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Effect of 9-(1,3-Dihydroxy-2-Propoxymethyl)Guanine on the Acute Local Phase of Herpes Simplex Virus-Induced Skin Infections in Mice and the Establishment of Latency†

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The effect of topical and systemic treatment with 9-(1,3-dihydroxy-2-propoxymethyl)guanine on the evolution of herpes simplex virus-induced skin infection in hairless mice was investigated. Systemic (subcutaneous) treatment with a 10-mg/kg dose and topical applications with a 5% cream started up to 48 h after infection prevented the development of severe skin lesions and a fatal outcome. However, the establishment of latent infections was prevented only by topical treatment started at 6 h after infection. Systemic (50 mg/kg) and topical treatments started 48 h after infection reduced virus titers in the skin and ganglia and promoted rapid clearance of virus from these sites. The clearance of infectious virus from ganglia during the acute phase of infection was followed by early establishment of latency. 9-(1,3-Dihydroxy-2-propoxymethyl)guanine (0.03 μg/ml) significantly inhibited the synthesis of infectious virus in explant cultures of latently infected ganglia, and at concentrations higher than 8 μg/ml no infectious virus was detectable in ganglia explant cultures.

The acyclic nucleoside 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG), synthesized by the procedures described by Ogilvie and Gillian (20), Martin et al. (19a), and Ashton et al. (1) has been shown to be an effective inhibitor of herpes simplex virus (HSV) and cytomegalovirus replication in cell cultures (1, 2, 4, 5, 19, 23, 26). DHPG proved to be superior to acyclovir (ACV) in the treatment of HSV-induced encephalitis and vaginitis in mice (1, 5, 6, 8, 26) and was effective in reducing the severity of primary and recrudescent lesions induced by genital infection of guinea pigs (9) and by eye infections in rabbits (27). Although 4 to 35 times more DHPG is required to inhibit cytomegalovirus in cell cultures than is necessary to inhibit HSV (26). DHPG is a more potent inhibitor of cytomegalovirus than is ACV. DHPG is an efficient substrate for HSV-induced thymidine kinase (1, 2, 26) and is more rapidly converted to the corresponding triphosphate derivative in virus-infected cells than is ACV (1, 10). The triphosphate of DHPG competitively inhibits incorporation of dGTP into DNA catalyzed by the DNA polymerase specified by HSV (7, 25).

In this study we have investigated the effect of DHPG on experimental HSV-induced skin infections in hairless mice and have examined the ability of the drug to prevent the colonization of sensory ganglia and the establishment of latency by the virus. In addition we have determined the concentration of DHPG required to prevent the reactivation of HSV in explant cultures of latently infected mouse trigeminal ganglia.

MATERIALS AND METHODS

Virus. The S strain of HSV type 1 was used in all experiments. The maintenance of the virus, the preparation of stock virus, and the quantification of inocula have been described in a previous publication (16).

Inoculation of mice. Female hairless mice of the fully immunocompetent HRS/J strain were obtained from Jackson Laboratories, Bar Harbor, Maine, and used in the experiments at the age of 6 to 8 weeks. The mice were inoculated percutaneously on a triangular area of the snout by rubbing a virus suspension containing 10^6 PFU/ml into the scarified skin. Approximately 10^4 PFU were applied on the scarified skin of each mouse.

Scoring skin lesions. The development of lesions was recorded daily for 14 days, and their intensity was graded on a scale from 0 to 4 as described elsewhere (16).

Monitoring infectious virus in sensory ganglia and skin specimens. At various intervals postinoculation (p.i.), groups of mice were exsanguinated by heart puncture under sodium pentobarbital anesthesia. The day on which each group of mice was to be sacrificed was randomly assigned after virus inoculation. Sensory ganglia were removed and homogenized immediately by sonication (Branson Sonifier Cell Disruptor 200). Skin specimens were removed from the inoculation area (ca. 0.25 cm^2) and similarly homogenized. The ganglia and skin suspensions were clarified by centrifugation, and their virus content was determined by a plaque assay on Vero cells.

Monitoring latent virus infections in sensory ganglia. At 3 to 4 weeks after virus inoculation, the surviving mice were sacrificed as described above. The trigeminal ganglia were removed, and each ganglion was maintained separately in explant culture. After 7 days in culture, the ganglia were homogenized by sonication and assayed for the presence of reactivated virus in monolayers of human fibroblasts (FS7 cells).

