

Orofacial Infection of Athymic Mice with Defined Mixtures of Acyclovir-Susceptible and Acyclovir-Resistant Herpes Simplex Virus Type 1

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Infection of athymic mice with defined populations of acyclovir-susceptible (thymidine kinase [TK]-positive) and acyclovir-resistant (TK-deficient or TK-altered) herpes simplex virus type 1 strains was used to simulate herpetic skin disease of the immunocompromised host. In vitro characterization of the defined virus mixtures revealed that the dye uptake method was quite sensitive in the detection of small amounts (3 to 9%) of acyclovir-resistant virus. Mice infected with homogeneous virus populations exhibited a good correlation between clinical response and the in vitro drug susceptibility of the infecting virus. Animals infected with defined mixtures of viruses exhibited varied patterns of infection and responses to acyclovir treatment. However, disease severity was useful in predicting the TK phenotype of virus recovered from lesions. Pathogenic, TK-altered virus was responsible for progressive disease in animals receiving low-dose (0.25-mg/ml) prophylactic acyclovir or high-dose (1.25-mg/ml) delayed therapy. Although this mutant was recovered infrequently, it was responsible for clinically significant disease in the animals from which it was isolated.

Since the discovery of the antiherpetic activity of acyclovir (ACV) (8, 25), the drug has been used extensively for a variety of human herpetic infections (7). Resistance to ACV has been reported to occur both in vitro (3, 5, 28) and in vivo (2, 4, 26, 31). In both settings, the most frequently encountered ACV-resistant herpes simplex virus (HSV) mutant is defective in its ability to induce thymidine kinase (TK). However, the substantial reduction in virulence that accompanies the loss of this important virus-coded enzyme renders these viruses less clinically significant (11, 12, 31). Other types of ACV-resistant mutants that retain TK activity were only a theoretical possibility until in vitro systems were devised for their selection and isolation (6). One such mutant contains alterations in the viral DNA polymerase that make it insensitive to ACV triphosphate (13, 17). A second type results from mutations in the TK gene that cause the induction of a TK enzyme with altered substrate specificity (6, 15, 16). ACV-resistant viruses representing both of these classes were initially isolated in vitro, and their retention of virulence was confirmed in animal systems (6, 17). More recently, similar mutants have been recovered from two patients receiving ACV therapy (10, 22). However, despite their pathogenicity, their clinical significance remains unclear.

Primary infection with HSV or reactivation of latent HSV can be life-threatening in patients whose immune systems are compromised: organ transplant recipients, patients receiving cancer chemotherapy, and patients infected with human immunodeficiency virus. The heterogeneity of HSV type 1 (HSV-1) clinical isolates (23) increases the possibility that established lesions in the immunocompromised host may contain both ACV-susceptible and ACV-resistant virus. Because such patients cannot depend on immune elimination of the initially few resistant viruses, ACV treatment could readily select less susceptible virus present in the lesions (1).

Several reports in the literature describe isolation of ACV-resistant virus from this patient group (2, 4, 26, 32).

In view of the potential clinical significance of pathogenic mixtures of ACV-susceptible and ACV-resistant viruses, we have evaluated their behavior in athymic mice. This model has proven to be clinically relevant in examining the pathogenicity of resistant isolates (26). This paper describes infection of athymic mice with defined mixtures of TK-positive (TK⁺) and TK-deficient (TK^D) or TK⁺, TK^D, and TK-altered (TK^A) viruses. Patterns of infection and responses to oral ACV are compared. Viruses isolated from infected mice are evaluated for ACV susceptibility and TK phenotype. Our results provide evidence of the clinical potential of TK^A virus.

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MATERIALS AND METHODS

Cell cultures. Vero (African green monkey kidney) cells (Flow Laboratories, Inc., McLean, Va.) were grown in Eagle minimal essential medium supplemented with 5% fetal calf serum (M. A. Bioproducts, Walkersville, Md.), 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (GIBCO Laboratories, Grand Island, N.Y.), sodium bicarbonate, 75 U of penicillin G, and 75 µg of streptomycin per ml.

Viruses. The virus strains used were 3-times-plaque-purified HSV-1 clinical isolates, designated here by virus laboratory (VL) numbers 9013 and 9014. The origin of these isolates has been reported previously (26). These viruses were obtained from an immunodeficient child and represent pre- and posttherapy virus isolates. VL 9014 is TK⁺ and ACV susceptible, while VL 9013 is TK^D and ACV resistant. A third HSV-1 strain (VL 8971) was used in these studies; originally designated SC16-S1, it is a TK^A laboratory strain that has been described previously (6).

