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Drug Resistance Patterns of Herpes Simplex Virus Isolates from Patients Treated with Acyclovir

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A decrease in the in vitro sensitivity to acyclovir (ACV) was observed in successive isolates of herpes simplex virus type 1 from three immunocompromised patients during intravenous therapy with this drug. The ACV-resistant isolate from patient 1 was cross-resistant to dihydroxypropoxymethylguanine and bromovinyldeoxyuridine, but still susceptible to three fluoro-substituted pyrimidines, 2′-fluoro-5-iodo-1-β-D-arabinofuranosylcytosine (FIAC), 2′-fluoro-5-iodo-1-β-D-arabinofuranosyluracil (FIAU), and 2′-fluoro-5-iodo-1-β-D-arabinofuranosylthymine (FMAU). The thymidine kinase (TK) from the resistant isolate showed a 50-fold or greater reduction in affinity for thymidine, FIAU, FMAU, and ACV, but the total enzyme activity was similar to that of the sensitive isolate. The ACV-resistant isolate from patient 2 was also resistant to dihydroxypropoxymethylguanine, bromovinyldeoxyuridine, and the fluoro-substituted compounds; TK activity for this isolate was less than 1% of the patient's pretherapy isolate. An isolate obtained during a subsequent recurrence in patient 2 was susceptible to ACV and the other TK-dependent agents. The ACV-resistant isolate from patient 3 was partially resistant to FIAC and FIAU but still suceptible to FMAU; the viral TK had a 10-fold-lower affinity for ACV, FIAU, and FMAU than did the sensitive pretherapy isolate, while the level of TK activity detected was reduced to 6%. In none of the isolates studied was a change in sensitivity to phosphonoformic acid observed. Compared with the corresponding pretherapy ACV-sensitive isolates, there was a 30-fold decrease in neurovirulence for mice of the two drug-resistant isolates with diminished levels of thymidine-phosphorylating activity and no change in virulence for the third isolate. These findings indicate that mixed patterns of drug-resistance to TK-dependent antiviral compounds can occur in clinical isolates, resulting from changes in either the amount or the affinity of viral TK activity.

Acyclovir (ACV) is being increasingly used for prophylaxis and therapy of herpes simplex virus (HSV) infections. An ill-defined liability of the use of this and other thymidine kinase (TK)-dependent drugs is the emergence and possible transmission of drug-resistant isolates (1, 9, 12, 14). Although in vitro passage in the presence of ACV can readily select for viruses with alterations in TK expression, the use of more stringent conditions is required to produce viruses resistant by virtue of changes in the viral DNA polymerase, the ultimate target of compounds such as ACV. The genetic locus (8) in which a change occurs to produce drug resistance may be important clinically, in that TK-deficient strains of HSV have decreased pathogenicity and a reduced ability to establish latency in a number of in vivo systems (13, 21), whereas DNA polymerase variants appear to retain virulence in vivo (13). A third class of resistant variants, with normal levels of TK but with altered TK-substrate specificities, has been studied less extensively. This class of variants appears to retain pathogenicity in vivo with only a 10-fold attenuation of neurovirulence (11). Although the definition of drug resistance may be debated in light of the finding of normal clinical recovery in immunocompetent patients whose HSV isolates show a decreased in vitro virus sensitivity to ACV inhibition, a number of clinical HSV isolates with in vitro 50% inhibitory doses (ID_{50}s) markedly above the normal range have been detected (2, 10, 12, 15, 19, 22). In many cases a shift in sensitivity was documented in sequential isolates collected during ACV treatment, but in other instances resistant isolates were obtained before ACV treatment (15, 17). With the exceptions of one partially characterized genital isolate which had a diminished ability to phosphorylate ACV, while retaining thymidine-phosphorylating activity (15), and a recent report of a TK-substrate variant from a clinical isolate (M. N. Ellis, P. M. Keller, S. E. Strauss, S. Nusinoff-Lehrman, and D. W. Barry, Abstr. Ninth International Herpesvirus Workshop, 1984, p. 225), all of the ACV-resistant HSV clinical isolates appear to have diminished TK expression. We report here on the properties of HSV isolates obtained from three immunocompromised patients treated with ACV, with special emphasis on the mixed pattern of drug resistance associated with different combinations of low TK expression and decreased TK affinity for both thymidine and the nucleoside analogs. A number of nucleoside analogs with a similar dependence on TK for activation have been proposed for the therapy of herpes infections and therefore were included in the present study; phosphonoformic acid (PFA) acts directly on DNA polymerase and was included as a non-TK-dependent drug.