Effect of DHPG on in vitro reactivation of HSV from latently infected ganglia. Latently infected ganglia were obtained from mice which survived a primary infection in the orofacial area. The mice were sacrificed as described above, and the trigeminal ganglia were maintained in explant culture in the presence of increasing DHPG concentrations. After 4

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TABLE 1. Effect of topical DHPG treatment[a] on HSV-induced orofacial skin infections in hairless mice

Expt no.	Start of treatment (h p.i.)	No. of mice with lesions/no. examined	No. of mice dead/no. tested	No. of latent infections/ no. of survivors
1	None	6/10	3/10	7/7
	6	0/10	0/10	0/10
	24	0/8	0/8	3/8
	48	1/10	0/10	8/10
2	None	10/10	10/10	
	6	0/10	0/10	0/10
	24	0/8	0/8	4/8
	48	2/10	0/10	9/10

[a] 5% DHPG cream was applied twice daily for 4 days.

days in culture, the ganglia were washed five times and sonicated, and their virus content was determined by a plaque assay on Vero cells.

Treatment of mice. Systemic treatment with various drug concentrations was administered by subcutaneous (s.c.) injection in the nape of the neck. Topical treatment was applied to the area of inoculation. It should be mentioned that the mice do not tend to lick the drug off the lesions. The treatments were initiated at various intervals p.i. and were given twice daily at an 8-h interval for 4 days or until the mice were sacrificed.

Antiviral compounds. DHPG and a cream containing 5% DHPG–propylene glycol oil in water were kindly provided by Syntex Research, Palo Alto, Calif. For systemic treatment DHPG was dissolved in phosphate-buffered saline.

RESULTS

Effect of DHPG on evolution of percutaneous HSV-induced skin infection and establishment of latency in sensory ganglia.

(i) Topical treatment. Topical treatment with a 5% DHPG cream completely prevented the development of skin lesions when the applications were begun up to 24 h p.i., and only 3 of 20 mice developed minimal skin lesions when the applications were started 48 h p.i. None of the treated mice died over an observation period of 3 weeks. The establishment of latent ganglionic infections was completely prevented when treatment was started 6 h p.i. Treatments started 24 h p.i. reduced the number of latently infected mice, but those started 48 h p.i. did not prevent the establishment of latent infection (Table 1). The effect of topical treatments was evaluated in two experiments: in one, untreated mice had a low mortality rate, and in another, a high mortality rate was observed. Nevertheless in both experiments the response of DHPG-treated mice was similar (Table 1).

(ii) Systemic treatment. The development of lesions was completely prevented with a low dose (10 mg/kg per day for 4 days) when s.c. treatment was started 6 h p.i. or with a high dose (100 mg/kg per day for 4 days) when s.c. treatment was started 48 h p.i. (Table 2). However, even the 10-mg/kg dose started with a delay of 48 h significantly reduced the number of mice which developed skin lesions. The lesions which developed in DHPG-treated mice were mild and healed rapidly. None of the treated mice died. The establishment of latent ganglionic infections could not be completely prevented by systemic treatment even when a high drug dose was used and treatment was initiated early. However, the 50-mg/kg dose reduced significantly the frequency of latent infections when treatment was started up to 6 h p.i., as did the 100-mg/kg dose when treatment was started up to 24 h p.i. (Table 2).

Systemic treatment with doses of 10, 50, or 100 mg of DHPG per kg which were initiated 3 or 4 days p.i. did not prevent the development of skin lesions. When treatment was started 3 days p.i., mild lesions developed and healed rapidly; the dose of DHPG had only marginal effects on the intensity of the lesions. When treatment was started 4 days p.i., the dose of DHPG had a more pronounced effect: recipients of a 10-mg/kg dose developed lesions whose severity paralleled that of untreated mice, whereas the lesions of mice treated with a 100-mg dose 4 days p.i. tended to follow the evolution of lesions observed in mice treated 3 days p.i. (Fig. 1).

All mice treated 3 days p.i. survived, whereas the mortality rate in untreated mice was 50%. Of 10 mice treated with the 10-mg and 50-mg doses, 2 and 1, respectively, died when the administration of DHPG was initiated 4 days p.i.

