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Research Article

Novel Animal Model for Evaluating Topical Efficacy of Antiviral Agents: Flux Versus Efficacy Correlations in the Acyclovir Treatment of Cutaneous Herpes Simplex Virus Type 1 (HSV-1) Infections in Hairless Mice

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This report describes the study of a novel animal model for the topical treatment of cutaneous herpes virus infections, with a focus upon the relationship between the dermal flux of the antiviral agent and the effectiveness of the topical therapy. A recently developed (trans)dermal delivery system (TDS) for controlling acyclovir (ACV) fluxes was employed in the treatment of cutaneous herpes simplex virus type 1 (HSV-1) infections in hairless mice. The TDS's were fabricated with rate-controlling membranes to provide nearly constant fluxes of ACV for up to 3 to 4 days. At the end of each experiment an extraction procedure was used to determine the residual ACV, validating the drug delivery performance of the TDS. Virus was inoculated into the skin of the mice at a site distant from the TDS area, and the induced lesion development was evaluated to distinguish between topical and systemic effectiveness of the therapy. In the main protocol, ACV therapy was initiated 0, 1, 2, and 3 days after virus inoculation and the lesion development "scored" on Day 5. The topical efficacies of 1- and 2-day-delayed treatments were essentially the same as that of a 0-day-delayed treatment, while the topical efficacy of a 3-day-delayed treatment was much poorer. Also, in the cases of 0-, 1-, and 2-day-delayed treatments, topical efficacy increased with increasing flux in the range of 10 to 100 μg/cm^2-day. When the ACV flux was 100 μg/cm^2-day or greater, a maximum 100% topical efficacy was obtained. The results for systemic efficacy were shifted to higher fluxes: approximately 10-fold greater ACV fluxes were necessary to provide efficacy equal to the topical efficacy results. The animals treated with a high ACV flux (350–500 μg/cm^2-day) lived significantly longer than those treated with a low ACV flux (10–125 μg/cm^2-day) and those of untreated (placebo) animals. Further, their mean survival time decreased with an increase in the time delay for ACV treatment. In contrast, the mean survival time for the animals which received a low ACV flux was similar to that of the control animals and remained unaltered with an increase in the time delay for ACV treatment. The approach developed in this study should be valuable in (a) the screening of new antiviral agents for the topical treatment of cutaneous herpes virus infections and (b) in the optimization of drug delivery systems (i.e., topical formulations).

KEY WORDS: acyclovir; controlled (trans)dermal delivery; hairless mice; herpes simplex virus type 1; topical and systemic antiviral efficacy; mean survival time.

INTRODUCTION

Experimental herpes simplex virus infections in hairless mouse skin produce a narrow band of lesion (1–4) that can be curtailed more or less by topical application of different antiviral agents (1,3–7). Taking advantage of this pattern of lesion development, we recently (8) developed a new method for efficacy evaluation of different antiviral agents in the treatment of cutaneous herpes simplex virus type 1 (HSV-1) infections in hairless mice. Acyclovir (ACV) was used as a model drug because of its high *in vivo* sensitivity against HSV-1 (9–11). In this hairless mouse model, shortly after virus inoculation, therapeutical amounts of ACV were delivered at an Azone-pretreated skin site using a (trans)dermal delivery system (TDS). At the end of a 5-day ACV treatment, the lesion was scored for each mouse and two distinct antiviral efficacy assessments were made: (a) "topical (local)" efficacy measured the antiviral activity of ACV delivered directly, topically to the local skin area covered by the TDS; and (b) "systemic" efficacy measured the antiviral activity of ACV delivered from the TDS via systemic circulation to the target site, presumably the epidermal basal layer (12).

An advantage of using a TDS over the conventional topical formulations is that the amount of ACV delivered to each infected animal can, in principle, be precisely con-

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trolled during the time period of the drug treatment through a rate-controlling membrane so that antiviral efficacy can be quantitatively correlated with the steady-state flux. In practice, the expected (theoretical) flux may be compared to the actual, average flux determined at the end of an *in vivo* experiment by carrying out an extraction of the residual drug in the TDS; this extraction assay then serves to validate the expected flux or, alternatively, provide the bounds of uncertainty in the particular experiment.

A shortcoming of the previous work (8) was that the method of residual ACV extraction from the TDS was by no means satisfactory, and therefore ACV mass balance could not be claimed for all *in vivo* experiments, especially those conducted with low ACV fluxes. As a consequence, only the theoretical fluxes (and not validated experimentally) were used in this earlier study. One of the goals of the present study was therefore to improve the existing procedure for ACV extraction so that the difference between the amount of ACV loaded and the amount extracted could fully account for the actual amount of ACV delivered to each animal.

The primary purpose of the present study was to investigate the treatment protocol. In the earlier work, all *in vivo* experiments involved the initiation of the TDS therapy on the same day (Day 0) immediately following virus inoculation and the continuation of the ACV treatment to Day 5 when the TDS was removed and the antiviral efficacy evaluated. In the present study, the effects of delaying the initiation of the ACV treatment (i.e., the application of the TDS) after virus inoculation has been investigated. Also, for the 2-day-delayed situation, the outcomes of a 1-, 2-, and 3-day application of the TDS are evaluated (on Day 5). These protocol studies provide insights to the usefulness of the animal model as a "screening" procedure for potential antiviral agents in the treatment of cutaneous herpes infections. Furthermore, the quantitative (or semiquantitative) nature of these studies should permit consideration of mechanistic aspects of the disease, the pharmacokinetics, and the pharmacodynamics of the ACV treatment.

MATERIALS AND METHODS

Drug and Permeation Enhancer

Acyclovir was obtained as its sodium salt (Zovirax) from Burroughs Wellcome Co., Research Triangle Park, NC. The salt was converted to its free acid before use according to the method described in a previous publication (8). The permeation enhancer Azone (1-dodecylazacycloheptan-2-one) was a gift from Nelson Research Corp., Irvine, CA. It was used as received.

