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RECURRENT HERPES SIMPLEX IN MICE; TOPICAL TREATMENT WITH ACYCLOVIR CREAM

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If applied frequently enough, topical treatment of the skin of latently infected mice with 2% acyclovir (ACV) cream had a significant ($P < 0.05$) prophylactic effect on the incidence of recurrent herpes simplex. Treatment of existing recurrent disease with 2% or 5% ACV cream shortened the time taken to healing of skin lesions, but did not eliminate latent infection in the ganglia.

acyclovir (topical) herpes simplex recurrence

INTRODUCTION

Acyclovir [9-(2-hydroxyethoxymethyl)guanine; ACV] has proved to be a selective, potent drug in the treatment of primary herpes simplex virus (HSV) infections when given systemically [1, 5, 9, 11, 13] or topically [1, 5, 9, 11, 12, 14, 15].

We have recently reported the efficacy of systemic treatment with ACV in preventing recurrent herpes simplex in mice [3]. We now report the results obtained with topical treatment of recurrent herpes simplex with ACV.

MATERIAL AND METHODS

Infection of mice

Four-week-old female outbred Swiss white mice were injected subcutaneously (s.c.) in the skin of the right ear with 3×10^5 plaque-forming units (p.f.u.) HSV-1 strain scl6 [7].

Drugs

Two preparations of acyclovir were supplied by Dr. P. Collins (Wellcome Research Laboratories, Beckenham, U.K.), one containing 2% of the drug, and the second 5% (Zovirax®), both in modified aqueous cream base.

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Administration of the drug

With a plastic spatula, about 4 mg of placebo cream or cream with drug was gently rubbed onto the right ear of mice. In all experiments, the drug and placebo were coded by a third party.

Induction of recurrent disease

Recurrent lesions were induced in the skin of the ear by stripping with cellophane tape [8].

Assessment of severity of clinical recurrent herpes simplex

After stripping, the ears were examined daily by naked eye for erythema, recurrent disease was defined by the criteria of Hill et al. [8], and in some experiments the severity of erythema was scored from 0 to +++. A score of + was recorded when the skin of the right ear was clearly more pink than that of the left. A +++ score implied that the right ear was bright red. In other experiments, mice were anaesthetised daily so that their ears could be examined under a dissecting microscope at ×6 magnification. The position and nature of individual lesions were recorded, and to avoid observer bias, examinations were made without access to the previous days' records. The scoring was four points per vesicle, three per pustule, two per early scabbed lesion, and one per late scabbed lesion or ulcer.

Isolation of virus from cervical ganglia

Mice were killed by intraperitoneal injection of pentobarbitone and the second, third and fourth cervical ganglia were removed, cultivated for 4 days in 0.5 ml growth medium (199 with 5% foetal calf serum), then ground. Two 50 μl samples were added to Vero cells grown in multi-dishes (Sterilin Ltd.). Cultures were examined for cytopathic effect (CPE) after 2 days.

Test for effect on hypersensitivity to HSV

The test was performed as described previously [4].

RESULTS

Definition of recurrent lesions using microscopy

Previously we used erythema as a convenient sign of recurrent disease. This correlated well with the reappearance of infectious virus in the skin after stripping [8], but was not a sufficiently quantitative measure of the severity, extent or progression of the disease to evaluate effects of treatment in detail. After stripping, the skin of 8-week-old uninfected mice usually appeared roughened when examined microscopically and there was often slight scabbing in the centre of the ear. These signs usually disappeared within 2 days.

The ears of a group of 77 mice infected 9–12 weeks previously were stripped with cellophane tape and observed daily for erythema. The expected incidence of latency in such mice would be 80–90% (Blyth, W.A., Harbour, D.A. and Hill, T.J., unpublished results). When new erythema developed, the ears were examined microscopically for herpetic lesions. Of the 29 mice which developed such erythema, 16 showed vesicles 3–8 days after stripping. In seven mice these were seen for 1 day only; in the remaining nine, vesicles were seen for 2 days. In three further animals, although vesicles were not recorded, pustules which progressed to scabbed lesions were seen. Erythema was often seen 1–5 days before vesicles and usually lasted until healing was almost complete. Thirteen of the 15 mice that had erythema sufficient to be defined as recurrent disease by the criteria of Hill et al. [8] also had vesicles or pustules. In the other two, even though the skin was red and swollen, vesicles did not develop. In six further mice, such lesions appeared even though the erythema did not meet these criteria. The mean number of each type of lesion per mouse recorded on each day is shown in Fig. 1A. The mean lesion score for the group is shown in Fig. 2A, and the percent mice with lesions is given in Fig. 3A.