(iii) Frequency of unilateral and bilateral latent HSV infections in trigeminal ganglia of mice after systemic and topical DHPG treatment. With the exception of topical applications of 5% DHPG cream, no treatment modality completely prevented the establishment of latent infection (Tables 1 and 2). Since the trigeminal ganglia of each mouse were examined individually for the presence of latent virus, we determined the relative proportion of mice with latent infections in either one or both ganglia.

Altogether, 73% of the surviving control mice developed latent infections in both trigeminal ganglia (Table 3). Systemic treatment with a 10-mg/kg dose reduced the frequency of bilateral latent infection to 50%, but the difference was not statistically significant. In mice treated 24 and 48 h p.i. topically or 24 h p.i. systemically with a 50- or 100-mg/kg DHPG dose, the number of bilateral latent infections was

TABLE 2. Effect of systemic DHPG treatment[a] on HSV-induced orofacial skin infections of hairless mice

Drug dose (mg/kg per day)	Start of treatment (h p.i.)								
	6			24			48		
	No. of mice with lesions/no. tested	No. dead/ no. tested	No. of latent infections/ no. of survivors	No. of mice with lesions/no. tested	No. dead/ no. tested	No. of latent infections/ no. of survivors	No. of mice with lesions/no. tested	No. dead/ no. tested	No. of latent infections/ no. of survivors
None	9/10	5/10	4/5	10/10	10/10		12/12	11/12	1/1
10	0/11	0/11	10/11	5/12	0/12	11/12	3/12	0/12	11/12
50	0/12	0/12	5/12[b]	1/11	0/11	7/11	2/12	0/12	12/12
100	0/10	0/10	3/10[b]	0/10	0/10	4/10[b]	0/10	0/10	7/10

[a] The drug dose was given in two daily s.c. injections for 4 days.
[b] The frequency of latent infections was significantly lower than in mice treated with the 10-mg/kg dose of DHPG.

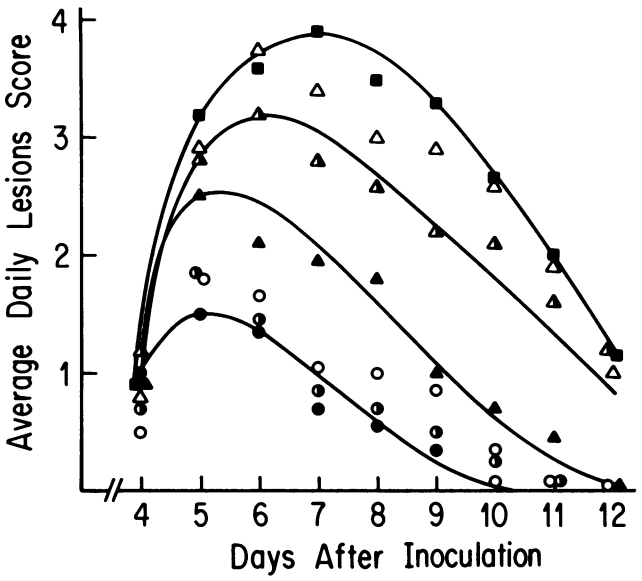

FIG. 1. Evolution of HSV-induced skin lesion in hairless mice treated s.c. with DHPG. Treatments started 3 days p.i. with a daily dose of 10 (○), 50 (◑), or 100 (●) mg/kg or 4 days p.i. with a daily dose of 10 (△), 50 (▲), or 100 (△) mg/kg. Treatments were given twice daily for 4 days. ■, Untreated mice.

reduced to a statistically significant extent. Due to the small number of mice with latent infections, a statistical significance for the decrease in the number of bilateral latent infections could not be demonstrated for all treatment groups (Table 3).

Effect of DHPG on virus titers in trigeminal ganglia and inoculation site during the acute phase of infection. Mice inoculated in the orofacial skin area were treated 48 h p.i. for 4 days with a 5% DHPG cream or a daily s.c. DHPG dose of 50 mg/kg. Starting with day 3 p.i., groups of four treated and four untreated mice were sacrificed, and the virus titers in the ganglia (separately in both trigeminal ganglia of each mouse) and skin were determined. Virus titers in the skin of untreated mice peaked by day 4 or 5 p.i. (about $10^5$to 10^6 PFU), and virus was still present in most skin specimens by day 8 or 9 p.i. (Fig. 2). Virus titers in the skin of mice treated systemically or topically with DHPG peaked by day 3 or 4 p.i. (about 10^3 PFU), and by day 6 p.i. virus could no longer be detected in skin specimens. Virus was detected in all four skin specimens only in mice treated systemically (50 mg/kg) and examined at 3 days p.i. In all other groups, virus was detected only in one or two of the four skin specimens examined on any single day p.i.

