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Effect of Acycloguanosine Treatment on Acute and Latent Herpes Simplex Infections in Mice

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Systemic treatment of mice with the nucleoside analog 9-(2-hydroxyethoxy-methyl)guanine (acycloguanosine [aciclovir]) was found to be highly effective against acute type 1 herpes simplex virus infection of the pinna. The drug ablated clinical signs and reduced virus replication both in tissue local to the inoculation site and within the nervous system. Provided that moderate-sized virus inocula were used, acycloguanosine treatment reduced or prevented the establishment of a latent infection in the dorsal root ganglia relating to the sensory nerve supply of the ear. However, although it aborted artificially produced infections in dorsal root ganglia, acycloguanosine was found not to be effective against the latent infection once established. This finding strongly indicated that latent herpes simplex virus in mice can exist in a nonreplicating form.

Mice, inoculated subcutaneously in the pinna with a suitable strain of herpes simplex virus (HSV), undergo an infection which closely resembles the human disease (7) and forms a convenient model for the study of antiviral chemotherapeutic agents. Concentrations of infectious virus in the ear may be followed by testing tissue homogenates, and the results of these titrations are found to give reproducible patterns of virus growth declining to undetectable levels in survivors 8 to 10 days after inoculation. Local inflammatory changes produced by the virus can be gauged by measuring ear thickness. Progression of virus into the nervous system evokes neurological signs; the most obvious of these is the development of flaccid paralysis of the inoculated pinna, which fails to respond to mild external stimuli (7). Subsequently latent virus may be reactivated by explanting and culturing the three cervical ganglia that relate to the sensory nerve supply of the pinna.

The nucleoside analog 9-(2-hydroxyethoxy-methyl)guanine (acycloguanosine [aciclovir]) has been reported to be an effective and extremely selective agent against several herpes viruses both *in vitro* and in a number of animal models (2, 13). However, previously published reports of acycloguanosine *in vivo* have not examined the effect of the drug on the establishment and maintenance of a latent infection with HSV. In our study we have examined in detail the effect of the agent on virus replication *in*

vivo during both the acute and the subsequent latent phase of the infection. To provide additional information on the effects of the drug on infected cells in the nervous system, some experiments were carried out on mice infected by intraneural inoculation.

MATERIALS AND METHODS

Mice. Three-week-old BALB/c female mice were obtained from Bantin and Kingman (Grimston, Aldbrough, Hull, England) and used when they were 4 weeks old.

Virus strains. The principal virus used was HSV-1 (SC-16), a clone of the type 1 strain of herpes simplex virus, isolated by S. K. R. Clarke (Public Health Laboratory, Bristol, England) from an oral lesion (7).

The strain of pseudorabies virus (N₁A-2) was originally obtained from J. B. McFerran, Veterinary Research Division, The Farm, Stormont, Belfast, N. Ireland, and shown to be highly virulent for mice (4).

The thymidine kinase-deficient mutant used in this study was Cl(101)TK⁻ (6). This is a mutant of HSV-1, originally isolated by Dubbs and Kit (1) and called by them B2006.

Cell line. BHK-21 cells were grown in Eagle medium supplemented with 10% (vol/vol) tryptose phosphate broth and 10% calf serum (ETC medium) (14). For reactivation experiments the proportion of calf serum was reduced to 2%.

Preparation and administration of the drugs. The compounds were received as dry powders from Wellcome Research Laboratories, Research Triangle Park, N.C. They were suspended in distilled water at 3.2 or 1.6 mg/ml; the suspension was briefly sonically disrupted before use and administered either subcutaneously (s.c.) or intraperitoneally (i.p.) in twice-daily

injections to give a total dose of 25, 50 or 100 mg/kg per day. Preliminary work had indicated that a daily dose of 50 mg of acycloguanosine per kg per day produced a marked effect on the clinical signs in HSV-infected mice. In earlier experiments the compounds were given s.c. into the scruff of the neck, but this was found inconvenient when handling large numbers of mice. Later, compounds were given i.p.; comparisons of the effectiveness of the two routes of administration showed no differences between the s.c. and the i.p. routes. No specific tests were carried out on the toxicity of acycloguanosine for mice. However, no abnormal signs were observed during 1 month of continuous daily treatment with 100 mg/kg per day i.p., the highest dose employed in these studies.