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Antiviral susceptibility assays. Virus isolates used in this study and virus recovered from infected mice were tested for their susceptibility to drug inhibition by both the standard plaque reduction (PR) assay (26) and the dye uptake (DU) method (19) in Vero cells.

[¹²⁵I]dC plaque autoradiography. Prior to inoculation of athymic mice, virus mixtures were analyzed by [¹²⁵I]iododeoxycytidine ([¹²⁵I]dC) plaque autoradiography, which has been described before (18). Similarly, viruses recovered from untreated and treated mice at various times after virus inoculation were evaluated for TK phenotype by this technique. For defined mixtures and mouse isolates, the TK phenotypes were determined by counting at least 1,000 plaques.

Mice. Female BALB/c athymic mice were used at 4 to 5 weeks of age. Mice were housed five to a cage.

Cutaneous infection. Groups of five mice, under light ether anesthesia, were inoculated on the snout by scarification with a 25-gauge needle, followed by rubbing for 10 s with a cotton-tipped applicator soaked in diluted virus. Appropriate virus dilutions were used for back titration in Vero cells and [¹²⁵I]dC plaque autoradiography. Mean lesion scores were recorded daily with the following scale: 0, no lesions; 1, fewer than five discrete lesions; 2, five or more lesions; 3, confluent lesions; 4, ulceration or necrosis.

Administration of ACV. The sodium salt of ACV was reconstituted at specified concentrations (0.25, 0.5, or 1.25 mg/ml) in sterile distilled water and administered to athymic mice in their drinking water. For animals receiving ACV at a dose of 0.5 mg/ml in the drinking water, this would correspond to approximately 100 mg/kg per day.

ACV therapy. Prophylactic ACV therapy was initiated 24 h prior to virus inoculation and continued for 10 days. Delayed ACV therapy was begun 96 h after virus inoculation and continued for 10 days.

Virus isolation from infected mice. At various times after virus inoculation (days 6, 10, and 17), the lesions of infected mice in each group were swabbed. Viruses obtained from snout swabs were grown in Vero cells to produce working stocks. Mice in treated and untreated groups were designated according to the number of mice in a particular treatment group. Viruses recovered from infected mice were then given a virus laboratory number.

Statistical analysis. The areas under the curve of mean lesion scores were compared for statistical significance by the Kruskal-Wallis test (27).

RESULTS

In vitro properties of cloned HSV-1 strains and defined mixtures. Viruses VL 9014 and VL 9013 were used in this experiment because previous work had shown that VL 9014 was ACV susceptible and TK⁺ while VL 9013 was ACV resistant and TK^D. Although both viruses produced lesions in athymic mice, VL 9014 was approximately 100-fold more virulent. Mutant VL 8971 was selected because its resistance to ACV appears to be due to the induction of an altered TK enzyme (6). This virus remained pathogenic for athymic mice (Table 1).

Cloned viruses or virus mixtures were prepared and assayed for ACV susceptibility by the DU and PR methods. Viruses with ACV 50% effective doses (ED₅₀s) of ≥ 3.0 $\mu\text{g/ml}$ as determined by the DU method are considered ACV resistant (19). However, in the PR assay, ACV-resistant viruses had ED₅₀s of ≥ 1.0 $\mu\text{g/ml}$. Additionally, the nature of

TABLE 1. In vitro properties of cloned HSV-1 strains and defined virus mixtures

Strain or mixture	TK phenotype (composition, %) ^a	ACV ED ₅₀ ^b ($\mu\text{g/ml}$)	
		DU	PR
VL 9014	TK ⁺ (100)	0.5	0.5
VL 9013	TK ^D (100)	8.9	3.2
VL 8971	TK ^A (100)	18.6	13.7
Mixture 1	TK ⁺ /TK ^D (91/9)	3.7 (7.4)	0.7 (1.4)
Mixture 2	TK ⁺ /TK ^D (70/30)	8.1 (16.2)	1.0 (2.0)
Mixture 3	TK ⁺ /TK ^D /TK ^A (76/17/7)	25.0 (50.0)	0.5 (0.0)

^a Percentage of each TK phenotype present in mixtures determined by [¹²⁵I]dC in Vero cells (see text).

