower (0.05 to 5 μM) concentrations of ACV used to determine the ID_{50} for each isolate, the size diminished with increasing ACV concentrations, although the survival of isolates was much greater than at high ACV concentrations (unpublished data). These results suggested that plaques detected at high ACV concentrations represented true ACV^r variants which were present in the unse-

TABLE 2. Plating efficiencies of HSV encephalitis strains in the presence of ACV

ACV (μM)	Plating efficiency for indicated strain[a]					
	Adult			Neonate		
	8-1973	7-370	0-1116	Patient 1	D-8575	7-2667
25	2.9	4.2	10.4	4.5	50.0	17.7
50	3.2	4.6	6.8	4.3	47.0	13.1
75	3.4	1.5	6.4	0.5	44.1	11.0
100	<0.001[b]	<0.01[b]	<0.01[b]	<0.1[b]	32.3	6.1

[a] The plating efficiencies are expressed as [(titer at 34°C with ACV)/(titer at 34°C without ACV)] × 10^4. Titers of the isolates in the absence of ACV were 2.8 × 10^8, 2.6 × 10^8, 2.5 × 10^8, 4.2 × 10^7, 3.4 × 10^6, and 6.2 × 10^7 PFU/ml for strains 8-1973, 7-370, 0-1116, Patient 1, D-8575, and 7-2667, respectively.

[b] No distinct plaques were detected.

lected virion populations. Therefore, three to four well-isolated plaques were picked from plates containing each concentration of ACV as well as from plates without ACV. Plaques were inoculated onto freshly seeded CV-1 cells, which were incubated at 34°C until 75 to 100% of the cells displayed cytopathology. To avoid additional ACV^r selection, we changed the media after 1 and 3 days to ensure that the plaques were grown in the absence of appreciable amounts of ACV. Cultures were harvested as described above, and the harvests were stored for virus titrations.

Resistance of variants to PAA. Each successfully isolated plaque was titrated by plaque assay on CV-1 cells in the absence and presence of the same concentration of ACV used for the initial selection to confirm that each isolate was resistant to ACV. Because the viral DNA polymerase gene locus is the site of some of the genetic alterations responsible for ACV resistance (1, 19), replicate dilutions of the isolates were assayed in the presence of PAA (100 μg/ml), an inhibitor of the HSV DNA polymerase. Although the loci conferring resistance to ACV and PAA in the DNA polymerase gene may be separable (2, 10), a number of ACV^r mutants with alterations in the DNA polymerase gene have been shown to be resistant to PAA as well (1, 8, 10, 19).

Nearly all of the plaques isolated in the presence of ACV maintained their resistance to ACV. The results for two encephalitis strains are presented in Tables 3 and 4. With few exceptions, the plating efficiency of each variant in the presence of the ACV concentration used for isolation was greater than 0.5. Because single exposures to high levels of ACV only were required for isolation, each plaque represented a variant in the unselected virion population resistant to high concentrations (25 to 100 μM) of ACV.

In contrast to their consistent resistance to ACV, isolates varied in their susceptibilities to PAA (Tables 3 and 4). All plaques isolated from cultures not exposed to ACV had plating efficiencies of $<10^{-3}$ in the presence of PAA (100 μg/ml), which agrees well with the values obtained for PAA-susceptible wild-type and *ts* mutants of strain KOS (14). Nevertheless, many of the ACV^r variants were significantly more resistant to PAA than were the parental ACV-susceptible isolates. Furthermore, 50 and 36% of the 0-1116 and 7-2667 isolates, respectively, had plating efficiencies in PAA in excess of 0.11 (Tables 3 and 4). Because each of these isolates simultaneously acquired increased resistance to PAA and ACV, these ACV^r variants may have had alterations in the DNA polymerase gene locus.

TABLE 3. Resistance of strain 7-2667 plaques to ACV and PAA

Isolate no.[a]	Plating efficiency in[b]:	
	ACV[c]	PAA (100 μg/ml)
0-1		<0.0001
0-2		<0.0001
0-3		<0.0001
25-1	≥1.0	0.0002
25-2	≥1.0	<0.0001
50-1	≥1.0	<0.0001
50-2	≥1.0	0.78
50-3	≥1.0	0.29
75-1	0.67	0.11
75-2	0.71	0.11
75-3	≥1.0	0.01
100-1	≥1.0	0.06
100-2	≥1.0	0.03
100-3	≥1.0	0.003

[a] Isolates are designated by the ACV concentration (μM) used for selection (first number) and an isolate number (second number).

[b] Expressed as (titer with drug)/(titer without drug).

[c] Concentration used was same as for selection.

Fluctuation analysis of encephalitis strains. To

TABLE 4. Resistance of strain 0-1116 plaques to ACV and PAA

Isolate no.[a]	Plating efficiency in[b]:	
	ACV[c]	PAA (100 μg/ml)
0-1		0.0008
0-2		0.0009
0-3		0.0004
25-1	0.77	0.001
25-2	0.95	0.012
25-3	0.28	0.043
25-4	≥1.0	<0.0001
50-1	0.12	≥1.0
75-1	0.78	0.19
75-2	0.52	0.76
75-3	0.81	0.30

[a] Isolates are designated by the ACV concentration (μm) used for selection (first number) and an isolate number (second number).
[b] Expressed as (titer with drug)/(titer without drug).
[c] Concentration used was same as for selection.

demonstrate conclusively that the ACV^r variants were clonally distributed in the unselected virus populations and did not reflect a constant possibility of adaptation to the drug, we performed a fluctuation analysis of the encephalitis strains 7-2667 and 0-1116 (Table 5). The theory of Luria and Delbruck (16) suggests that individual cultures produced from a small inoculum will display a larger variation in the amount of ACV^r virus than that obtained by multiple samplings of a bulk culture if the ACV^r variants are clonally distributed in the original virus population. In other words, with a small enough inoculum, those cultures which were inoculated with at least one ACV^r variant have a higher plating efficiency in ACV than do cultures which received no ACV^r virus. If the ACV variants simply reflected a constant possibility of adaptation to the drug, the variations observed among the individual cultures and among multiple samples of a bulk culture would be similar.