Virus titers ranging between 10^2 and 10^3 PFU were found in the ganglia of untreated mice from days 3 to 7 p.i. From 85 to 100% of the ganglia examined contained infectious virus, and most specimens were still virus positive on day 8 or 9 p.i. In systemically treated mice infections virus was no longer detected at 5 days p.i. Virus was still present on day 8 p.i. in some of the topically treated mice. In both topically and systemically treated mice, infectious virus was present in ca. 25% of the ganglia examined on any single day p.i. Latent infections were demonstrated by explanting ganglia from all mice treated at 48 h p.i. with 50 mg of DHPG per kg or with a 5% DHPG cream (Tables 1 and 2). Therefore, we determined the presence of virus in ganglia obtained from systemically treated mice sacrificed on day 4 or 8 p.i. (three mice per group) and maintained in explant culture for 7 days; after explantation, at least one trigeminal ganglion from each of these mice contained infectious virus.

Effect of DHPG on in vitro reactivation of HSV from latently infected mouse trigeminal ganglia. A DHPG concentration of 0.03 μg/ml in the explant culture medium reduced the mean titer of infectious virus detectable in latently infected ganglia ca. 100-fold (Fig. 3). The proportion of ganglia containing infectious virus and the amount of virus in the ganglia decreased as the concentration of DHPG increased. Only 1 of 10 and 1 of 8 ganglia contained small amounts of virus at DHPG concentrations of 2 and 8 μg/ml, respectively. No infectious virus was detected in ganglia explant cultures in the presence of 32 μg of DHPG per ml.

It should be mentioned that the amount of reactivatable virus present in latently infected ganglia varied. The virus titers in ganglia maintained for 4 days in drug-free medium showed differences of up to 1.7 $\log_{10}$ units. Similar differences in virus titer were also detected in individual ganglia maintained in the presence of DHPG (Fig. 3). It can also be seen that at DHPG concentrations of 0.125 and 0.5 μg/ml, about 50% of the ganglia were virus free, and at concentrations of 2 and 8 μg/ml, reactivated virus was detected in only ca. 10% of the ganglia.

DISCUSSION

Systemic and topical treatment of HSV-induced skin infections in hairless mice with DHPG prevented the development of severe lesions and, in most cases, a fatal outcome of infection. These beneficial effects were observed even when treatment was started as late as 2 days p.i. Like ACV, phosphonoformate, and phosphonoacetate, topical DHPG prevented the establishment of latent infections in sensory ganglia only when treatment was initiated during the first 6 h p.i. (11, 14, 18).

Although late initiation of DHPG treatment did not prevent the establishment of latent virus, delayed treatment had a definite influence on whether only one or both trigeminal ganglia became latently infected. Whereas 73% of the untreated mice had latent infections in both ganglia, none of the mice treated either topically or systemically (100 mg/kg) at 24 h p.i. had bilateral ganglionic infections. Significant differences were also observed in mice treated s.c. with 50 mg/kg at 24 h p.i. and in mice treated topically at 48 h p.i. The relative frequency of unilateral or bilateral latent infections might serve as an additional parameter in evaluating antiviral drugs for their ability to prevent or reduce the colonization of ganglia by HSV.

The effectiveness of DHPG in preventing the evolution of the experimental HSV infection can be related to its ability to reduce the accumulation of infectious virus in trigeminal ganglia through the inhibition of virus synthesis at the inoculation site. DHPG appears to be superior to phosphonoacetate (17). Both systemic and topical treatment with DHPG was effective in preventing dissemination of virus in the ganglia.