^b Numbers in parentheses represent the increase (fold) in ACV ED₅₀ compared with the control value as determined by each assay method.

the mixtures was confirmed by [¹²⁵I]dC plaque autoradiography as shown in Table 1.

When plaque-purified viruses were tested for ACV susceptibility, there was no difference in the ACV ED₅₀ values when determined by the two assay methods. However, as the amount of TK^D virus in the mixture was increased, the DU method was more sensitive to these changes in composition. An increase to 9% TK^D resulted in a 7.4-fold increase (0.5 to 3.7 $\mu\text{g/ml}$) in ACV ED₅₀ by the DU assay, an additional 21% TK^D virus resulted in another 16.2-fold increase (0.5 to 8.1 $\mu\text{g/ml}$) in the ACV ED₅₀. When the same mixtures were tested for ACV susceptibility by the PR method, there was a 1.4-fold increase (0.5 to 0.7 $\mu\text{g/ml}$) and 2-fold increase (0.5 to 1.0 $\mu\text{g/ml}$), respectively, in the ACV ED₅₀s. This difference may reflect the greater amount of input virus used in the DU assay (≈ 500 PFU) than in the PR assay (100 to 200 PFU/ml) or may be the result of the different endpoints used in the assay.

Pathogenicity of plaque-purified viruses for athymic mice. Mice were infected on the snout with approximately 10⁵ PFU of the appropriate virus strain. Herpetic lesion scores and virus titers in snout tissue are shown in Table 2. TK⁺ and TK^A viruses showed similar abilities to produce progressive cutaneous disease in athymic mice. Lesions were most severe 6 to 8 days after virus inoculation, with an approximate 4-log increase in virus titers in snout skin between days 0 and 4. On the other hand, TK^D virus had greatly reduced ability to cause cutaneous disease, with peak lesion scores of 1.2 on day 5 and a 0.6-log increase in virus skin titer between days 0 and 4. These results confirm earlier reports (11, 12) of reduced virulence of TK^D viruses for athymic mice.

Response to ACV therapy of athymic mice infected with plaque-purified virus. Athymic mice were infected with approximately 10⁵ PFU of the appropriate virus strain (TK⁺, TK^D, or TK^A). The mice infected with a particular virus strain were divided into three groups of 7 to 10 mice each. One group received no treatment. A second group received prophylactic oral ACV (drinking water) at a concentration of 0.5 mg/ml, begun 24 h before infection and continued for 10 days. The final group received delayed oral ACV at 0.5 mg/ml, begun 96 h after virus inoculation and continued for 10 days. Clinical responses to oral ACV therapy are shown in Table 3.

Untreated mice infected with 100% TK⁺ virus had rapidly progressive, fatal disease. Prophylactic ACV was quite effective in suppressing virus infection, but lesions did reappear and progress when therapy was terminated. Delayed ACV treatment reduced lesion scores and associated mortality, but lesion scores again progressed when treatment

TABLE 2. Viral replication and lesion induction in athymic mice infected on the snout with different HSV-1 strains

HSV-1 strain	Virus titer ^a (log ₁₀ PFU/ml) in snout skin on day postinoculation:					Mean lesion score ± SD on day postinoculation:				
	1	2	3	4	7	1	3	5	7	
VL 9014 (TK ⁺)	3.3	4.1	5.5	6.6	5.6	0.0 ± 0.00	0.4 ± 0.55	1.0 ± 0.00	3.0 ± 0.00	
VL 9013 (TK ^D)	4.3	4.4	4.5	4.9	4.2	0.0 ± 0.00	0.1 ± 0.32	1.2 ± 0.45	1.4 ± 0.30	
VL 8971 (TK ^A)	3.0	4.9	5.1	6.7	5.8	0.0 ± 0.00	1.1 ± 0.60	2.6 ± 0.53	3.6 ± 0.53	

^a Snout skins from three animals were pooled at 1, 2, 3, 4, and 7 days after virus inoculation. Titers were determined by plaque assay in Vero cells.

was stopped. Mean lesion scores for both treated groups were significantly reduced compared with those for controls ($P < 0.05$).

Mice infected with 100% TK^D virus had indolent disease with slow progression of lesions. Prophylactic ACV significantly reduced lesion scores but did not prevent lesions. Delayed ACV treatment did not produce a significant reduction in lesion scores compared with untreated mice.