Virus and Animals

Samples of the same batch herpes simplex virus type 1, strain E-377, with a titer of 1.35×10^8 plaque-forming units (PFU)/ml, were used throughout this study. They were stored in aliquots at $-70°C$ until used. The preparation and assay of the virus have been previously (13) described.

Female hairless mice (strain SKH/HR-1), 5–6 weeks old with body weights of 20 ± 2 g, were purchased from Temple University, Philadelphia, PA, and used in this study.

Fabrication of the (Trans)Dermal Delivery System

As in the previous study (8), polymer hydrogel membranes were used for controlling ACV fluxes through the Azone-pretreated hairless mouse skin. The preparation and characterization of these hydrogel membranes have been described previously (8,14) in detail.

The fabrication of the TDS has also been described previously (8). The drug reservoir in each TDS was loaded with a suspension of ACV sufficient for constant-rate release over the experimental period.

In Vitro ACV Flux Determinations for the TDS

The *in vitro* ACV flux from the TDS was determined by HPLC using the method described previously (8).

Residual ACV Extraction from the TDS

At the end of a release experiment, each TDS was removed from the acetate buffer and placed in a scintillation vial containing 5 ml diethyl ether to disassemble the TDS. The vials were left uncovered at room temperature in a hood. After complete evaporation of ether, the residual ACV in the TDS was extracted with 10 ml 0.1 *N* NaOH at 80°C for 8 hr. The ACV extract was filtered through a 0.45-μm Gelman Acrodisc membrane filter. An aliquot of the filtrate was neutralized with an equal volume of 0.1 *N* HCl and then diluted with deionized water before injection into a HPLC column for ACV analysis.

In Vivo Antiviral Efficacy Studies with ACV Delivered from the TDS

The *in vivo* animal experiments have been described previously (8). In the previous work ACV treatment with the TDS was started immediately after virus inoculation. The present research investigates the effects of delaying the initiation of the TDS (ACV) treatment. Tables IA and IB are the timetables for execution of the different time-delayed ACV treatments. In part A of the *in vivo* experiments, treatment with ACV was delayed for either 1, 2, or 3 days after the virus inoculation. In part B, ACV treatment was carried out for 1, 2, or 3 days following a 2-day delay period after virus inoculation. For control, the TDS's containing only Carbopol gel were applied to animals of the placebo group in each experimental run.

Inoculation of virus followed the method of Lieberman *et al.* (3). Details were given in the previous publication (8).

For ACV treatment, the skin area dorsal to the virus inoculation site and in the predicted path of lesion development was chosen as the application site for the TDS (see Fig. 1). The distance from the center of the TDS application site to that of the virus inoculation site was about 1.5 cm. To enhance the permeation of ACV through the skin, the designated TDS application site was pretreated with 25 mg Azone (8). The gauze sponge (1.5×1.5 cm) containing Azone was firmly attached to the skin by strips of OpSite Incise drape (Smith and Nephew Medical Limited, Hull, England) and Dermiform hypoallergenic knitted tape (Johnson & Johnson, New Brunswick, NJ). After application for 24 hr, the Azone patch was removed and the TDS was immediately applied and held in place in the same manner as de-

Table IA. Timetable for Conducting *in Vivo* Experiments with Different Time-Delayed ACV Treatments

Treatment	Day −1	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
0-day-delayed[a]	Pretreatment with 25 mg Azone for 24 hr	Inoculation of virus & application of TDS					Removal of TDS & observation of results
1-day-delayed		Inoculation of virus & pretreatment with 25 mg Azone for 24 hr	Application of extra 10 mg Azone & application of TDS				Removal of TDS & observation of results
2-day-delayed		Inoculation of virus	Pretreatment with 25 mg Azone for 24 hr	Application of TDS			Removal of TDS & observation of results
3-day-delayed		Inoculation of virus		Pretreatment with 25 mg Azone for 24 hr	Application of TDS		Removal of TDS & observation of results

[a] Experiments carried out in the previous study (8).

scribed above. Following findings from our previous study (8), an extra 10 mg Azone was applied prior to the attachment of the TDS in the animals with a 1-day delayed treatment where the ACV therapy was intended for 4 consecutive days, this to maintain an elevated skin permeability for ACV throughout the experiment.

Observations were made 5 days after inoculation in all experiments. Lesion developments were categorized and used for quantification of antiviral efficacy. The TDS's removed from the experimental groups were extracted for residual ACV as described earlier in the *in vitro* experiments. The survival time of each animal was continuously monitored.

Categorization of Lesion Development and Measurement of Antiviral Efficacy

The lesion development in hairless mice infected cutaneously with HSV-1 can be classified into five categories: "not reach (NR)," "stop (St)," "jump (J)," "through (Th)," and "miss (M)." These categories have been clearly illustrated and described in detail in our previous work (8). Briefly, they represent the cases where the lesion development does not reach, stops at the edge of, jumps over, passes through, and misses entirely the TDS-covered skin area, respectively. These lesion categories, except for M, are indications of the effectiveness of ACV delivered systemically and/or topically in curtailing virus replication and virus-induced skin lesion.

Based on these lesion categories, the two antiviral efficacies may be defined as

$$\text{topical efficacy (\%)} = \frac{N_{St} + N_J + N_{NR}}{N_{St} + N_J + N_{Th} + N_{NR}} \times 100\% \quad (1)$$

$$\text{systemic efficacy (\%)} = \frac{N_{NR}}{N_{St} + N_J + N_{Th} + N_M + N_{NR}} \times 100\% \quad (2)$$

where N_{St}, N_J, N_{NR}, N_{Th}, and N_M are the numbers of animals corresponding to each of the five lesion categories in each experimental group.