The effectiveness of topically applied antiviral compounds in experimental HSV-induced skin infections is strongly influenced by the nature of the vehicle incorporating the drug. De Clercq (3) has shown that the effectiveness of (*E*)-5-(2-bromovinyl)-2′-deoxyuridine is significantly enhanced when the drug is incorporated in a vehicle consisting of Azone and dimethyl sulfoxide. ACV is also more potent when incorporated in dimethyl sulfoxide instead of standard vehicles (25). It is therefore likely that the full potential of topical treatment with DHPG was not achieved by the

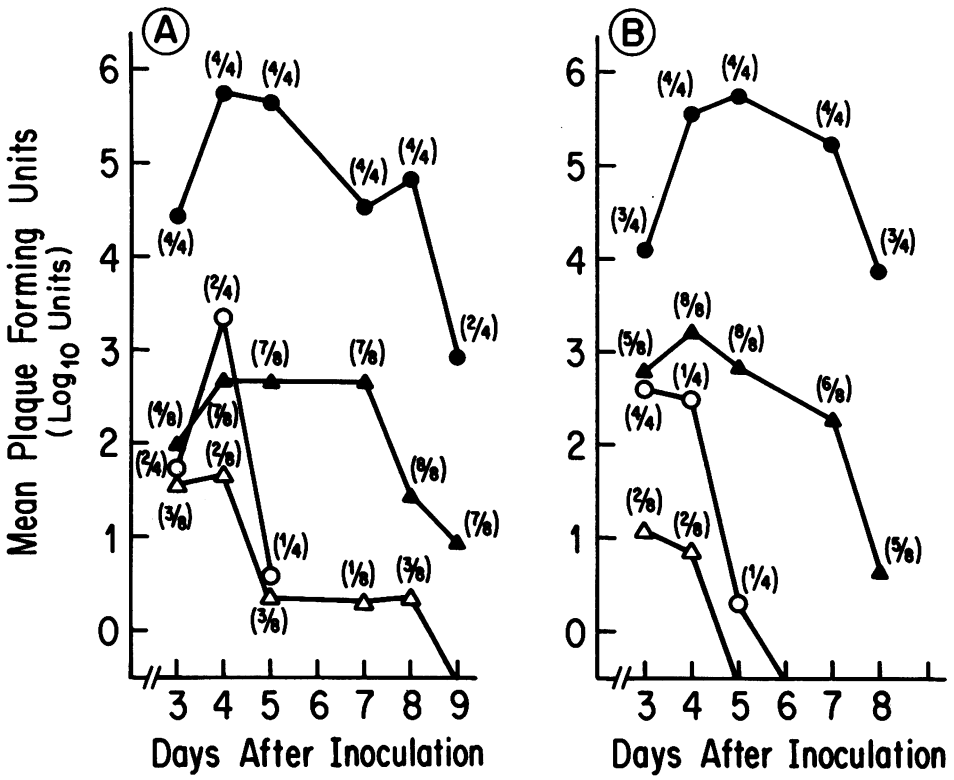

FIG. 2. Virus titers in skin and ganglia of mice given topical (A) or systemic (B) DHPG treatment. Topical treatment with a 5% DHPG cream and s.c. injections with a daily dose of 50 mg of DHPG per kg were given twice daily for 4 days, and virus titers were determined in the skin of untreated (●) and DHPG-treated (○) mice and in the ganglia of untreated (▲) and DHPG-treated (△) mice. At indicated intervals, groups of four mice were killed, and the virus titers in skin and ganglia homogenates were determined. The number of virus-positive specimens over the number of specimens examined is shown in parentheses for each data point.

vehicle used in our experiment. The vehicles used for the incorporation of antiviral drugs in topical preparations do not have any therapeutic effect and may sometimes even enhance the severity of the infection in placebo treatments (3, 14, 18).

Our data confirm the potential of DHPG in the treatment of various other experimental HSV infections, including intravaginal infections in mice treated orally (23) and guinea pigs treated systemically (9), intraperitoneal and orofacial infections in mice treated systemically (5, 8), and intranasal infections of mice treated orally and systemically (6). DHPG is active whether given orally, by s.c. or intraperitoneal injection, or, as shown in our experiments, by topical application at the inoculation site.

Studies on the ability of drugs active against HSV to prevent the synthesis of infectious virus in explant cultures of latently infected ganglia may serve as an indicator for their therapeutic use in preventing recurrent herpetic disease in humans (12, 13, 15, 21, 22, 28). Our experiments have shown that at a concentration of 0.03 μg/ml, DHPG reduced

TABLE 3. Frequency of unilateral and bilateral latent HSV infections in trigeminal ganglia of hairless mice after systemic or topical DHPG treatment

Treatment[a]	Start of treatment (h p.i.)											
	6				24				48			
	No. of mice with latent infections	% of infections that were:		P[b]	No. of mice with latent infections	% of infections that were:		P	No. of mice with latent infections	% of infections that were:		P
		Unilateral	Bilateral			Unilateral	Bilateral			Unilateral	Bilateral	
Systemic (mg/kg per day)												
10	10	50	50	NS[c]	11	55	45	NS	11	45	55	NS
50	5	80	20	NS	7	86	14	<0.03	12	67	33	NS
100	3	67	33	NS	4	100	0	<0.03	6	67	33	NS
Topical (5%) cream)	0				7	100	0	<0.01	17	71	29	<0.03

[a] Treatment was administered twice daily for 4 days.