Untreated mice infected with 100% TK^A virus exhibited a severe disease pattern similar to that in the mice infected with wild-type virus. Neither prophylactic nor delayed ACV treatment at 0.5 mg/ml had any clinical effect on athymic mice infected with this mutant.

Clinical responses of athymic mice infected with defined mixtures. Mice were infected with approximately 10⁵ PFU of defined virus mixtures. The actual amount of TK⁺-TK^D or TK⁺-TK^D-TK^A in the population was determined by [¹²⁵I]dC plaque autoradiography in Vero cells (see Materials and Methods).

Mice infected with a 70% TK⁺-30% TK^D mixture were divided into three groups. The first group received no antiviral therapy. The second group received prophylactic oral ACV (0.5 mg/ml), begun 24 h before virus infection and continued through day 9. The last group received delayed (96 h postinoculation) ACV therapy at a concentration of 0.5 mg/ml. Only mice with lesions were used for this last group.

Untreated animals receiving the 70% TK⁺-30% TK^D mixture had progressive disease similar to the lesion patterns observed in untreated animals receiving 100% TK⁺ virus (Table 4). Mice responded to prophylactic oral ACV with lesion scores of less than 0.30 through day 10. These reductions in lesion scores were statistically significant ($P < 0.05$). However, mice receiving delayed treatment continued to form new lesions while on therapy; disease stabilized only after 3 days of treatment. Animals receiving either treatment

regimen had a recrudescence of disease when therapy was stopped.

We then examined the effect of a lower dose of oral ACV (0.25 mg/ml) on mice infected with a more complex mixture of virus strains (Table 4). This mixture contained approximately 76% TK⁺, 17% TK^D, and 7% TK^A. Untreated animals had severe and rapidly fatal disease, reflecting the ability of both TK⁺ and TK^A viruses to replicate in the skin. Prophylactic oral ACV caused a slight delay (96 h) in the appearance of lesions. Once lesions were present, however, they continued to increase in severity despite antiviral therapy at this limited dose.

This clinical outcome is quite different from the effect of prophylactic ACV on the TK⁺-TK^D mixture and is the result of both the lower ACV dose (0.25 versus 0.5 mg/ml), which allows some breakthrough of TK⁺ virus, and the relative virulence of the TK^A virus. Mice receiving ACV treatment at a dose of 0.25 mg/ml begun 96 h postinoculation had delayed appearance of lesions, brief stabilization of lesion scores on days 8 through 14, and continued lesion progression after treatment was terminated on day 14. Mean lesion scores for the two low-dose treatment regimens were significantly different from those for controls ($P < 0.05$).

The clinical responses of mice infected with either mixture (TK⁺-TK^D or TK⁺-TK^D-TK^A) and receiving delayed treatment with 0.5 or 0.25 mg/ml were equivalent. Withholding treatment allowed the TK⁺ and TK^A components of the mixtures to overgrow the TK^D virus, which replicates poorly in the skin. A small amount of TK^A virus (~7%) had little effect on clinical response to delayed treatment because of the undiminished growth of wild-type viruses within the initial 96 h.

A fivefold increase in ACV dose (to 1.25 mg/ml) in the drinking water greatly enhanced the benefit of prophylactic therapy in mice infected with the TK⁺-TK^D-TK^A mixture

TABLE 3. Effect of oral treatment with ACV on athymic mice infected with cloned HSV-1 strains