The variation observed for multiple samplings of a bulk culture is due entirely to plating error, which is reflected by the standard deviation (σ^2) of the bulk cultures. Although the observed mean for the individual cultures of each strain fell within one σ^2 of the mean for the bulk culture samples, the variation among the individual cultures was much greater than that among bulk culture samples. It is assumed that for the bulk cultures, a plating efficiency in 25 μM ACV in excess of three σ^2 above the mean is extremely rare. In fact, none of the samples from the bulk cultures exceeded this value. However, 25 and 15% of the individual cultures inoculated with strains 7-2667 and 0-1116, respectively, had plating efficiencies in 25 μM ACV in excess of 3 σ^2 above the bulk culture mean. Thus, the ACV^r variants must have been clonally distributed in the original virus populations.

DISCUSSION

We believe this to be the first report to demonstrate that variants resistant to high concentrations of ACV are present in HSV populations from clinical infections. Our ability to detect plaques from strains that had no prior exposure to ACV was not due to a higher resistance of the majority of the virion population, as evidenced by the fact that the ID_{50} values of ACV for these strains were not appreciably different from those of common laboratory strains or from published values for HSV-1 and HSV-2 (2, 4, 8). Instead, these resistant variants represented a small proportion of the viable virions in the population, as demonstrated conclusively by fluctuation analysis. Nevertheless, the frequency with which they were isolated (ca. 1/10^4 PFU) was extremely high. This frequency was not restricted to encephalitis strains; a similar proportion of variants resistant to 25 to 75 μM ACV could be detected in all of the genital strains and most of the oral strains (unpublished data). It is unlikely

TABLE 5. Fluctuation analysis of HSV encephalitis strains 7-2667 and 0-1116

Strain	Type of samples	No. of samples	Mean plating efficiency ($\bar{Y}$)[a]	$\sigma^2 \times 10^3$	$\bar{Y} + 3\sigma^2$ for bulk samples $\times 10^3$	No. of samples with plating efficiencies $> \bar{Y} + 3\sigma^2$
7-2667	Bulk	9	2.82	1.18	6.36	0
	Individual	20	3.96	3.64		5 (25)[b]
0-1116	Bulk	10	2.17	1.44	6.49	0
	Individual	20	2.84	3.59		3 (15)[b]

[a] Expressed as (titer with 25 μM ACV)/(titer without ACV).
[b] Percentages of total given in parentheses.

that the high frequency of isolation was due to a selective growth advantage over the wild type conferred during propagation because no ACV was introduced until the initial virus titrations. In fact, we found that most of the ACV^r variants grew more slowly than did the parental strains (unpublished data). The high frequency of isolation may reflect the fact that two distinct genetic loci encode ACV resistance (1, 19). Viral TK, the product of one of these loci, is not essential for replication in vitro. Thus, there is a much larger number of nonlethal mutations possible for this gene than there is for genes encoding products required for viral replication. The majority of ACV^r mutants which have been characterized appear to have altered TK phenotypes, as demonstrated by lack of TK induction (1, 8, 19) or altered TK substrate specificity (5). However, some of the naturally occurring ACV^r variants we isolated may have alterations in the DNA polymerase gene, as demonstrated by the ability to grow in the presence of PAA.

The in vivo significance of the relatively large number of ACV^r variants found in the isolates used in this study is still unknown. Animal model studies have indicated that TK$^-$ mutants of HSV are avirulent when injected intracerebrally, presumably owing to the lack of cellular TK in the undividing cells (9). Therefore, the majority of variants that we found may not be virulent in adults. However, it is not clear whether such mutants would be virulent in human neonates with encephalitis, owing to the presence of immature and dividing brain cells in these hosts. In contrast, PAA-resistant mutants are virulent when injected intracerebrally (7; B. Roizman, personal communication). Therefore, ACV^r-PAA variants with alterations in the DNA polymerase gene locus would be expected to be virulent. Furthermore, such mutations are codominant with the wild-type alleles (1) and could survive mixed infections with the wild-type virus.

Thus, naturally occurring ACV^r-PAA variants may pose a problem in ACV therapy. In vivo studies are necessary to determine whether the host immunological defenses are capable of eliminating the routinely found concentrations of these variants. In support of the theory that selection of natural ACV^r variants occurs during ACV treatment is a recent clinical report demonstrating acquired resistance of HSV-1 to ACV after extensive therapy (3). In this study, HSV-1 isolated from the lip lesions of an immunosuppressed patient in the middle of the third course of intravenous ACV therapy exhibited an ACV ID_{50} that was significantly greater than that for each previously obtained isolate from the same patient (3). More clinical data of this type, however, will be required before the true impact of selection of ACV^r variants in treated patients can be assessed.

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