MATERIALS AND METHODS

Virus strains. The isolation and initial characterization of the clinical strains was as reported previously (2). Briefly, virus isolates collected before or in the early stages of ACV therapy of cancer patients were compared for shifts in in vitro sensitivity to ACV with isolates collected in the latter stages. Isolates collected from three patients during therapy showed a significant increase in the concentration of ACV required to produce a 50% inhibition in plaque number (ID_{50}), compared to earlier isolates, and were selected for further study. Representative sensitive and resistant isolates were plaque

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purified (two cycles), and virus pools were prepared in Vero (African green monkey kidney) cells.

The clinical details of the patients were as follows: Patient 1 was a 29-year-old female with acute myelocytic leukemia. A "pretherapy" isolate of HSV type 1 (HSV-1) was obtained from her throat before a 1-week course of intravenous ACV treatment (750 mg/m^2 per day). She received no further ACV until 1 year later, when she received another course of ACV; after 2 weeks, the dosage was increased to 1,500 mg/m^2 per day. Esophagitis, sore mouth, and HSV shedding from oral lesions persisted, and a "therapy" isolate was collected by esophageal endoscopy 3 weeks later. Her lesions cleared over the following month.

Patient 2 was a 24-year-old male with acute lymphocytic leukemia who had received ACV (750 mg/m^2 per day) intravenously for 2 weeks and a lower dose (250 mg/m^2 per day) for 1 week before isolation of the ACV-sensitive pretherapy isolate of HSV-1 from his throat. An ACV-resistant therapy isolate was recovered from his throat 10 days later while the patient was still on the lower dose of ACV. His lesions cleared soon afterward, and ACV was discontinued 1 week after the isolate was obtained. Virus was isolated from a subsequent recurrence 4 months later.

Patient 3 was a 36-year-old male with acute lymphocytic leukemia. The pretherapy HSV-1 isolate was made 15 months before marrow transplantation. ACV (750 mg/m^2 per day) was administered intravenously starting 5 days after marrow transplantation, when HSV-1 was shed from the throat. Oral lesions persisted, and after 1 month the ACV dose was increased to 1,500 mg/m^2 per day; an ACV-resistant therapy isolate was collected after 3 days on the higher drug dose. Oral herpetic lesions persisted until the patient's death 3 days later of cytomegalovirus pneumonia.

A laboratory reference HSV-1 strain (KOS; obtained from Kendal O. Smith, San Antonio, Texas) was included in some of the experiments.

Virus inhibition assays. The sensitivity of HSV isolates to inhibition by a series of antiviral compounds was tested in a quantitative microtiter cytopathic effect (CPE) reduction assay (16). The assay was modified in that culture fluid was removed from the microtiter wells before 200 μl of neutral red dye (0.15% in Hanks balanced salt solution) was added; this procedure eliminated neutral red crystal formation. 2′-Fluoro-5-iodo-1-β-D-arabinofuranosylcytosine (FIAC), 2′-fluoro-5-iodo-1-β-D-arabinofuranosyluracil (FIAU), and 2′-fluoro-5-iodo-1-β-D-arabinofuranosylthymine (FMAU) were synthesized according to the method of Fox et al. (23); ACV (acyclovir sodium; Zovirax) was purchased from Burroughs Wellcome Co. (Research Triangle Park, N.C.); 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG) was a gift from J. Verheyden (Syntex, Palo Alto, Calif.); (E)-5-(2-bromovinyl)-2′-deoxyuridine (BVdUrd) was a gift from I. S. Sim (G. D. Searle, High Wycombe, United Kingdom); PFA was a gift from B. Oberg (Astra Lakemedel, Sodertolje, Sweden). The sensitivity of each viral isolate was expressed as an ID_{50} value, that is, the concentration of drug (in micrograms per milliliter) reducing viral CPE by 50%. ID_{50}s determined in this laboratory by quantitative dye uptake were similar to those obtained in a standard plaque reduction assay, in contrast to the 5- to 10-fold-higher dye uptake values obtained previously (16).