HSV-1 strain	Treatment ^a	No. of mice	ACV concn in drinking water (mg/ml)	Mean lesion score ± SD on day postinoculation:					Area under the curve (±SD) ^b
				1	3	5	7	10	
VL 9014 (TK ⁺)	None	7		0.0 ± 0.00	0.4 ± 0.55	1.0 ± 1.00	3.0 ± 0.00	4.0 ± 0.00	44.2 (±1.78) ¹
	Pro	7	0.5	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.3 ± 0.49	12.8 (±4.12) ²
	Del	7	0.5	0.0 ± 0.00	0.0 ± 0.00	0.9 ± 0.38	1.4 ± 0.98	1.7 ± 1.50	23.3 (±12.9) ²
VL 9013 (TK ^D)	None	10		0.0 ± 0.01	0.1 ± 0.33	1.2 ± 0.48	1.4 ± 0.32	1.7 ± 0.67	15.5 (±4.33) ¹
	Pro	10	0.5	0.0 ± 0.00	0.4 ± 0.52	0.3 ± 0.42	0.3 ± 0.52	0.2 ± 0.82	3.3 (±4.80) ²
	Del	10	0.5	0.0 ± 0.00	0.3 ± 0.48	0.7 ± 0.48	0.9 ± 0.48	1.3 ± 0.42	11.5 (±5.90) ¹
VL 8971 (TK ^A)	None	10		0.0 ± 0.00	1.1 ± 0.60	2.6 ± 0.58	3.6 ± 0.53	4.0 ± 0.00	50.4 (±1.94) ¹
	Pro	10	0.5	0.0 ± 0.00	1.0 ± 0.47	2.3 ± 1.06	3.1 ± 1.20	3.7 ± 0.67	50.5 (±2.74) ¹
	Del	10	0.5	0.0 ± 0.00	1.2 ± 0.44	2.7 ± 0.50	3.6 ± 0.53	4.0 ± 0.00	47.6 (±8.30) ¹

^a Pro, Prophylactic oral ACV therapy begun 24 h before virus inoculation and continued for 10 days; Del, delayed oral ACV therapy begun 96 h after virus inoculation and continued for 10 days.

^b The areas under the curve were compared by the Kruskal-Wallis test. Means with the same numerical superscript are not significantly different ($P \geq 0.05$).

TABLE 4. Effects of oral treatment with ACV on athymic mice infected with defined mixtures of HSV-1 strains^a

Mixture	Treatment	No. of mice	ACV concn in drinking water (mg/ml)	Mean lesion score ±SD on day postinoculation:						Area under the curve (±SD)
				1	3	5	7	10	17	
70% TK ⁺ -30% TK ^D	None	8		0.0 ± 0.00	0.5 ± 0.58	1.1 ± 0.64	1.7 ± 1.14	3.2 ± 1.04	3.6 ± 1.06	36.9 (±11.92) ¹
	Pro	8	0.5	0.0 ± 0.00	0.0 ± 0.00	0.3 ± 0.49	0.0 ± 0.53	0.3 ± 0.52	3.0 ± 0.63	12.1 (±2.44) ²
	Del	8	0.5	0.0 ± 0.00	0.5 ± 0.53	1.8 ± 1.10	1.9 ± 1.83	2.5 ± 1.07	2.9 ± 1.13	30.4 (±12.09) ¹
76% TK ⁺ -17% TK ^D -7% TK ^A	None	18		0.0 ± 0.00	0.8 ± 0.43	2.6 ± 0.70	3.3 ± 0.96	3.7 ± 0.97	3.6 ± 1.10	45.6 (±13.19) ¹
	Pro	9	0.25	0.0 ± 0.00	0.0 ± 0.00	0.9 ± 1.13	1.7 ± 1.04	2.5 ± 1.07	3.9 ± 0.35	34.6 (±9.99) ²
	Pro	9	1.25	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.4 ± 0.88	1.1 ± 1.69	6.8 (±11.07) ³
	Del	9	0.25	0.0 ± 0.00	0.3 ± 0.49	1.4 ± 1.13	2.1 ± 1.57	2.4 ± 1.74	2.7 ± 1.70	30.5 (±20.33) ²
	Del	9	1.25	0.0 ± 0.00	0.3 ± 0.49	1.2 ± 0.90	2.0 ± 0.00	2.0 ± 1.15	2.9 ± 0.90	27.2 (±12.79) ²

^a See Table 3, footnotes a and b.

and limited lesion scores to <1.0 through day 9. Delayed therapy with the high dose was also of considerable benefit. Our results indicated that higher doses of ACV were clearly effective in suppressing infections initiated by defined virus mixtures containing ACV-resistant HSV-1 strains.

Clinical responses of individual mice and characterization of virus recovered from infected mice. Disease patterns noted in individual mice were dependent on the infecting mixture and the component of the mixture that grew in a particular mouse (Table 5). For example, untreated mice infected with the TK⁺-TK^D mixture exhibited two types of disease. The first, a rapidly progressive fatal disease, as observed in mouse M4, was associated with 100% TK⁺ and ACV-susceptible virus (ACV ED₅₀, 0.8 µg/ml by DU). The second was indolent disease associated with 100% TK^D, ACV-resistant (ACV ED₅₀, 12.0 µg/ml by DU) virus, as noted in mouse M8. Prophylactic treatment of mice infected with this mixture selected for TK^D virus (ACV ED₅₀, 16.0 µg/ml), as exemplified by mouse M2 on day 10. Removal of this selective influence, however, allowed the reemergence of the more "natural" virus, and by day 17, 8 days after therapy was stopped, virus isolated from the same mouse was 97% TK⁺ and 3% TK^D, with an ACV ED₅₀ of 0.9 µg/ml.