Table IB. Timetable for Conducting *in Vivo* Experiments of a 2-Day-Delayed ACV Treatment with the TDS Applied for Different Time Periods

TDS application time	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
1 day	Inoculation of virus	Pretreatment with 25 mg Azone for 24 hr	Application of TDS	Removal of TDS		Observation of results
2 days	Inoculation of virus	Pretreatment with 25 mg Azone for 24 hr	Application of TDS		Removal of TDS	Observation of results
3 days	Inoculation of virus	Pretreatment with 25 mg Azone for 24 hr	Application of TDS			Removal of TDS & observation of results

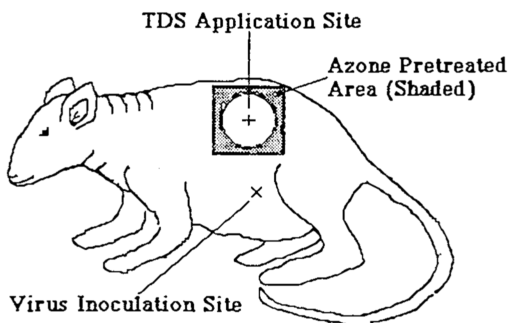

Fig. 1. A schematic illustration of the spatial relationship between the TDS application site and the virus inoculation site in the lumbar skin area of a hairless mouse.

Equation (1) is a modification of the previous expression used (8) for topical efficacy. Here we have incorporated N_{NR} in both the numerator and the denominator because we consider an NR to mean that therapeutic levels of ACV were likely achieved locally as well.

Relationships Used in Data Analysis

To calculate the accumulated amount (Q_n) of ACV released from a TDS during an *in vitro* experiment, the following equation was used:

$$Q_n = V_R \cdot C_n + \sum_{i=1}^{n-1} V_S \cdot C_i \tag{3}$$

where C_n is the ACV concentration of the *n*th sample last taken from the receiver solution, C_i is the ACV concentration of the *i*th sample taken from the receiver solution, and V_R and V_S are the volumes of the receiver solution and the sample, respectively.

By plotting the accumulated amount of ACV released against time, the steady-state ACV flux ($\Delta Q_n/\Delta t$) can easily be derived from the linear portion of the plot. The ACV permeability coefficient for the rate-controlling membrane (P_M) can then be calculated, assuming sink conditions, from Eq. (4):

$$P_M = \frac{\Delta Q_n/\Delta t}{A \cdot C_D} \tag{4}$$

where A is the effective area (0.636 cm^2) of the rate-controlling membrane for ACV diffusion and C_D is the solution ACV concentration in the TDS. Since ACV suspension was employed to fill the TDS in this study, a C_D value equivalent to the solubility of ACV in water at 37°C (2.5 mg/ml) was used (8).

In *in vivo* experiments where the rate-controlling membrane is in series with the Azone-pretreated skin, the total permeability coefficient (P_T) of ACV through this series is described by

$$\frac{1}{P_T} = \frac{1}{P_M} + \frac{1}{P_S} \tag{5}$$

where P_S is the permeability coefficient for ACV through the Azone-pretreated skin. This value has been determined previously (8) in hairless mice to be 1.1×10^{-5} cm/sec. The calculated P_M values in this study are of the order of 10^{-7}–10^{-6} cm/sec and are thus much smaller than the P_S value. Therefore, $P_T \approx P_M$ and the ACV flux is nearly entirely controlled by its rate of diffusion across the rate-controlling membrane. The theoretical amounts of ACV released ($A_{rel,\, theo}$) from the TDS over the experimental period (t) are then estimated for the *in vivo* experiments according to the following:

$$A_{rel,\, theo} = P_T \cdot A \cdot C_D \cdot t \tag{6}$$

where Eq. (5) is used for P_T, Eq. (4) for P_M, and $P_S = 1.1 \times 10^{-5}$ cm/sec.

To validate our current method for residual ACV extraction, mass balance was checked with the TDS's in *in vitro* experiments. We may write the following relationship:

$$F = \frac{A_{rel}}{A_{ld} - A_{ext}} \tag{7}$$

where A_{rel} is the amount of ACV released from the TDS over the experimental period and is equivalent to Q_n of Eq. (3), A_{ld} and A_{ext} are the amounts of ACV loaded in and extracted from the TDS, respectively, and F is a unitless ratio. F values obtained from the *in vitro* experiments were used as correction factors to estimate the actual amounts of ACV released from the TDS in the corresponding *in vivo* experiments ($A_{rel,\, exp}$) according to Eq. (8):

$$A_{rel,\, exp} = (A_{ld} - A_{ext}) \cdot F \tag{8}$$

Statistical Analysis

Student's *t* test was used in this study to compare the difference between two means.

RESULTS AND DISCUSSION

Validation of the Extraction Method Based on Mass Balance

One of the aims of this study was to improve the procedure currently in use to extract residual ACV from the TDS so that mass balance could be claimed for all the experiments including those conducted with low ACV flux. Table II presents the results of mass balance check with the TDS's from all *in vitro* experiments. The calculated F values are, in general, within 10% errors from the predicted value of 1.00. This indicates a good mass balance in all the TDS's regardless of the amounts of ACV loaded. Since consistent F values were obtained throughout this study, they may be used later to correct for the actual amount of ACV released in the *in vivo* experiment.