Vesicles appeared as discrete raised domes, 0.3–0.5 mm in diameter, often in an erythematous area; sometimes they occurred in clumps so that they coalesced. Their number varied greatly (from 1 to 30) and by the day after their appearance they became yellow and often less domed and sometimes umbilicated. This appearance resulted from infiltration of polymorphonuclear leucocytes (D.M. Altmann, personal communication), so that they were described as pustules. By the following day, scabbing usually occurred. Ulcers were often seen where scabs had become detached before healing was complete. In nine of the 16 mice that showed vesicles, two successive crops of lesions were seen, usually on consecutive days. In these 16 mice, the skin became normal by microscopic examination on average 4.3 days after the vesicles were first seen, and in 11 of them the skin was normal by 8 days after stripping.

Effect of application of ACV cream on healing after stripping ears with cellophane tape

Two percent ACV cream was applied to the right ear of five uninfected 8-week-old mice twice daily for 3 days, starting treatment immediately after stripping the ear. All mice were normal by 2 days after stripping, although there was immediate, short-term erythema after each application of cream. Two percent ACV cream or placebo cream was also applied to the stripped ears of eight uninfected mice 6 times daily at 3 h intervals. The ears of a further similar group of animals were stripped and left without further treatment. Difficulties due to the short-term erythema were avoided by examining animals before the first treatment of the day. There was a slight increase in the duration and severity of erythema in mice treated with placebo cream compared to control mice, but this increase was less marked in mice treated with ACV cream. However, in neither case was the erythema severe enough to influence assessment of clinical recurrences by the criteria of Hill et al. [8]. On histological examination, the thickness of the epidermal

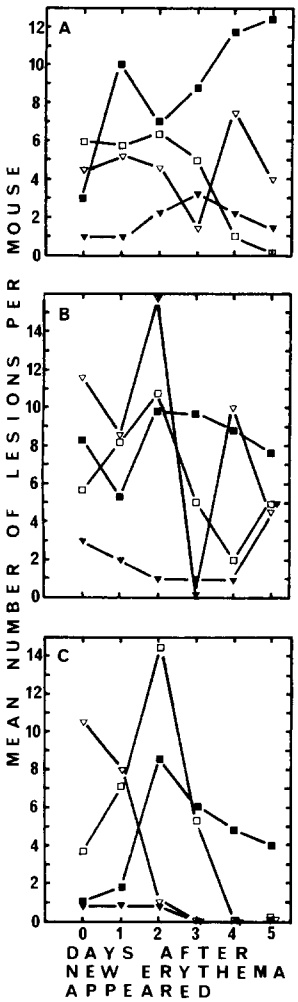

Fig. 1. Effect of topical treatment with ACV on existing recurrent herpes simplex: number of lesions. Placebo or cream with drug was applied to the ear 6 times daily from the day when new erythema appeared. A) Untreated. B) Placebo. C) 5% ACV. Vesicles (▽), pustules (□), scabbed lesions (■), ulcers (▼).

layer was increased by stripping [8] and further increased by treatment with ACV or placebo cream. Mast cells were particularly evident in the ears of mice treated with placebo cream.

Effect of treatment with ACV or placebo cream on hypersensitivity to HSV

Application of ACV cream to the ear 6 times daily, starting 2 h before s.c. injection of heat-killed HSV, reduced the thickening produced in the ears of latently infected