Delayed ACV treatment of mice infected with the TK⁺-TK^D mixture produced variable clinical responses dependent on lesion severity at the time of treatment. For example, mouse M3 had a lesion score of 1.0 on day 4, when treatment was started. On day 6, virus recovered from this mouse was 100% TK⁺ and ACV susceptible (ACV ED₅₀, 0.4 µg/ml), and the lesion had progressed to a score of 3.0. When the same mouse was tested on days 10 and 17, the virus recovered continued to be predominantly TK⁺ and ACV

susceptible. Therefore, in severe infections, delayed antiviral treatment did not eradicate established wild-type viruses. Mice with lesion scores of <1.0 at the time of treatment had better responses to delayed therapy (data not shown), suggesting that virus load at the time of treatment was important in determining clinical outcome.

Individual mice infected with the TK⁺-TK^D-TK^A mixture also had varied patterns of infection and responses to oral ACV therapy. Untreated animals had severe disease associated with TK⁺ viruses (usually dead by day 7) or indolent disease caused by TK^D virus, as shown for mouse M3 in Table 6. In contrast, mouse M6, treated prophylactically with ACV at 0.25 mg/ml, had severe disease 6 days after infection, with a lesion score of 3.0. Virus recovered from this animal was ACV resistant (ACV ED₅₀, 34.4 µg/ml by DU) and consisted of 5% TK⁺, 5% TK^D, and 90% TK^A. This animal died shortly after this mixture of viruses was isolated. Another animal (mouse M9) had a more satisfactory response to ACV, with ACV-resistant virus (ACV ED₅₀, 9.1 µg/ml by DU) isolated on day 10. By day 16, the ACV ED₅₀ of virus recovered from this animal had decreased to 4.8 µg/ml, and this change was associated with a shift from 60% TK⁺-40% TK^D on day 10 to 97% TK⁺-3% TK^D on day 16. Mice receiving a higher dose of prophylactic ACV (1.25 mg/ml) had more complete responses to treatment. Viruses obtained from these mice were 100% TK^D with one exception: on day 17, the isolate from mouse M9 contained 10%

TABLE 5. In vitro properties of viruses isolated from athymic mice infected with a mixture of 70% TK⁺-30% TK^D

Isolate	Mouse no.	Treatment ^a	Day postinfection	ACV ED ₅₀ (µg/ml)		Composition of isolate ^b (%)		
				DU	PR	TK ⁺	TK ^D	TK ^A
VL 10929	M4	None	8	0.8	0.2	100	0	0
VL 10937	M8	None	17	12.0	14.0	0	100	0
VL 10934	M2	Pro	10	16.0	16.0	0	100	0
VL 10939	M2	Pro	17	0.9	0.3	97	3	0
VL 10930	M3	Del	6	0.4	0.4	100	0	0
VL 10935	M3	Del	10	1.0	0.4	99	1	0
VL 10941	M3	Del	17	3.0	0.5	98	2	0

^a See Table 3, footnote a.

^b Determined by plaque autoradiography in Vero cells (see text).

TABLE 6. In vitro properties of viruses isolated from athymic mice infected with a 76% TK⁺-17% TK^D-7% TK^A mixture^a