ACV Release from the TDS in *in Vivo* Experiments

Table III summarizes the experimental results of ACV release from the TDS ($A_{rel,\, exp}$) in *in vivo* experiments with different time-delayed treatments and these data are compared to the theoretical predictions ($A_{rel,\, theo}$). Because of the large number of experiments, these results provide a clear picture for the *in vitro* ($A_{rel,\, theo}$) versus *in vivo* ($A_{rel,\, exp}$) relationship over the entire range of interest. The shorter the time of TDS application (e.g., 2 days), the closer the

Table II. Mass Balance Check with the TDS's from All *in Vitro* Experiments

Expt group	TDS No.	A_{ld} (μg)	A_{ext} (μg)	$A_{ld} - A_{ext}$ (μg)	A_{rel} (μg)	F[a]	Mean $F \pm$ SD
1-1	1	982	400	582	652	1.12	
	2	977	392	585	620	1.06	1.08 ± 0.04
	3	1011	389	622	652	1.05	
1-2	1	666	379	287	302	1.05	
	2	637	378	258	258	1.00	1.06 ± 0.06
	3	682	443	239	268	1.12	
2-1	1	596	388	208	237	1.14	
	2	598	380	218	241	1.11	1.09 ± 0.06
	3	597	371	226	233	1.03	
2-2	1	929	476	453	480	1.06	
	2	1050	505	545	576	1.06	1.09 ± 0.05
	3	911	472	439	504	1.15	
3-1	1	437	336	101	106	1.05	
	2	422	318	104	90	0.87	0.95 ± 0.09
	3	434	333	101	95	0.94	
3-2	1	2729	658	2071	2227	1.08	
	2	2735	378	2357	2328	0.99	1.03 ± 0.04
	3	2700	445	2255	2303	1.02	
4-1	1	498	301	197	187	0.95	
	2	458	303	155	168	1.08	1.01 ± 0.07
	3	501	287	214	214	1.00	
4-2	1	1383	207	1176	1163	0.99	
	2	1282	238	1044	1012	0.97	1.00 ± 0.04
	3	1281	352	929	966	1.04	
5	1	2748	251	2497	2650	1.06	1.06 ± 0.01
	2	2490	192	2298	2418	1.05	
6	1	679	457	222	233	1.05	
	2	653	408	245	239	0.98	0.98 ± 0.07
	3	667	392	275	253	0.92	

[a] Calculated according to Eq. (7).

agreement between *in vivo* and *in vitro* results. For the longest-duration experiments (i.e., 1-day delay and 4-day TDS application), the *in vivo* value is only 50 to 65% of the *in vitro*. These findings are consistent with the experiments in an earlier (8) limited study. The difference was attributed partially to a sharp drop of the skin permeability coefficient occurring on the fifth day after the termination of the Azone treatment.

Table IV presents another set of experiments which essentially point out the same conclusion. Here all experiments were carried out with a 2-day delay and the ACV TDS's were removed after 1, 2, or 3 days. It is seen that the $A_{rel,\ exp}$ agreed well with $A_{rel,\ theo}$ only for the 1-day application of the TDS. For the 2- and 3-day TDS application cases, $A_{rel,\ exp}$ values were only 65 to 80% of $A_{rel,\ theo}$ (see Fig. 2), in agreement with the experiments reported in Table III.

Table III. ACV Release from the TDS in *in Vivo* Experiments with Delayed Treatments

Expt group	Delayed Treatment	$10^7 \times P_M$ (cm/sec)	$10^5 \times P_S$ (cm/sec)	$10^7 \times P_T$ (cm/sec)	$A_{rel,\ theo}$ (μg)	n	A_{ld} (μg)	A_{ext} (μg)	$A_{ld} - A_{ext}$ (μg)	F	$A_{rel,\ exp}$ (μg)
1-1	1 day	11.8	1.1	10.7	586/4 days	10	994 ± 32[a]	714 ± 42[a]	280 ± 31[a]	1.08	302 ± 33/4 days[a]
1-2	1 day	5.2	1.1	5.0	272/4 days	8	638 ± 15	469 ± 47	168 ± 43	1.06	178 ± 45/4 days
2-1	2 days	4.5	1.1	4.3	179/3 days	9	615 ± 17	528 ± 33	87 ± 32	1.09	95 ± 35/3 days
2-2	2 days	9.4	1.1	8.6	356/3 days	9	912 ± 39	736 ± 57	176 ± 52	1.09	191 ± 57/3 days
3-1	2 days	1.8	1.1	1.8	74/3 days	10	436 ± 18	389 ± 22	48 ± 14	0.95	45 ± 13/3 days
3-2	2 days	40.9	1.1	29.8	1229/3 days	10	2743 ± 104	1963 ± 149	780 ± 113	1.03	804 ± 117/3 days
4-1	3 days	3.3	1.1	3.2	88/2 days	10	494 ± 16	414 ± 45	80 ± 39	1.01	81 ± 39/2 days
4-2	3 days	18.0	1.1	15.5	426/2 days	10	1341 ± 46	919 ± 124	421 ± 114	1.00	421 ± 113/2 days

[a] Values reported as mean ± SD.

Table IV. ACV Release from the TDS Applied for Different Time Periods in *in Vivo* Experiments with 2-Day-Delayed Treatments

Expt group	Application time of TDS	$10^7 \times P_M$ (cm/sec)	$10^5 \times P_S$ (cm/sec)	$10^7 \times P_T$ (cm/sec)	$A_{rel, theo}$ (μg)	n	A_{ld} (μg)	A_{ext} (μg)	$A_{ld} - A_{ext}$ (μg)	F	$A_{rel, exp}$ (μg)
5-1	1 day	50.2	1.1	34.5	474/1 day	8	2526 ± 165[a]	2068 ± 145[a]	458 ± 96[a]	1.06	484 ± 101/1 day[a]
5-2	2 days	50.2	1.1	34.5	947/2 days	8	2503 ± 110	1918 ± 225	586 ± 210	1.06	619 ± 221/2 days
5-3	3 days	50.2	1.1	34.5	1421/3 days	8	2553 ± 86	1691 ± 293	863 ± 267	1.06	912 ± 283/3 days
6-1	1 day	4.4	1.1	4.2	58/1 day	10	690 ± 28	630 ± 30	60 ± 15	0.98	59 ± 15/1 day
6-2	2 days	4.4	1.1	4.2	115/2 days	10	678 ± 12	578 ± 23	101 ± 18	0.98	100 ± 18/2 days
6-3	3 days	4.4	1.1	4.2	173/3 days	10	687 ± 16	559 ± 39	128 ± 30	0.98	125 ± 30/3 days

[a] Values reported as mean ± SD.