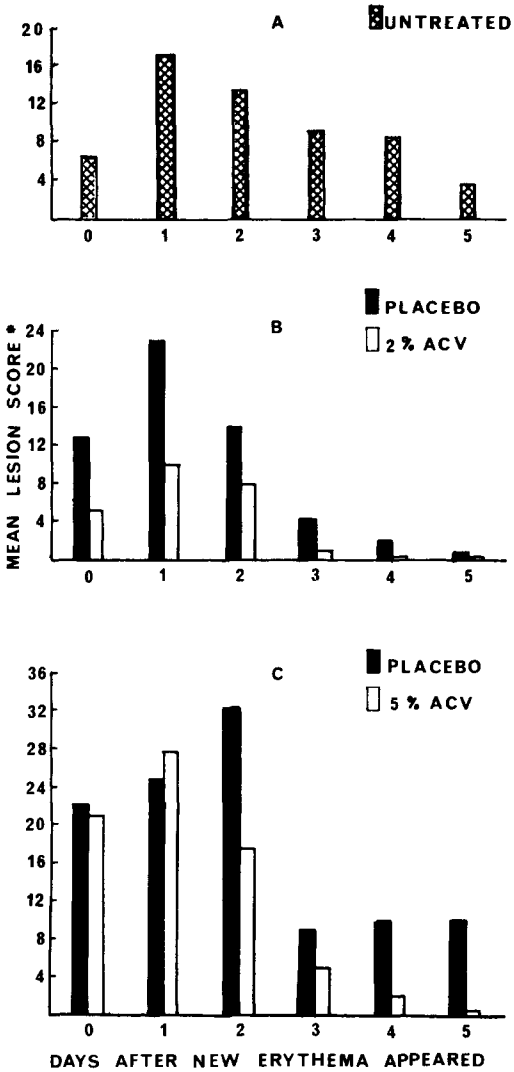

Fig. 2. Effect of topical treatment with ACV on existing herpes simplex: severity of disease. Placebo or cream with drug was applied to the ear 6 times daily from the day when new erythema appeared. * Four points per vesicle, three per pustule, two per early scabbed lesion, one per late scabbed lesion or ulcer.

mice [4] by 30% and slightly increased the erythema. Mice treated with placebo had slightly increased erythema, but the ear thickness was not affected.

Effect of treatment with placebo cream on unstripped mice

Since the placebo cream caused inflammation when applied to the skin of the mouse ear, it might itself stimulate recurrent disease. Therefore, a group of latently infected

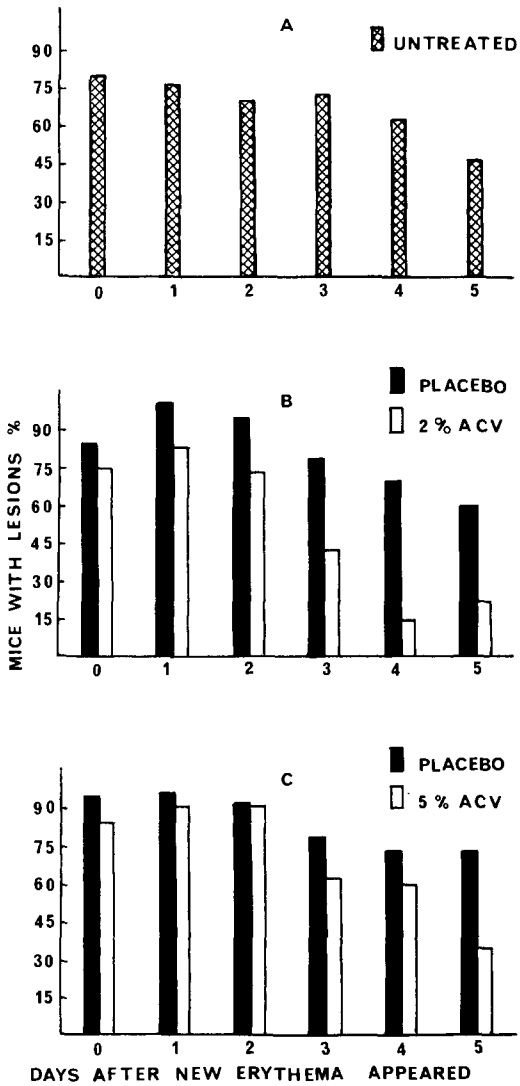

Fig. 3. Effect of topical treatment with ACV on existing recurrent herpes simplex: persistence of lesions. Placebo or cream with drug was applied to the ear 6 times daily from the day when new erythema appeared.

mice was treated 6 times daily for 5 days with placebo cream. A second, control group was handled in the same way in order to stress them similarly, but their ears were not treated. Both groups were examined daily by the naked eye for erythema. One of the 30 control mice developed recurrent disease, a figure similar to the incidence of spontaneous recurrent erythema [8]. Eight of the 25 mice treated with placebo (32%) developed recurrent disease, which started on average 4.5 days after the first treatment and lasted on average 2.8 days.