TK assay. Thymidine-thymidylate kinases from LMTK$^-$ cells infected with HSV-1 were purified as described by Cheng and Ostrander (7) and Chen and Prusoff (5). Before being used in kinetic studies, enzymes were changed to the ATP · Mg^{2+} form by passage through a Sephadex G-25-40 column (Sigma Chemical Co., St. Louis, Mo.), equilibrated with 1 mM ATP · Mg^{2+}–10 mM Tris (pH 7.5)–10% glycerol, and eluted with the same buffer. The assay for enzyme activities and analysis of kinetic data were done as described previously (4, 6).

Neurovirulence determinations. Groups of 12 4-week old female BALB/c mice (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) were inoculated intracerebrally with approximately 50 μl of serial 10-fold dilutions of pools of each virus isolate; replicate samples were also plaque titrated on Vero cell monolayers. The cumulative mortality for each virus was determined over a 21-day observation period. Deaths occurring in the initial 24-h postinoculation period were not included in the mortality score. The median mouse lethal dose for each virus isolate was calculated according to the method of Reed and Muench (18).

RESULTS

Sensitivity of virus isolates to antiviral compounds. Quantitative CPE reduction assays on HSV-1 isolates from three immunocompromised patients treated with ACV revealed significant increases in the ID_{50}s to ACV of later isolates compared to pretherapy isolates. Similar ID_{50}s were obtained with low-passage, non-plaque-purified pools compared with the plaque-purified pools reported here.

The virus isolated during ACV therapy from patient 1 (Table 1), showed significant increases in ID_{50} for ACV and to the structurally related DHPG and BVdUrd. The sensitivity to the fluoropyrimidines and to PFA was not diminished compared with the pretherapy isolate. For patient 2, the pretherapy isolate had an ID_{50} of ACV of 1.3 μg/ml, while the therapy isolate showed a marked decrease in sensitivity to inhibition by ACV, with an ID_{50} of 52 μg/ml. This isolate was also resistant to all of the TK-dependent compounds tested, but did not show any increase in ID_{50} for PFA. An isolate from a recurrence in patient 2 16 weeks after ACV therapy had a sensitivity profile similar to that of the initial pretherapy isolate. The posttreatment isolate from patient 3 displayed a pattern of resistance with a marked shift in ID_{50} for the compounds ACV, DHPG, and BVdUrd and a smaller, five- to sixfold increase in ID_{50} for FIAC and

TABLE 1. Inhibition of HSV isolates by antiviral compounds

Patient and isolate[a]	ID_{50} (μg/ml)[b] of antiviral compounds						
	ACV	DHPG	BVdUrd	FIAC	FIAU	FMAU	PFA
1							
Pretherapy	1.6	0.59	0.17	0.38	0.60	0.29	25
Therapy	8.7	4.6	25.0	0.70	0.39	0.41	29
2							
Pretherapy	1.3	1.8	1.6	1.0	0.43	3.1	49
Therapy	52	107	>166	149	>166	59	42
Post-therapy	1.7	1.6	0.79	1.0	0.58	3.7	52
3							
Pretherapy	1.3	0.22	0.15	0.56	0.34	0.76	38
Therapy	9.2	5.8	21	3.0	2.2	1.1	40

[a] Isolate: pretherapy, collected before ACV therapy was initiated or during initial phase of therapy; therapy, collected during, or immediately after, ACV therapy; post-therapy, collected from a subsequent recurrence 16 weeks after ACV therapy.

[b] ID_{50}, concentration of compound producing 50% inhibition of viral CPE as measured by uptake of neutral red dye.

FIAU. No change was observed in the response to FMAU and PFA.

Comparative neurovirulence of HSV isolates. The pretherapy and drug-resistant therapy isolates from each patient were compared for neurovirulence in BALB/c mice in a single experiment. In two of the three pairs, the decreased sensitivity to ACV inhibition observed in the therapy isolates was associated with a reduction in neurovirulence compared with the ACV-sensitive pretherapy isolate. Thus, for the isolates from patient 2, 27-fold more of the therapy isolate was required to produce 50% mortality than for the pretherapy isolate, while the recurrence isolate was similar in neurovirulence to the pretherapy isolate. A 33:1 ratio was found between the virulence of the pretherapy and therapy isolates from patient 3 (Table 2). The isolates from patient 1 were the most virulent of those examined, and no difference in virulence was observed between the pretherapy and therapy isolates.