We hesitate to speculate on the probable causes of the differences between the *in vivo* and the *in vitro* results after more than 1 day of TDS application. However, it is worthwhile to note that, although the *average* rates of drug delivery diminished with time after Day 1, many *individual* TDS's maintained high fluxes during Day 2 and Day 3. For example, 5 of 18 TDS's with a 2-day application time (1 from Expt Group 5 and 4 from Expt Group 6) and 3 of 18 TDS's with a 3-day application time (1 from Expt Group 5 and 2 from Expt Group 6) had nearly perfect ACV release performance predicted from theory. This suggests that, if a combination of conditions remains favorable (e.g., good Azone pretreatment and good contact of TDS with site of drug absorption), constant ACV delivery could be sustained for 3 to 4 days.

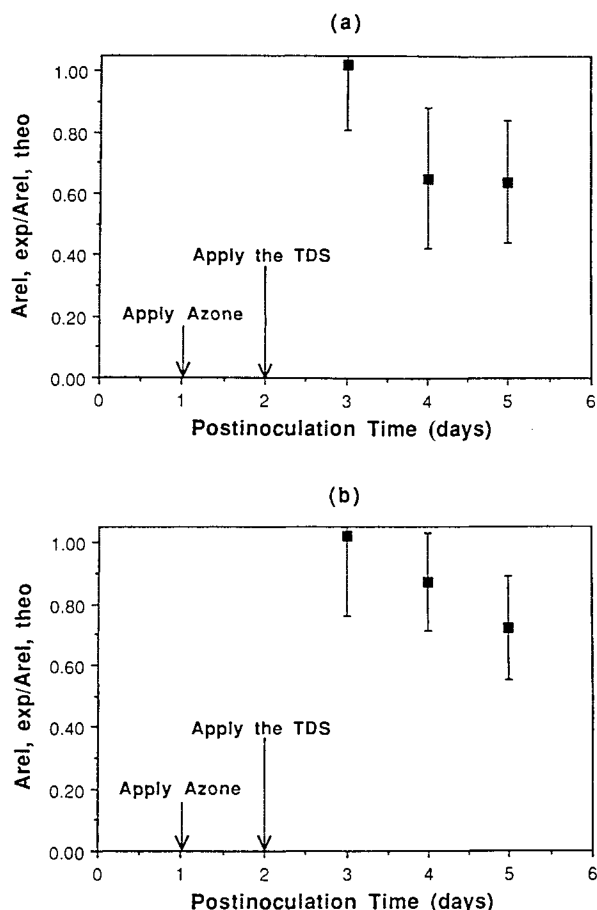

Fig. 2. A time-dependent change in the ratio of $A_{rel, exp}/A_{rel, theo}$ following the application of TDS on the skin area pretreated with 25 mg Azone for 24 hr: results obtained (a) from experimental group 5 and (b) from experimental group 6.

In Vivo Antiviral Efficacy

Table V presents the results of antiviral efficacy calculated based on the numbers of animals corresponding to each of the five lesion categories in each experimental group from *in vivo* studies conducted with different time-delayed ACV treatments. Lesion "scoring" was performed on Day 5 postinoculation; this choice is based on our previous (8) experience that 5 days represented an optimum when a fully developed lesion (25 mm or longer) could be identified in all the control animals while the mortality rates of the animals were still low. Included in Table V for comparison are the results of placebo groups in each experimental run. As can be seen, all animals in the placebo groups developed a Th lesion category 5 days after virus inoculation.

To illustrate clearly the relationship between the antiviral efficacy and the amount of ACV released and to appreciate better the differences and similarities in the antiviral effectiveness between the different time-delayed ACV treatments, both the topical and the systemic efficacies are plotted in Fig. 3 as a function of the experimental flux.

It is clear from Fig. 3 (each data point represents the calculated antiviral efficacy based on a group of 8–10 animals) that the topical efficacy curve from the 1- and 2-day-delayed treatments is displaced by about one log unit in terms of required flux from the 3-day-delayed treatment. In comparison to the previously published 0-day-delayed treatment data (8), the topical efficacy of the 1- and 2-day-delayed treatment is not inferior to the no-delay treatment. Further, there is a strong relationship between ACV flux and topical efficacy. At low ACV flux from 10 to 100 μg/cm^2-day, the topical efficacy increases with increasing flux; when the ACV flux is 100 μg/cm^2-day or greater, the topical efficacy reaches a maximum of 100%. However, when the ACV treatment was delayed for 3 days, the topical efficacy appeared to be much poorer than that of 0-day-delayed treatments. This outcome is reasonable considering that we have observed in several cases in the control animals fully developed skin lesions (25 mm or longer) by Day 3.5 postinoculation and ACV therapy initiated on Day 3 could likely be too

Table V. Antiviral Efficacies in *in Vivo* Experiments with Delayed ACV Treatments

Expt group	Delayed treatment	ACV flux, theo (μg/cm²-day)	ACV flux, exp (μg/cm²-day)	*n*	No. of animals with lesion category of St	J	Th	NR	M	Topical efficacy (%)	Systemic efficacy (%)
1-0	1 day	Placebo	Placebo	9	0	0	9	0	0	0	0
1-1		231	119	10	6	2	0	2	0	100	20
1-2		107	70	8	3	3	1	1	0	88	13
2-0	2 days	Placebo	Placebo	6	0	0	6	0	0	0	0
2-1		94	50	9	0	5	2	2	0	78	22
2-2		187	100	9	1	6	0	2	0	100	22
3-0	2 days	Placebo	Placebo	10	0	0	10	0	0	0	0
3-1		39	24	10	0	3	6	1	0	40	10
3-2		644	421	10	0	1	0	9	0	100	90
4-0	3 days	Placebo	Placebo	10	0	0	10	0	0	0	0
4-1		69	64	10	0	1	9	0	0	10	0
4-2		334	331	10	1	2	1	4	2	88	40

late to curtail the further development of a skin lesion, especially when the ACV flux is low.