Inoculation of the mice and virus growth in mouse ear. A 20- μ l volume of virus suspension was inoculated intradermally into the left pinnae of anaesthetized 4-week-old mice (7). To study the replication of virus in ears, the left pinnae were cut off from groups of mice, and the tissue from each mouse was then minced with scissors and ground in 1 ml of ETC in a small glass grinder. The suspensions were then diluted for independent assay using BHK cells (12).

Measurement of ear thickness. Ear thickness was measured with a Mitutoyo engineer's micrometer screw gauge. The thickness was measured at a point on the anterior edge, 5 mm from the root of the ear. The estimate has proved to be a rapid and reproducible indication of the severity of infection. It has enabled repeated estimates, presumably of the inflammatory processes, on the same set of animals and has been valuable in following the course of acute disease in the presence or absence of different treatments. The normal 4-week-old mouse ear is 220 μ m thick, and thickness increases to 400 to 500 μ m by 5 days after infection. The increased thickness has been expressed in micrometers as the difference in thickness between the infected and uninfected ears of individual mice.

Reactivation of latent virus from dorsal root ganglia. The second, third, and fourth cervical dorsal root ganglia were dissected from mice into 0.5 ml of ETC with serum reduced to 2%. The ganglia were maintained in this medium in closed bottles at 37°C for 5 or 6 days. The tissue was then homogenized and titrated for the presence of infectious virus as described above.

Intraneural inoculation. Mice were anaesthetized, and the sciatic nerve was exposed by blunt dissection of the thigh muscles at a point 1 cm from the root of the tail. Approximately 1 μ l of virus suspension was then injected into the sciatic nerve using a finely drawn pipette. The inoculum was later calibrated by expressing the same volume of a similar virus suspension into medium and titrating the number of plaque-forming units (PFU) present.

RESULTS

Effects of acycloguanosine on primary infection in the pinna. Mice were inoculated with 10^4 or 10^5 PFU of virus and divided into four groups treated with the following compounds: (i) acycloguanosine; (ii) 2,6-diaminopu-

rine; (iii) 2,6-diaminopurine arabinoside, approximately equal in effect to 9- β -D-arabinofuranyladenine in animal models (3), and (iv) untreated. In each case the drug was administered s.c. in twice-daily doses (at 9 a.m. and 6 p.m.) commencing the day before virus inoculation and continuing for 10 days thereafter. The larger inoculum produced a more severe disease in untreated mice with overt signs of neurological involvement. The lower inoculum of 10^4 PFU per mouse usually produced a nonlethal disease and thus resembled more closely the natural human infection. Acycloguanosine ameliorated the course of the disease, as judged by clinical signs and the titers of infectious virus obtained from ear homogenates on day 3 after inoculation, which was the expected peak of virus multiplication (Table 1, columns I, II, and III). Only samples from the acycloguanosine-treated mice showed a striking reduction in virus titer from the untreated controls. For comparison, the drug was given in similar doses to mice inoculated with 10^4 PFU of the herpes virus pseudorabies (which was found to be nonsusceptible to the drug *in vitro*) into the pinna, but it had no effect on the course of the disease in this case, and all the mice died.

The effectiveness of acycloguanosine was followed throughout the period of the acute disease by taking samples of ear tissue from mice on successive days (Fig. 1a). The development of ear thickness in the same mice is shown in Fig. 1b; twice-daily treatment was continued throughout the experiment. The measurement of ear thickness was also used to examine the effects of commencing or terminating drug treatment during the acute phase (Fig. 2). After an input of 10^4 PFU, treatment begun 3 days after infection prevented further marked increase in ear thickness (Fig. 2a). When treatment (started before inoculation) was terminated 2 days after virus inoculation, this short period of administration still produced a considerably ameliorating effect. Similar results were obtained using the larger inoculum of 10^5 PFU (Fig. 2b). The rapid ameliorating effects of acycloguanosine suggested that there might be nonspecific effects on inflammation; similar effects of reduced ear thickness, however, could not be demonstrated in acycloguanosine-treated mice in which ears were inflamed by injection of endotoxin, heat-killed HSV-1 (SC-16), or the thymidine kinase-deficient mutant Cl(101)TK⁻, or by topical treatment with the contact-sensitizing agent oxazalone. From these tests it was concluded that the effects of the drug described above resulted principally from reduced virus multiplication.