Determination of TK activity of HSV isolates. TK enzymes purified from cultures of TK^- cells infected with the three pairs of HSV clinical isolates and the reference KOS HSV-1 strain were tested for their affinities to thymidine and to the antiviral compounds ACV, FIAU, and FMAU (Table 3). FIAC was not tested because previous studies have shown that its metabolite, FIAU, is the active antiviral agent (3). The total TK activity of the enzyme preparations was also determined at saturating concentrations of thymidine.

Resistance to only ACV, DHPG, and BVdUrd was observed for the therapy isolate from patient 1. The level of TK expression did not appear to be reduced when assayed in conditions of excess thymidine, but the affinities of the enzyme from the therapy isolate were markedly reduced for all of the substrates examined, ranging from a 47-fold reduction with thymidine to 77-fold for FIAU (no inhibition was detected with 2.7 mM ACV, the highest attainable concentration in solution). The K_is for FIAU and FMAU of the TK of the ACV-resistant isolate were still lower than those of the pretherapy isolate for ACV.

The therapy isolate from patient 2 was resistant to all the TK-dependent compounds tested and demonstrated a virtual absence of TK activity, with only 0.4% thymidine phosphorylation compared with the corresponding ACV-sensitive isolate. In association with the decreased activity, a diminished affinity for thymidine and the nucleoside analogs was also observed. The decrease in affinities ranged from 3.4-fold for thymidine to 31-fold for FIAU.

A different combination of results was found with the isolates from patient 3. A decrease in TK expression was found with the therapy isolate compared with the pretherapy isolate, but this decrease was not total and approximately 6% of the initial level of TK activity was present. A 5- to 10-fold reduction in enzyme affinity for all of the substrates tested was observed in the therapy isolate, which was resistant to ACV, DHPG, and BVdUrd and partly resistant to FIAC and FIAU, but was susceptible to FMAU. The K_is of the therapy isolate for FIAU and FMAU were similar and were significantly lower than that of the pretherapy isolate to ACV.

Enzyme from the laboratory strain of HSV-1 (KOS) showed greater affinity for the substrates than did TK from any of the clinical isolates tested; this virus is sensitive to all of the compounds used in this study.

TABLE 2. Neurovirulence of HSV-1 isolates

Patient and isolate[a]	Mouse infectivity (PFU/LD_{50})[b]	Therapy/pretherapy ratio
1		
Pretherapy	5	1
Therapy	5	
2		
Pretherapy	10	27
Therapy	270	
Post-therapy	27	
3		
Pretherapy	6	33
Therapy	200	

[a] See Table 1, footnote *a*.
[b] Mice were inoculated intracerebrally with 50 μl of serial 10-fold dilutions of virus, and the mortality was recorded over a 21-day period. Portions of the inoculum were plaque titrated in Vero cell monolayers.

TABLE 3. Affinities of TK from HSV-1 isolates for antiviral compounds

Patient and isolate[a]	Substrate affinity (μM)[b]				TK activity ($\times 10^{-4}$ pmol/μg per min)[c]
	TdR	ACV	FIAU	FMAU	
1					
Pretherapy	0.54	252	0.43	0.89	668
Therapy	25.5	>2,700[d]	33.0	50.0	763
T/P ratio	47.2	>10.7	76.7	56.2	1.14
2					
Pretherapy	0.57	196	0.25	0.42	2,308
Therapy	1.96	1,629	7.75	5.00	10.5
T/P ratio	3.4	8.3	31	11.9	0.004
3					
Pretherapy	0.29	189	0.32	0.46	2,668
Therapy	1.41	1,033	3.12	4.06	172
T/P ratio	4.9	5.5	9.8	8.8	0.064
Control					
HSV-1 (KOS)	0.14	45	0.14	0.18	263

[a] See Table 1, footnote *a*. T/P ratio, therapy/pretherapy ratio.
[b] Substrate affinity: K_m (thymidine [TdR]) and K_i (ACV, FIAU, FMAU).
[c] TK activity: $\times 10^{-4}$ pmoles of thymidine phosphorylated to TMP per micrograms of protein per minute.
[d] K_i could not be determined at maximum solubility of ACV.