The systemic efficacy results support the view (see Fig. 3) that higher fluxes are required to treat cutaneous herpes infections via the systemic route in hairless mice than for treatment via the direct topical route. These data indicate that in topical therapy, as opposed to systemic therapy, there would be an ACV concentration gradient from the epidermis toward the dermis at the site of lesion development; therefore, lower ACV fluxes would suffice to maintain a steady-state ACV concentration in the epidermis that would be sufficiently high to inhibit virus replication *in vivo*. In systemic therapy, higher fluxes would generally be needed to achieve the same steady-state epidermis ACV levels, as high steady-state systemic blood levels would be required to equilibrate adequately the target epidermis from the systemic blood compartment.

The results of antiviral efficacy calculated for the 2-day-delayed ACV treatments with the TDS's applied for different time periods (1 to 3 days) are presented in Table VI and in Fig. 4 as a function of the experimental flux. Included in Fig. 4 for comparison are the topical efficacies (solid line) obtained earlier (Fig. 3) for the 1- and 2-day-delayed treatments. As expected for a 2-day-delayed treatment, the data points obtained for the TDS's applied for 3 days are superimposable with the solid line. When the TDS application times were less than 3 days, the performances of the TDS's were poorer. Even at very high fluxes (480 μg/cm²-day), the treatment results were poor for a 1-day TDS application time.

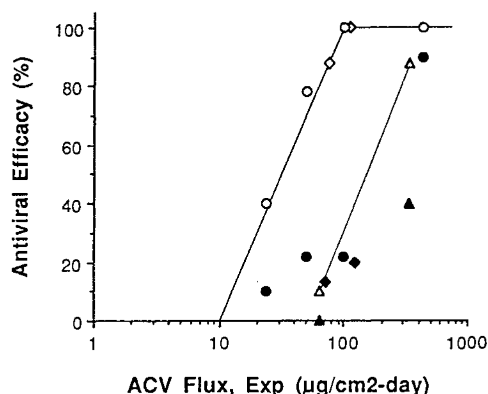

Fig. 3. The topical (open symbols) and the systemic (filled symbols) antiviral efficacy presented as a function of the experimental ACV flux in *in vivo* experiments with 1-day (◇, ◆)-, 2-day (○, ●)-, and 3-day (△, ▲)-delayed ACV treatments. Each data point represents the calculated antiviral efficacy based on a group of 8–10 animals.

The Mean Survival Time of Animals

In addition to the lesion development, the survival time of each animal was also monitored and used as an indication whether different ACV treatments would result in different antiviral activities. In Fig. 5, the survival time of each animal from the 2-day-delayed, 3-day ACV treatments (which was the protocol for the majority of the experiments in the present study) is plotted as a function of the experimental flux. The data points in Fig. 5 are scattered over a wide range of ACV fluxes. However, upon close examination, they seem to fall into one of the three groups: (1) those of the placebo group, (2) those with a low ACV flux (10–125 μg/cm²-day), and (3) those with a high ACV flux (350–500 μg/cm²-day). Group 2 corresponds to a region in Fig. 3 where the topical efficacy is sensitive to the ACV flux; group 3 is in the region where the topical efficacy is not sensitive to the ACV flux but where systemic efficacy is sensitive to the flux. It was therefore decided to conduct a statistical analysis on the mean survival times of the animals in these three groups using the pooled data points. The calculated mean survival times are 6.6 ± 0.9 days (n = 29), 7.0 ± 1.0 days (n = 36), and 8.3 ± 1.1 days (n = 14) for the placebo group, the low-flux group, and the high-flux group, respectively. A one-tailed t test shows that the mean survival time of the low-flux group is not significantly ($P < 0.1$) higher than that of the placebo group. However, the mean survival time of the high-flux group is significantly ($P < 0.0005$) higher than that of the placebo group and that of the low-flux group. Similar conclusions can be drawn for the 1- and 3-day-delayed treatments and they are in general agreement with the findings of

Table VI. Antiviral Efficacies in *in Vivo* Experiments with 2-Day-Delayed ACV Treatments Using the TDS for Different Application Times

Expt group	Application time of TDS	ACV flux, theo (μg/cm²-day)	ACV flux, exp (μg/cm²-day)	n	No. of animals with lesion category of St	J	Th	NR	M	Topical efficacy (%)	Systemic efficacy (%)
5-0	3 days	Placebo	Placebo	7	0	0	7	0	0	0	0
5-1	1 day	745	761	8	2	0	4	1	1	43	13
5-2	2 days	745	487	8	1	0	0	7	0	100	88
5-3	3 days	745	478	8	0	0	0	8	0	100	100
6-0	3 days	Placebo	Placebo	6	0	0	6	0	0	0	0
6-1	1 day	91	93	9	0	0	7	2	0	22	22
6-2	2 days	91	79	9	1	0	5	3	0	44	33
6-3	3 days	91	66	10	2	5	1	2	0	90	20

our previous study (8) with 0-day-delayed treatments. Table VII summarizes the mean survival times of the three groups for these studies.

As noted in Fig. 5, most of the animals in the placebo group died on Day 6 or Day 7 postinoculation in the present study. This translates into a mean survival time of 6.6 ± 0.8 days for all the control animals ($n = 48$), a value very close to the previously (8) reported 6.5 days. With a low-ACV flux treatment (10–125 μg/cm²-day), the animals did not seem to live longer than those in the placebo group, and they all had the same mean survival time regardless of how many days the therapy was delayed. On the other hand, with a high-ACV flux treatment (350–500 μg/cm²-day), (a) the animals had significantly prolonged mean survival times compared to the other two groups, and (b) most importantly, this increase in the mean survival time seemed to decrease with an extension in the time delay for ACV treatment. The meaning and the significance of these findings are discussed below in relation to the pathogenesis of HSV-1 infections.