It was noted (as shown in Fig. 1a) that infec-

TABLE 1. *Clinical signs and virus isolation from pinnae and cervical dorsal ganglia of mice after inoculation with HSV-1 (SC-16)*

Treatment ^a (dose)	Virus dose (PFU per mouse)	I Mortality ^b	II Ear paralysis ^b	III Virus titer on day 3 p.i. (log ₁₀ PFU per ear ± SD) ^c	IV Virus detected in ganglia on day 3 ^d	V Mice shown to be latently infected ^d
None	10 ⁵	4/13	9/9	5.5 ± 0.3	2/2	7/7
2,6-Diaminopurine (25 mg/kg per day)	10 ⁵	3/10	7/7	5.5 ± 0.4	3/3	7/7
2,6-Diaminopurine arabinoside (50 mg/kg per day)	10 ⁵	0/10	8/10	4.7 ± 0.7 (<i>P</i> > 0.1) ^e	2/2	9/9
Acycloguanosine (50 mg/kg per day)	10 ⁵	0/10	2/10	3.5 ± 0.8 (<i>P</i> < 0.005)	4/4	6/10
None	10 ⁴	1/15	14/14	4.6 ± 0.2	ND ^f	7/8
2,6-Diaminopurine (25 mg/kg per day)	10 ⁴	0/15	14/14	4.6 ± 0.2	ND	8/9
Acycloguanosine (50 mg/kg per day)	10 ⁴	0/15	0/15	1.7 ± 0.6 (<i>P</i> < 0.001)	ND	7/9

^a Drugs were administered in twice-daily s.c. injections from day before inoculation until 10 days thereafter.

^b Ratio of number of mice dying or showing ear paralysis to total number in the group or number surviving.

^c Geometric mean titers of separate samples obtained from five mice for each treatment. p.i., postinfection; SD, standard deviation.

^d Ratio of number of mice from which virus was isolated to total number tested.

^e Student's *t* test applied to difference in geometric mean between treated group and untreated controls.

^f ND, Not determined.

tious virus was not completely eliminated from the ears of treated mice and indeed showed a rise 4 to 6 days after inoculation, also reflected in the ear thickness curves (Fig. 1b). This late rise was found to be reproducible; similar curves were obtained in two repeats of this late stage of the acute infection. Three explanations have been considered. The first was the possibility that drug-resistant mutants had emerged during the course of acute infection in the presence of acycloguanosine. This was tested by dividing the ear homogenates from treated and untreated mice and assaying for infectivity in the absence or presence of 10 μM acycloguanosine. This concentration is approximately 10 50% inhibitory doses in plaque reduction assays using HSV-1 (SC-16) in BHK-21 cells; it has little activity on the thymidine-kinase deficient mutant Cl(101)TK⁻. The results (Table 2) showed no evidence of the emergence of resistant strains of virus.

The second possibility was that the suspension of acycloguanosine used for treating the mice was gradually deteriorating during storage. Comparisons of the efficiency of stored and fresh suspensions showed no evidence of deterioration either in plaque-reduction assays or when tested *in vivo*. This could not have accounted for the secondary rise in virus activity in the later stages of the acute infection.

The third possibility is that the virus interacts

with a population of cells which are less susceptible to the drug. This is discussed later.