Effect of topical treatment with 2% ACV cream on development of recurrent herpes simplex

In the first experiment, ACV or placebo cream was applied twice daily, at 9 a.m. and 5 p.m., for a total of 6 days commencing 1 day before the right ears of the mice were stripped with cellophane tape. In the second experiment, ACV or placebo cream was applied 6 times per day, at 3 h intervals (6:30 a.m. to 9:30 p.m.) for 6 days. These mice also were stripped with cellophane tape on the second day of treatment. Recurrent disease was defined by the development of erythema [8].

Of 31 mice that received placebo cream twice daily, 15 (48%) developed recurrent clinical disease, and the same percentage developed disease in the group of 27 mice that were treated with placebo cream 6 times a day (Table 1). In mice that received ACV cream twice daily, there was virtually the same incidence of recurrent disease as in their control group, although the mean duration of such disease was shortened from 4.5 days in the control group to 3.1 days. Of the 24 mice treated with ACV cream 6 times daily, only four (17%) developed recurrent clinical disease and the mean duration of erythema was reduced from 3.6 days in the control group to 2.25 days. In the mice treated 6 times daily, the reduction in incidence of recurrent disease in the group treated with ACV as compared with controls was significant ($P < 0.05$) by χ^2 test.

TABLE 1

Effect of topical treatment with ACV on clinical recurrence of herpes simplex in mice stripped with cellophane tape

Treatment	No. with recurrent disease[a] / No. mice stripped	Duration of erythema in days (mean ± S.D.)
Placebo cream base twice daily for 6 days (9 a.m. and 5 p.m.)	15/31 (48%)	4.50 ± 2.87
2% ACV cream twice daily as above	13/32 (41%)	3.08 ± 1.26
Placebo cream base every 3 h for 6 days from 6:30 a.m. to 9:30 p.m.	13/27 (48%)	3.57 ± 1.22
2% ACV cream every 3 h as above	4/24 (17%)[b]	2.25 ± 1.89

[a] As judged by erythema [8].

[b] χ^2 : $P < 0.05$ compared to placebo.

Effect of topical treatment with 2% ACV cream on existing recurrent disease

The ears of latently infected mice were stripped with cellophane tape and observed daily for erythema. When new erythema developed, the mice were randomly assigned to groups and thereafter received placebo cream or ACV cream. Treatment was commenced at 9 a.m. on the day of admission to the groups, and was repeated every 3 h until 12 midnight for 6 days. Mice were examined daily by the naked eye for erythema, and by microscope for specific lesions.

Of the 32 mice treated with placebo cream, 22 (69%) developed vesicles. A further two animals had pustules, though vesicles were never seen. Of 29 mice treated with ACV cream 11 (38%) showed vesicles. In an additional nine animals, vesicles were not seen, but lesions at later stages – pustules or early scabbed lesions – were recorded.

In mice that developed vesicles the average time before the skin became normal by microscopic examination was 4.4 days for mice treated with placebo cream and 2.6 days for mice treated with ACV cream. There was no correlation between the number of vesicles and the time taken for lesions to disappear.

The mice in the two groups showed different patterns of development of disease (Figs. 2B, 3B). However, the group chosen for treatment with ACV had fewer specific lesions when they were first examined microscopically (on the day after entry to the experiment) than the group treated with placebo cream. No vesicles were present in the ACV group after the 2nd day of treatment, whereas mice treated with placebo were not free of vesicles until the 4th day. In addition, the mice treated with ACV had fewer ulcers than those in the other two groups, and scabs formed and disappeared more rapidly. The skin of the ear of only six of the 29 (21%) mice treated with ACV had lesions by microscopic examination by the 6th day of treatment, whereas this proportion was 19/32 (59%) in mice treated with placebo cream (Fig. 3B).

Twenty-three of the mice treated with placebo that had developed recurrent disease on the first occasion of stripping were again stripped 6 weeks later. Five had a further, less severe episode of recurrent disease. The mean number of vesicles was 5.5 instead of 10.4 and on average the skin of the ear became normal in 2.5 days compared with the 3 days taken previously. The erythema was sufficiently severe to be judged as a clinical recurrence by the criteria of Hill et al. [8] in only two animals, whereas previously this had occurred in all five.