Some of the animals which received a high ACV flux in the 0-day-delayed treatments eventually recovered from virus infections and survived for a very long time. Four of the animals from the experimental groups with a 2-day-delayed ACV treatment in the present study also escaped from the fatal outcome of cutaneous HSV-1 infections. These animals are excluded from the above statistical analysis.

In the experiments on 2-day-delayed ACV treatments with the TDS's applied for different time periods, a general conclusion cannot be reached from the results (Table VIII), i.e., whether a high ACV flux has a beneficial effect on the mean survival time of the animals over a low ACV flux and a placebo, whether a 3-day TDS application is better than a 2-day application, and whether the latter is better than a 1-day TDS application in improving the life span of the animals. This is probably due to both the marginality of the effects and the very small sample size in each of the test groups.

Pathogenesis of HSV-1 Infections and Its Implications on ACV Therapy

To understand better the topical and systemic antiviral efficacy and the prolongation of the mean survival times upon ACV therapy using a TDS, it would be necessary to

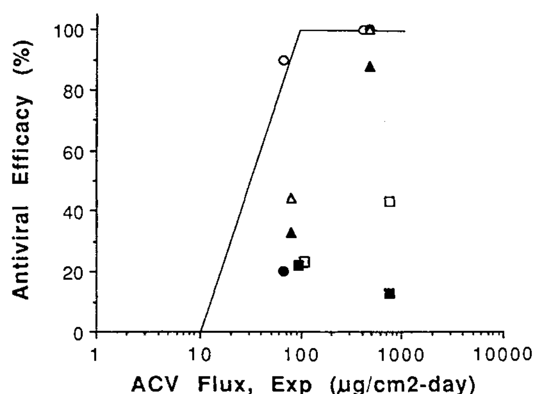

Fig. 4. The topical (open symbols) and the systemic (filled symbols) antiviral efficacy presented as a function of the experimental ACV flux in *in vivo* experiments of a 2-day-delayed treatment with the TDS applied for 1 day (□, ■), 2 days (△, ▲), or 3 days (○, ●) following the initiation of an ACV therapy. The solid line represents the topical efficacy of 1- and 2-day-delayed treatments from Fig. 3. Each data point represents the calculated antiviral efficacy based on a group of 8–10 animals.

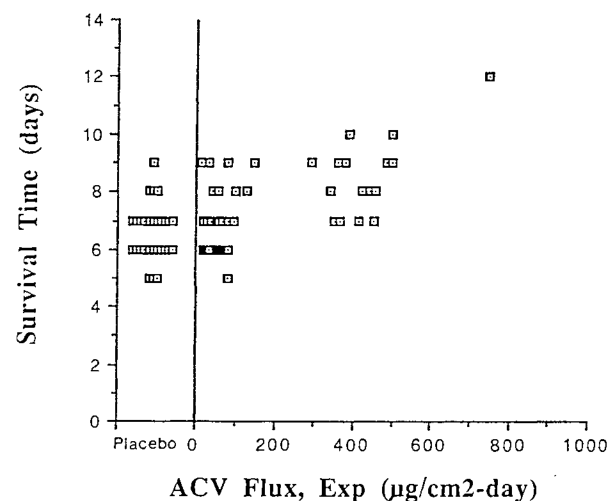

Fig. 5. The survival time of each individual animal plotted as a function of the experimental ACV flux in *in vivo* experiments with a 2-day-delayed 3-day treatment. Included for comparison are the survival times of the animals in the corresponding placebo groups.

Table VII. The Mean Survival Times of the Animals in the Placebo, the Low-Flux, and the High-Flux Groups in *in Vivo* Experiments with Different Time-Delayed ACV Treatments

Treatment	Group		
	Placebo	Low flux (10–125 μg/cm²-day)	High flux (350–500 μg/cm²-day)
0-day-delayed[a]	6.5 ± SD (n = 34)	6.8 ± SD (n = 47)	9.2 ± SD (n = 30)
1-day-delayed	6.2 ± 0.4 (n = 9)	6.8 ± 0.8 (n = 17)	—[b]
2-day-delayed	6.6 ± 0.9 (n = 29)	7.0 ± 1.0 (n = 36)	8.3 ± 1.1 (n = 14)
3-day-delayed	6.9 ± 0.6 (n = 10)	6.9 ± 0.7 (n = 10)	7.7 ± 1.6 (n = 9)

[a] Results from the previous (8) study.
[b] No data point falls in the high-flux region.

have a full grasp of the sequence of pathological events that take place once the animals are acutely infected with HSV-1. The pathogenesis of HSV infections both in humans and in animals has been the focus of several review articles (12,15–20). The following discussion is based on the consensus of viewpoints from different researchers.

HSV-1 is a neurotropic virus which infects principally the neuroectodermal tissues including the skin, the peripheral nerves, and the central nerve system (CNS). When the viruses are inoculated in the skin, they first replicate at the inoculation site to increase the local virus titers which are necessary for effective exposure of nerve endings. Therefore, the size of the virus inoculum used to infect the animal should have a definite effect on the time required for virus titer to increase to its effective level. In this study, a virus titer of 1.35×10^8 PFU/ml was used. This number is higher than those used by other researchers (3,7,21–23) and may in part contribute to a more severe lesion development, a higher mortality rate, and a shorter mean survival time in the animals of the present study.

Following their seeding at the nerve terminals, the viruses preferentially penetrate and migrate in the axon to the sensory ganglion where they colonize. Controversy exists regarding whether the viruses can use alternate routes, such as the systemic circulation, to reach the ganglion. If the infection does not spread further to the spinal cord and the brain stem, the animal may survive and become a carrier of a latent viral infection. However, if the infection disseminates into the CNS and causes severe neurological complications, such as encephalitis, the animal develops paralysis and eventually dies. These events take place very quickly. Earlier studies (12,21–23) have shown that free viruses are detectable in the lumbosacral ganglia by the second day postinoculation of HSV at the footpads of the mice; the viruses could also be isolated from the spinal cord 2 days after inoculation (21,22), and they were present in the brain by the seventh day (23).