Effects of acycloguanosine on the establishment of neurological infection. After replication in the epidermal tissue of the pinna, virus progresses probably by axonal flow (5, 8) to the sensory ganglia supplying the ear, in particular the second, third, and fourth cervical ganglia. In the experiment in which 10⁵ PFU was inoculated the drug was seen to prevent the appearance of clinical signs of neurological involvement (Table 1, columns I and II). Nevertheless, virus was detected qualitatively in ganglia removed from these mice on day 3 (Table 1, column IV) in both treated and untreated mice. Acycloguanosine treatment was continued until day 10 after inoculation, and then, 2 weeks later, the second, third, and fourth cervical ganglia were tested qualitatively for the presence of latent virus. The results of these isolations are shown in Table 1 (column V). The remaining mice inoculated with 10⁴ PFU were also checked for the presence of latent virus by quantitative assay 6 days after explanation; the animals were sampled 7 weeks and 10 weeks after inoculation. These results and those of another experiment in which the drug was given *i.p.* are shown in Table 3. There was evidence for reduction in the establishment of latency in these three experiments, particularly marked in the last.

The observation that latent infection could be

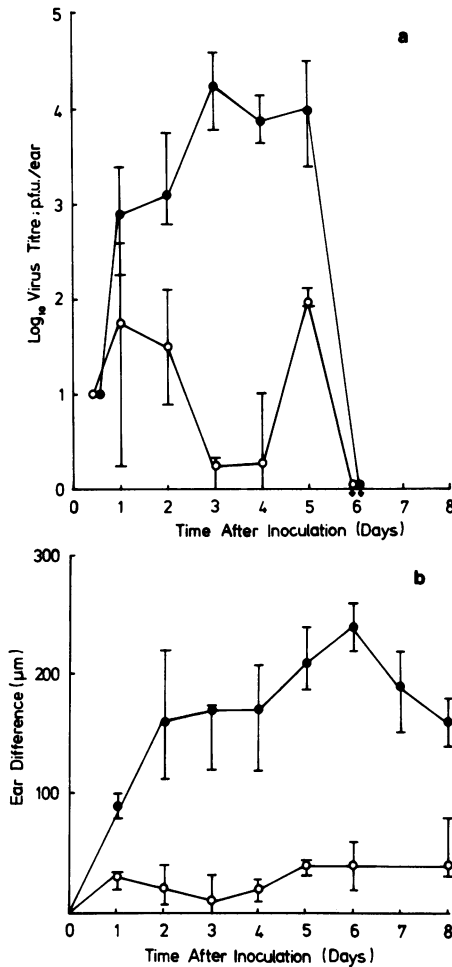


FIG. 1. (a) Infectious virus monitored in the ears of mice after inoculation with 10^4 PFU of HSV-1 (SC-16) into the left ear. (●) Untreated; (○) acycloguanosine, 50 mg/kg per day i.p., from the day before inoculation. Points represent geometric mean titers with ranges obtained from three mice at each time. (b) Development of ear thickness in mice inoculated with 10^4 PFU of HSV-1 (SC-16) into the left ear. Ear difference is the thickness of left ear minus that of right ear, in micrometers. (●) Untreated; (○) acycloguanosine, 50 mg/kg per day i.p., from the day before inoculation.

established despite continuous treatment during the acute phase of the infection suggested that acycloguanosine is not effective against the latent virus. To further this investigation, mice in which a latent infection was already established (mice surviving an inoculum of 10^5 PFU into the ear 1 to 6 months previously) were treated with acycloguanosine with a number of different regimens. These included s.c. for i.p. administration given continuously for 1 month (50 mg/kg per

day), or in some cases short discontinuous treatments of 50 or 100 mg/kg per day were employed. Mice were then killed, and their cervical dorsal root ganglia were assessed for latency by the *in vitro* test. Some mice were sampled during a course of treatment, others at various times after treatment was completed. None of these experiments showed any significant reduction in the number of animals harboring the latent virus, or in the titers of virus obtained from the ganglia of such mice (Table 4). The wide varia-