DISCUSSION

In the present study we found two classes of ACV-resistant clinical HSV isolates from patients receiving ACV therapy for herpesvirus infections during immunosuppressive cancer treatment. The occurrence of drug-resistant virus was associated with a longer-than-normal excretion of virus during ACV treatment, but the clinical course was otherwise unremarkable. The first class of variant virus was isolated from patient 1 and showed normal levels of TK expression, albeit at saturating concentrations of thymidine. This variant was as neurovirulent as the ACV-sensitive pretherapy isolate from the same patient. Although the affinity of the enzyme for all of the substrates tested was uniformly decreased, the virus was resistant in vitro to only ACV, DHPG, and BVdUrd. The K_is of the variant TK were more than 50-fold lower to FIAU and FMAU than to ACV, and this affinity was sufficient to allow phosphorylation of inhibitory levels of FIAU or FMAU.

The second class of variants was found in patients 2 and 3

and demonstrated reduced levels of TK expression with diminished neurovirulence for mice. The reduction in virulence was not as great as reported with other TK-deficient clinical strains of HSV (19); cutaneous virulence was not examined in the present study. The affinities of the TK enzymes from the ACV-resistant viruses from patients 2 and 3 did not demonstrate alterations as great as those found with the virus from patient 1. However, the combination of barely detectable TK activity and the shift in substrate specificity was great enough to render the therapy isolate of patient 2 resistant to all of the nucleoside analogs tested. In the case of the isolate from patient 3, the slightly higher TK activity (6% of that of the pretherapy isolate) was apparently sufficient to produce partial inhibition with FIAC and FIAU and unchanged susceptibility to FMAU. Thus, resistance to a particular nucleoside analog is a function of both the affinity of the TK enzyme for the analog (with ACV and BVdUrd having weak affinities and the fluoropyrimidines having strong affinities) and the level of TK activity. Accordingly, viruses with a small amount of TK enzyme can still be inhibited with a highly avid substrate. In this context the laboratory strain KOS did not have as high a level of TK expression as the clinical isolates, but the affinity of the KOS enzyme was the highest for the substrates tested, and the virus was therefore very susceptible to inhibition by these compounds.

Although a number of HSV strains with diminished sensitivity to in vitro inhibition by ACV have been isolated from patients, the impact of this phenomenon is still difficult to assess. It has been pointed out that in most cases the clinical recovery appeared normal during ACV therapy despite the finding of "resistant" strains in lesions (12). In the immunocompromised patients from whom such resistant isolates were obtained, the disease was of an indolent rather than a rapidly progressive nature (9, 19). Assessment of the problem of the clinical resistance of viruses is still hampered by the relative novelty of this area of therapy, as reflected by the absence of a standardized assay and by a lack of consensus as to what constitutes a resistant strain. The demonstration of mixed populations of ACV-resistant and -sensitive strains of HSV in clinical isolates from patients who have not been exposed to ACV (15, 17, 20) suggests that natural mutation of the TK gene is relatively frequent and that such mutant viruses are selected for during treatment with TK-dependent drugs such as ACV. Whether this will ultimately result in the widespread transmission of drug-resistant strains is not known, but the findings of a decreased ability of TK-negative strains to establish ganglionic latency (13, 19, 21) and the need for higher titers of virus to produce cutaneous infection (19) might indicate that such strains are poorly adapted for transmission.

The ACV-resistant clinical strains of HSV reported to date have been predominantly of the TK-deficient type, which are also produced readily in vitro by passage in the presence of low concentrations of ACV. One strain with diminished ability to phosphorylate ACV while maintaining thymidine-phosphorylating activity was reported in a study of genital HSV isolates (patient 316, Table 3, in reference 15), and a further isolate with altered TK substrate specificity has been recently reported (Ellis et al., Abstr. Ninth International Herpesvirus Workshop, 1984). The latter isolate was more neurovirulent than TK^- virus, but the neurovirulence compared to the ACV-sensitive parent was not stated. An in vitro-derived strain of HSV with altered TK-substrate specificity showed slight attenuation of neurovirulence (11). Clinical experience so far suggests that viruses almost devoid of TK activity may be more frequent than those with only partially reduced levels of this enzyme. Our demonstration of mixed patterns of drug resistance in this limited sample of isolates suggests that alternative therapies might be an option in instances of resistant HSV infections. However, alternative safe and efficacious drugs and rapid methods for assessing viral resistance are required for this option to be realized.

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