In the present study, the hairless mice in the placebo groups developed no pathological symptoms for the first 3 days postinoculation. As reported earlier, a fully developed skin lesion (25 mm or longer) could be observed in some of the control animals as early as 3.5 days postinoculation and was surely seen in all the placebo groups by the end of Day 5. These results are consistent with the earlier findings of Lieberman *et al.* (3). The animals subsequently became flaccid on Day 6, and most of them would die on the same day or 1 day later.

Treatments with topical ACV preparations (1 and 5% gels and ointments) have been shown (7) capable of preventing the lesion development and death from HSV-1 infections of the lumbosacral skin area in hairless mice. Delay in the initiation of ACV treatment from 3 to 24 and, further, to 48 hr postinoculation was characterized by a growing average lesion score and a higher latent infection frequency in the test animals (7). The results of this earlier study (7) strongly suggested that topical treatment with ACV during the acute phase of HSV-1 infections in the hairless mice prevented the invasion and the subsequent colonization of virus in the sensory ganglia by halting the continuous virus supply from the site of the primary infection. Topical treatment with ACV had little or no effect on the virus which had already invaded the ganglia (7,12).

In the present study, the TDS loaded with ACV was applied at a skin area away from the virus inoculation site for the purposes of easy lesion scoring and of clear differentiation between the topical and the systemic antiviral efficacy. However, this practice denied a chance for direct action of ACV on its intended target site. Therefore, the ACV released from the TDS must travel through systemic circulation to the primary infection site to prevent the virus from building up its local titer. This is believed to be manifested as the systemic efficacy. It is then expected that treatment with a high ACV flux should result in a higher systemic efficacy than treatment with a low ACV flux. The results of systemic efficacy in Fig. 3 seemed to indicate the presence of such a trend. It is further expected that an earlier initiation in the ACV therapy should bring about a higher systemic efficacy than a delayed treatment. Such a clear-cut conclusion could not be reached based on the same results of systemic efficacy in Fig. 3 due to the scatter in the data points. Nevertheless, the results of mean survival time in Table VII substantiated our predictions on the systemic efficacy of topical ACV treatments in relation to the drug flux and the initiation time of drug therapy. Clearly, when the animals were treated

Table VIII. The Mean Survival Times of the Animals in the Placebo, the Low-Flux, and the High-Flux Groups in *in Vivo* Experiments of a 2-Day-Delayed ACV Treatment with the TDS Applied for Different Time Periods

TDS application time	Group		
	Placebo	Low flux (10–125 μg/cm²-day)	High flux (350–500 μg/cm²-day)
1 day	—	7.2 ± 1.2 (n = 9)	7.0 ± 0.5 (n = 8)
2 days	—	7.9 ± 2.0 (n = 9)	8.4 ± 0.5 (n = 7)
3 days	6.4 ± 0.9 (n = 13)	7.0 ± 1.0 (n = 9)	8.3 ± 2.0 (n = 6)

with a high ACV flux, they lived significantly longer than those treated with a low ACV flux and those untreated. Furthermore, in the case where the animals received a high ACV flux, the mean survival time was the longest (9.2 days) if the treatment was initiated without any delay following the virus infection. The mean survival time dropped to 8.3 days when the treatment was delayed for 2 days and to 7.7 days when the treatment was delayed for 3 days. These findings are consistent with the viewpoint that ACV delivered topically exerts its systemic antiviral effect by inhibition of the active viral replication at the primary infection site. In contrast, the mean survival time for the animals which received a low ACV flux was not significantly higher than that of the control animals and did not change with a time delay in the treatment. This is probably because the ACV in systemic circulation never reach its therapeutical level in these animals.

The ACV released from the TDS can act locally to prevent the evolution of skin lesion. In this regard, the TDS with a high ACV flux (100 $\mu g/cm^2$-day or higher) were capable of halting the further development of a skin lesion and demonstrated the same 100% topical efficacy for the 0-, 1-, and 2-day-delayed treatments, even though the systemic efficacy might be significantly different in these three cases. On the other hand, a flux-dependent increase in the topical efficacy was observed for the TDS with a low ACV flux (10–100 $\mu g/cm^2$-day) and the data points for the 1- and 2-day-delayed treatments were found to be superimposable and no different from those for the 0-day-delayed treatment; this is not surprising when we realize that the ACV in the systemic circulation may be far below therapeutic levels for these three cases, and the prevention of lesion development is then solely dependent on the local ACV concentration. In the case of 3-day-delayed treatments, the topical efficacy was lower than in the other three cases, especially with a low ACV flux. This is probably because the skin lesion has already evolved and, in some animals, is nearly fully developed 3 days after infection.

Once the ACV treatment was terminated, the NR, St, and J lesion categories might develop into a Th category within days, depending on the surviving virus titer and the residual amount of ACV remaining in the animals. Eventually, all these animals which received ACV treatment delayed for 1 day or longer after infection were expected to die, as did the control animals.

CONCLUSIONS

The present study demonstrates the validity of a novel method involving the use of a (trans)dermal drug delivery system to describe quantitatively the relationship between the antiviral efficacy and the acyclovir (ACV) flux. The results obtained clearly show that how soon the topical treatment is initiated after virus inoculation, the duration of the treatment, and the dermal ACV flux during treatment may be related to the effectiveness of the therapy in HSV-1 cutaneous infections. Also, the present approach provides a means to assess the local effectiveness of topical drug therapy versus systemic efficacy.

It is believed that the method described in the present study should be valuable in (a) the screening of new antiviral agents for topical treatment of cutaneous herpes virus infections and (b) the optimization of drug delivery systems (topical formulations).

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