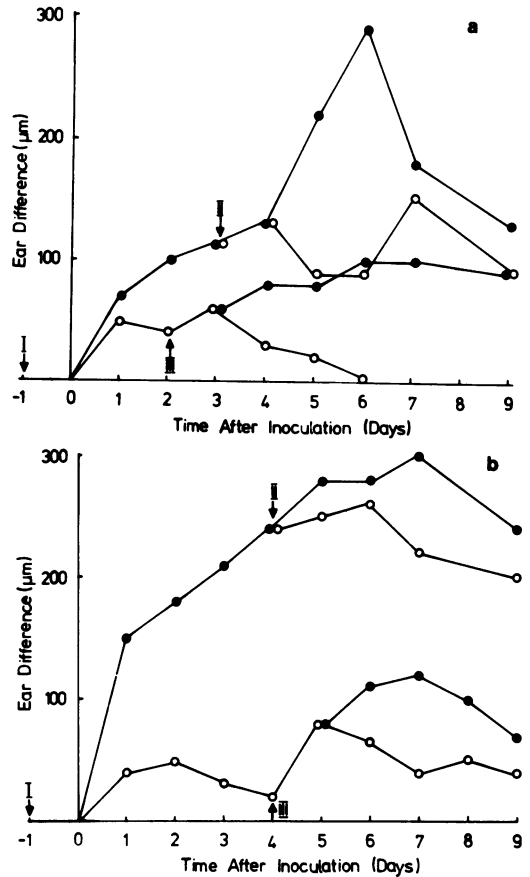


FIG. 2. Development of ear thickness in mice inoculated with HSV-1 (SC-16) and treated with acycloguanosine, commencing or terminating treatment at various times. (●) Untreated or treatment ceased; (○) acycloguanosine, 50 mg/kg per day i.p., or treatment commenced. (a) Virus inoculum was 10^4 PFU per mouse. (↓ I) Treatment commenced 1 day before inoculation; (↓ II) treatment commenced 3 days after inoculation. (b) Virus inoculum was 10^5 PFU per mouse. (↓ I) Treatment commenced 1 day before inoculation; (↓ II) treatment commenced 4 days after inoculation; (↑ III) treatment ceased 4 days after inoculation.

TABLE 2. Development of acycloguanosine resistance^a

Treatment	Assayed ^b	Mouse no.	Virus titer (log ₁₀ PFU per ear) on day p.i. ^c			
			4	5	6	7
Acycloguanosine (50 mg/kg per day) ^d	A	1	<0.0	2.1	<0.0	<0.0
		2	1.3	2.2	0.8	<0.0
		3	0.3	2.3	0.8	<0.0
	B	1	<0.0	<0.0	<0.0	<0.0
		2	<0.0	0.6	<0.0	<0.0
		3	<0.0	<0.0	<0.0	<0.0
Untreated	A	1	3.4	2.4	3.0	2.7
		2	3.8	2.9	3.0	<0.0
		3	4.3	2.1	2.5	<0.0
	B	1	<0.0	<0.0	<0.0	<0.0
		2	<0.0	1.6	<0.0	<0.0
		3	<0.0	0.0	<0.0	<0.0

^a Titers of virus in ears of individual mice obtained during treatment after inoculation of 10⁴ PFU of HSV-1 (SC-16) into the pinna.

^b A, Assayed in absence of drug; B, assayed in presence of 10 50% inhibitory doses of acycloguanosine.

^c p.i., Postinoculation.

^d Twice-daily doses i.p. commencing the day before virus inoculation and continued throughout the experiment.

TABLE 3. Levels of infection in homogenates of cervical dorsal root ganglia after explantation and incubation *in vitro*^a

Expt. I: virus titer ^b		Expt. II: virus titer ^b	
Untreated mice	S.c.-treated mice ^c	Untreated mice	I.p.-treated mice ^c
>4.5	>4.5	5.0	<0.0
>4.5	>4.5	4.8	<0.0
>4.5	3.5	4.3	<0.0
>4.5	2.9	4.3	<0.0
>4.5	1.5	3.8	<0.0
>4.5	1.0	0.0	
>4.5	0.8	<0.0	
<0.0	<0.0		
	<0.0		

^a Mice were inoculated with 10⁴ PFU of HSV-1 (SC-16) into the pinna several weeks previously.

^b Log₁₀ virus in second, third, and fourth cervical ganglia, combined for individual mice, after 6 days of incubation. Experiment I, cf. Table 1.