[b] Probability that the difference between the frequency of unilateral and bilateral latent infection in treated and untreated mice is due to chance (Fisher exact test). For untreated mice, 11 had latent infections; 27% were unilateral, and 73% were bilateral.

[c] NS, Not statistically significant.

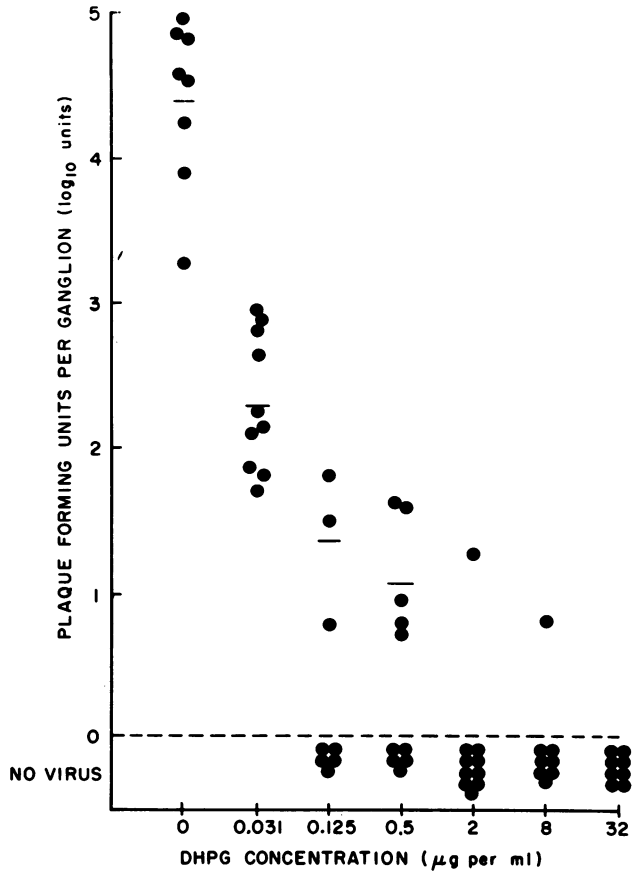

FIG. 3. Effect of DHPG on reactivation of HSV in explant cultures of latently infected mouse trigeminal ganglia. After 4 days of in vitro cultivation in the presence of various drug concentrations, the ganglia were washed and homogenized, and the virus titer in individual ganglia was determined by a plaque assay. Horizontal bars, Mean virus titer for virus-positive ganglia at the indicated drug concentration.

the titer of infectious virus in explant cultures of latently infected ganglia ca. 100-fold. This concentration is 10- to 100-fold lower than that required for other antiviral compounds that are also phosphorylated by the viral thymidine kinase that have been tested in a similar manner (13).

It is interesting that when only small amounts of virus accumulated in the trigeminal ganglia of DHPG-treated mice during the acute phase of infection, transition to the latent phase appeared to take place rapidly. Indeed, during the first 8 days p.i. only about 25% of the ganglia examined on any subsequent day p.i. contained infectious virus. However, when parallel groups of ganglia were removed during the early phase of infection and maintained in explant culture for 7 days, they all proved to contain infectious virus. It is tempting to speculate that, after penetrating a neuron, the virion becomes established quite rapidly in its latent state. As long as the virions from the inoculation site can gain access to neurons permissive for latency, the infection will remain confined to the ganglia and the mouse will survive. However, if the virions outnumber the available neurons, infectious virus will accumulate in the ganglia and eventually migrate to the brain, causing fatal encephalitis.

Therefore, even if antiviral drugs cannot in each case prevent the invasion of ganglia and the establishment of latent HSV infection, they can limit the degree of acute ganglionic infection and thereby promote a better chance for the survival of the animal.

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