Isolate	Mouse no.	Treatment and dose (µg/ml)	Day postinfection	ACV ED ₅₀ (µg/ml)		Composition of isolate (%)		
				DU	PR	TK ⁺	TK ^D	TK ^A
VL 11216	M3	None	10	19.2	10.4	0	100	0
VL 11225	M3	None	17	14.8	4.6	0	100	0
VL 11212	M6	Pro (0.25)	6	34.4	16.7	5	5	90
VL 11218	M9	Pro (0.25)	10	9.1	1.4	60	40	0
VL 11224	M9	Pro (0.25)	16	4.8	0.1	97	3	0
VL 11215	M10	Pro (1.25)	7	10.1	5.6	0	100	0
VL 11217	M9	Pro (1.25)	10	38.1	13.6	0	100	0
VL 11223	M9	Pro (1.25)	17	26.4	2.1	10	90	0
VL 12213	M9	Del (0.25)	6	0.7	0.4	99	1	0
VL 11220	M3	Del (0.25)	10	0.4	0.5	100	0	0
VL 11219	M1	Del (1.25)	10	12.2	13.1	1	0	99
VL 11214	M3	Del (1.25)	6	9.2	6.2	1	98	1
VL 11226	M3	Del (1.25)	16	10.1	1.4	69	31	0

^a See Table 3, footnote a, and Table 5, footnote b.

TK⁺ and 90% TK^D virus, although it was still highly resistant (ACV ED₅₀, 26.4 µg/ml).

In animals treated prophylactically with the lowest dose of ACV, TK^A virus could emerge and cause clinical disease (as in mouse M6). At higher ACV doses, no animals had lesions attributable to TK^A virus.

Low-dose, delayed ACV treatment produced responses in mice infected with the TK⁺-TK^D-TK^A mixture similar to those observed in the previous experiments with the less complexly defined population. Animals with severe lesions at the time of treatment responded poorly and succumbed to overwhelming TK⁺ infection (e.g., mouse M9). Mice with less severe disease (lesion score on day 4, <1.0) had their disease stabilized during treatment. Virus recovered from these animals was predominantly TK⁺ and ACV susceptible, e.g., VL 11220 isolated from mouse M3.

Higher doses of delayed ACV therapy (1.25 mg/ml) caused striking differences in the viruses recovered from individual mice. For example, mouse M1 had a lesion score of 2.0 when therapy was started on day 4. His lesions continued to progress while on therapy, and on day 10 his lesion score was 3.0. The virus recovered on day 10 was ACV resistant (ACV ED₅₀, 12.2 µg/ml by DU) and was 1% TK⁺ and 99% TK^A by [¹²⁵I]dC plaque autoradiography. A second mouse, M3, had insignificant disease when treatment was started. Lesions progressed to a score of 1.0 on day 5, when they began to stabilize. Virus recovered from lesions on day 6 was ACV resistant (ACV ED₅₀, 9.2 µg/ml by DU) and 1% TK⁺ and 98% TK^D. When therapy was stopped (day 14), lesions again progressed. On day 16, virus isolated from the lesions of mouse M3 was still ACV resistant (ACV ED₅₀, 10.1 µg/ml by DU), but the amount of TK⁺ virus had increased from 1 to 69%.

In summary, disease patterns and clinical outcome varied somewhat even within mice of the same group. Although mice in the two separate experiments were infected in similar manners with the defined mixtures, individual patterns of disease were largely dependent on the TK phenotype of the virus that predominated in individual mice. TK⁺ virus and TK^A virus were associated with progressive disease or "breakthrough" lesions in low-dose-treated mice and in animals receiving delayed treatment for severe lesions. In contrast, TK^D virus caused indolent, chronic lesions in untreated mice and in prophylactically treated animals. Lesions were quickly recolonized by TK⁺ virus after treatment was stopped.

DISCUSSION

The recent reports of pathogenic, ACV-resistant mutants isolated from normal and immunocompromised patients (10, 22) have caused renewed concern about clinical resistance to ACV. This issue has resurfaced because of the current acquired immunodeficiency syndrome epidemic and the subset of these patients who are receiving ACV for HSV infections (21).

Numerous reports have appeared recently that evaluate methods for the detection of ACV-resistant virus. These methods include an enzyme assay which determines the uptake of labeled drug in HSV-infected cells (28), plaque autoradiography with labeled substrates (8, 30), and various drug susceptibility assays with different endpoints of viral growth. Our data indicate that assays that require higher concentrations of virus as well as increased culture time (>48 h) may be more sensitive for detecting small amounts of resistant virus.

Harmenburg et al. (14) reported that the enzymatic method can detect approximately 6.2% TK^D virus after 24 h of incubation; with increasing incubation times, it can detect even smaller amounts of TK^D virus. Our results with the DU assay indicate that it can usually detect between 3 and 9% of ACV-resistant virus and, when coupled with [¹²⁵I]dC plaque autoradiography, is a very precise method for the detection and identification of ACV-resistant mutants. For our purposes, the absolute numerical value of the ED₅₀ is less important than the detection and definition of small numbers of less-sensitive viruses.