^c Mice were treated s.c. or i.p. with 50 mg of acycloguanosine per kg per day from the day before to 10 days after inoculation.

tion in titers observed in explants from both treated and untreated groups is to be expected, because once the virus reactivates in the culture there is a rapid multiplication of infectious virus. The experiments show, however, that there was no marked reduction of the latent infection as judged by the *in vitro* reactivation test.

Effect of acycloguanosine on infection by intraneural inoculation. The experiments described above had not proved that acycloguanosine is effective within the peripheral nervous system. Although reduced neurological involvement was observed in the primary infection, this might have been explained by the effects of the drug on local virus replication in the skin, reducing the supply of virus into the nervous system. Experiments were therefore carried out in which virus was injected intraneurally. By inoculation directly into sciatic nerve it was hoped to produce a neurological infection that did not require prior virus replication in non-neurological tissue. The results of these experiments, in which treatment was commenced either the day before virus inoculation or 3 h after inoculation (Table 5), showed that acycloguanosine was highly effective in reducing both clinical signs and levels of infectious virus in several neurological tissues: sciatic nerve, spinal cord, and the first sacral and IV, V, and VI lumbar dorsal root ganglia. It was concluded, from the marked reductions in virus titer observed in these tissues from drug-treated mice, that adequate levels of the drug were available within these tissues, and in particular within the dorsal root ganglia, to affect virus-infected cells within the peripheral nervous system. Eight intraneural-infected, drug-treated mice were also tested for latency by subsequent explantation of the lumbar-sacral ganglia, and

TABLE 4. Latent infection experiments^a

Expt.	Treatment regimen	Time after treatment before explantation	Acycloguanosine-treated mice		Untreated mice	
			Cultures yielding virus ^b	Log ₁₀ virus ^c	Cultures yielding virus ^b	Log ₁₀ virus ^c
1	1 month, 50 mg/kg per day s.c.	3 weeks	8/10	≥5.0, ≥5.0, ≥5.0, ≥5.0, 2.7, 2.7, 2.3, 1.0	19/20	≥5.0, ≥5.0, ≥5.0, ≥5.0, ≥5.0, ≥5.0, ≥5.0, ≥5.0, ≥5.0, 5.0, 4.9, 4.8, 4.6
2	1 month, 50 mg/kg per day s.c.	3 weeks	5/6	5.4, 5.2, 4.1, 4.0, 0.8	7/12	5.0, 4.8, 4.6, 3.3, 2.7, 1.7, 1.7
3	5 days, 50 mg/kg per day i.p.	1 day	5/5	4.7, 3.3, 2.9, 0.0, 0.0	5/5	5.2, 5.0, 4.8, 1.6, 1.4
		3 days	5/5	5.5, 4.9, 3.0, 2.0, 1.5	4/4	5.8, 5.7, 5.7, 5.6
		11 days	4/4	5.5, 4.5, 3.6, 1.3	4/4	5.5, 4.5, 3.6, 1.3
4	1 month, 50 mg/kg per day i.p.	1 week	7/7	5.1, 5.0, 4.8, 4.7, 3.5, 2.7, 1.4	7/7	5.7, 5.4, 5.3, 4.9, 4.6, 4.0, 1.4
5	1 week, 50 mg/kg per day i.p.	Explanted during treatment	6/8	5.6, 5.3, 5.2, 4.6, 4.1, 2.7	2/10	4.0, 1.3
Totals			40/45 (89%)	3.7 ± 1.7 ^d	48/62 (77%)	4.4 ± 1.3 ^d

^a Five experiments in which mice with established latent HSV infection were treated with acycloguanosine and their cervical dorsal root ganglia were then explanted and assessed for the presence of the latent infection. Treatment of groups of mice commenced 1 to 6 months after inoculation with 10⁵ PFU of HSV-1 (SC-16). They had recovered from the acute disease and now carried the infection in latent form. Acycloguanosine was administered daily with the dose and treatment period shown.

^b Ratio of number of explant cultures (CII, CIII, and CIV combined for each mouse) yielding virus/number of mice tested.

^c Log₁₀ virus in the virus-yielding explants after 5 to 6 days of incubation.

^d Geometric mean titer ± standard deviation.

all were shown to be harboring latent virus. Thus, these experiments were similar to the ear model in that treatment had not prevented the establishment of a latent infection in spinal ganglia.

DISCUSSION

The important observations made in this study of acycloguanosine in mice infected with HSV were as follows. (i) Continuous treatment with the drug had a marked effect on the clinical signs produced in infected animals. Inflammation was reduced as judged by ear thickness even when treatments were of brief duration, although it did not reduce similar inflammation induced by other agents. (ii) Although clinical signs were reduced, low levels of virus replication persisted for several days. (iii) Treatment that commenced before inoculation and was continued throughout the acute disease reduced, but did not always prevent, the establishment of a latent infection. (iv) Treatment appeared to have no effect on the latent infection once established. (v) Acycloguanosine was effective against active infection in the nervous system after in-

traneural injection of virus, but again did not prevent the establishment of latency.

The persistence of virus in the ear and the nature of the late peak of virus activity observed on day 5 was not elucidated, but it could not be attributed to the development of resistance to acycloguanosine. Of various possible mutations to drug resistance, mutants lacking thymidine kinase have been studied, and two of these, obtained artificially have been found by us to show extremely low virulence in the mouse model (6). Perhaps a more likely explanation is that there are scattered cells in which the virus replicates in the ear but which are less susceptible to the action of the drug either because of reduced permeability, because of differences in their thymidylate metabolism, or because the virus in them is not undergoing the lytic replication cycle.

The observation that latency was established despite treatment would be consistent with and probably reflects the reduction of virus titers observed in the ear during week 1 of the infection. We believe that latency can be established (though less reproducibly) with comparatively

TABLE 5. Effect of acycloguanosine treatment on virus titers in neural tissues^a

Treatment	Log ₁₀ PFU in individual mice at day postinoculation:						
	1		3			6	
	A ^b	B	A	B	C	A	B
Untreated			5.8	5.3	2.0		
			5.7	5.4	4.3		
			5.6	4.1	3.0		
			5.5	3.9	3.3		
			4.9	4.4	5.2		
			3.7	2.1	1.3		
Acycloguanosine ^c			1.6	<0.0	<0.0		
			0.3	1.0	0.6		
			0.0	<0.0	<0.0		
			<0.0	<0.0	<0.0		
			<0.0	<0.0	<0.0		
Untreated	3.5	2.4	5.3	4.2			
	2.9	2.3	5.2	4.9			Mice dead
	1.8	1.8	3.8	3.4			
Acycloguanosine ^d	2.3	1.3	1.9	0.0		<0.0	<0.0
	2.2	1.4	<0.0	<0.0		<0.0	<0.0
	<0.0	<0.0	<0.0	0.7		<0.0	<0.0

^a Mice received 5×10^5 PFU of HSV-1 (SC-16) in the left sciatic nerve.

^b A, Left lumbar sacral dorsal root ganglia; B, left sciatic nerve, 1-cm length, from close to spinal cord; C, lumbar sacral spinal cord, 1-cm length.

^c Acycloguanosine, 100 mg/kg per day i. p. in twice-daily injections, beginning 1 day before virus inoculation and continuing for 10 days

^d Acycloguanosine, 100 mg/kg per day i. p. in twice-daily injections, beginning 3 h after virus inoculation and continuing for 10 days

small inocula, e.g., 10^2 or 10^3 PFU (unpublished data). This would explain why the drug had little effect on the establishment of latency after the inoculation of 10^5 PFU and a variable effect using the smaller dose of virus. Our results suggest, however, that prompt treatment of the primary infection would minimize the likelihood of establishment of latency, and that, if latency is not completely prevented, could reduce the number of latently infected foci in the ganglia.

It was of great interest to investigate the potential of acycloguanosine treatment of mice in which the latent infection was already established. This failure to cure latently infected mice is consistent with the static model for latency. It is possible that recurrence of disease in humans or in animal models is preceded by active virus replication in ganglion cells (9). In this situation, some latently infected cells may become susceptible to the activity of the drug, and it is interesting to speculate that after several such recurrences the reservoir of latently infected cells might be destroyed. Work is now in progress to test these possibilities in the mouse ear model.

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