Cutaneous HSV infection of athymic mice is a reasonable model of severe virus infection in the immunocompromised host. Defects in T-cell-mediated immunity of humans appear to increase the risk of HSV infection (20, 24, 33), and in athymic mice, HSV infection is almost uniformly fatal, while mice with intact immune responses appear to recover completely. In addition, these mice are quite susceptible to infection with a range of ACV-resistant mutants, whereas ACV-resistant TK^D viruses replicate poorly in normal mice (26).

Infections of athymic mice with plaque-purified virus strains confirmed earlier reports of decreased pathogenicity of the TK^D virus. Wild-type (TK⁺) and TK^A viruses grew equally well in snout skin. The titer of virus in snout skin reached a maximum approximately 3 days before the most severe herpetic skin lesions were seen.

When mice infected with plaque-purified viruses were treated with orally administered ACV, the clinical responses correlated quite well with *in vitro* data for the respective virus strains. Mice infected with ACV-susceptible virus generally had excellent clinical responses to prophylactic therapy and good responses to delayed treatment. Mice infected with TK^D virus had less complete responses, but their cutaneous disease, even untreated, was much less severe. ACV treatment of mice infected with virulent TK^A viruses was of no benefit at the dose used (0.5 mg/ml). These findings confirm the potential for clinically significant illness as a result of infection with pathogenic ACV-resistant TK^A HSV strains in immunocompromised patients.

Our results with viruses recovered from individual mice infected with defined mixtures emphasize the difficulty associated with the characterization of heterogeneous clinical isolates. It was not always possible to predict the types of viruses that would be recovered from individual mice infected with the same mixture. As noted in a previous report (11), virus mixtures recovered from infected mice tended to be more heterogeneous and generally more resistant than virus mixtures prepared *in vitro*. Clearly, the selective conditions differ *in vivo* and *in vitro*. For instance, it has been reported (9) that nucleosides are metabolized quite differently in cells from different species, which can lead to quite different levels of the active molecules. Also, it is possible that more subtle changes in the viruses, somehow associated with changes in other genes, may create additional disadvantages *in vivo*. However, observation of the disease pattern in infected mice enables a reasonable prediction of the TK phenotype that predominates in individual mice.

TK^D viruses were found in chronic, indolent ulcers of surviving, untreated mice that had been infected with predominantly TK⁺ virus. These TK^D mutants were also found in prophylactically treated mice late in the course of therapy. Our observations are similar to reports of TK^D virus patterns of infection in marrow transplant patients (2, 4). Interestingly, when therapy was stopped, TK⁺ virus rapidly rees-

tablished itself in the lesion. This suggests that in some non-life-threatening clinical situations, it might be possible to stop treatment, allow regrowth of TK⁺ virus, and then restart therapy.

In agreement with earlier reports, delayed therapy (96 h or longer) for severe herpetic disease was of little clinical benefit (9), and established lesions harboring wild-type virus were slow to respond to low doses of oral ACV. However, a fivefold increase in ACV dose enhanced the response to delayed therapy.

Pathogenic TK^A virus was responsible for progressive disease in mice receiving low-dose (0.25-mg/ml) prophylactic ACV or high-dose (1.25-mg/ml) delayed ACV treatment. Although this mutant was recovered from only a few mice, it caused severe disease and death in the animals from which it was isolated. At present, there are no clinical reports of progressive herpetic disease in patients harboring similar mutants, and some studies indicate that these viruses may respond to other nucleoside analogs (10).

There are a number of significant differences between cutaneous disease in athymic mice and HSV disease in immunocompromised patients. For example, in athymic mice virus is introduced as a primary infection that, because of a lack of T-cell-dependent immunity, progresses to the central nervous system and kills the animals. In immunocompromised humans, HSV is usually a recurrent infection, with little central nervous system involvement. Recovery from infection generally depends on reestablishment of normal T cell function (24). Additionally, most immunocompromised patients receive parenteral ACV, which yields levels in plasma considerably higher than can be achieved in athymic mice receiving oral ACV therapy (26). Despite these differences, the athymic mouse is a useful model for studying the interactions of different components of a viral population, and this model may predict the selection, pattern of infection, and clinical outcome of HSV infections in immunocompromised patients.

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