Effect of topical treatment with 5% ACV cream on existing recurrent disease

Mice were stripped and taken into the experiment when new erythema first appeared in the skin of the ear. Before animals were assigned to treatment groups, their ears were examined microscopically so that, as far as possible, the animals in each group were equivalent in terms of the nature and numbers of lesions (Fig. 2C). Mice were treated with either ACV or placebo cream for 6 days, 6 times per day at 3 h intervals, and their ears were examined daily by microscope.

In the group of 32 mice treated with placebo, 21 (66%) developed vesicles. In three others pustules but no vesicles were seen. In the 29 animals treated with ACV, 19 (66%) developed vesicles, and one showed pustules without vesicles. In each group, the average time from the beginning of treatment to complete healing (by microscopical examination) was 3.4 days.

Although there was no difference in the time to complete healing, the disease progressed differently in the two groups (Figs. 1B, C, 2C, and 3C). One mouse in the ACV group had a single vesicle on the 3rd day of treatment compared with seven mice (with a total of 11 vesicles) in the placebo group. In addition, five mice in the placebo group developed vesicles on the 5th and 6th days of treatment, whereas none were seen in the ACV group after the 3rd day. The mean lesion score of mice treated with ACV cream was less than that for mice given placebo from the 2nd day of treatment onwards (Fig. 2C). By White's ranking test [17] this difference was significant ($P < 0.01$) on the 5th day. By the 6th day the skin was abnormal by microscopic examination in only 10 of the 29 (34%) mice treated with ACV compared to 23 of 32 (72%) mice treated with placebo (Fig. 3C). There was little difference between the groups with regard to erythema.

From the group originally treated with ACV, 23 mice which had shown recurrent disease were again stripped 9 weeks later. Six of them had a further recurrence and HSV was isolated from the ear of five of them. Also in this experiment, 28 mice were stripped that had shown recurrent disease while in the group treated with placebo. Of these, seven had a further recurrence and HSV was isolated from the ear of five of them.

Isolation of virus from the cervical ganglia

Eleven weeks after being treated with 5% ACV or placebo cream, mice were killed. The second and third cervical ganglia were removed and tested for the presence of latent virus. Of 23 mice from the ACV treatment group 17 yielded virus (74%). Virus was isolated from 21 of 28 (75%) mice from the group treated with placebo.

DISCUSSION

We have shown that in the mouse systemic treatment with acyclovir almost completely prevents recurrent herpes simplex when the drug is given daily from the day before the reactivating stimulus [3]. However, for clinical use in humans it is likely that the drug will be administered topically only after early symptoms of recurrent herpes simplex have developed. Therefore we have tested two formulations of cream, containing 2% or 5% ACV, the former for its ability to prevent clinical recurrent herpes simplex, and both for their effects on existing vesicles.

The cream used as a vehicle for ACV itself caused short-term inflammation in the skin of the mouse. This inflammation may have played a part in the induction of recurrent disease that followed application of placebo cream alone, since many stimuli that cause inflammation in the skin also induce recurrent disease [2, 8]. Inclusion of ACV

decreased the inflammation caused by the placebo cream and also the severity of the delayed hypersensitivity reaction of HSV in the skin of latently infected mice. In human skin, mild erythema is induced by the placebo cream only under most severe conditions of test (P. Collins, personal communication) so that this should present no problem.

Application of 2% ACV cream twice daily, even when treatment was started before lesions were obvious, did not reduce the incidence of recurrent disease. However, the duration of erythema was shortened. Application of the cream 6 times a day reduced the incidence and mean duration of recurrent disease. It is possible that self-dosage by a patient would be even more effective, since it is likely that the cream would be more efficiently rubbed into the skin than was possible in the mice, and that the cream could be applied more frequently. Had the mice licked the whole of the cream from their ears when given six doses daily they could have ingested 1.2 mg per day of ACV. (This was 80% of a dose in the drinking water that affected, but did not completely cure primary infection (T.J. Hill, W.A. Blyth and D.A. Harbour, unpublished observations).) However, since animals were not seen to wash their ears after application of the cream, such ingestion was considered unlikely.

Experiments to test the effect of ACV on existing lesions required more detailed definition of recurrent lesions than that used previously [8]. From this it became clear that recurrent herpes in mouse skin closely resembled the lesion in humans. The one or more crops of vesicles were each short-lived and became pustules which progressed to scabs, and within 5–6 days the lesions healed. Observation of specific lesions confirmed the value of erythema as an indicator of recurrent disease in that the presence of erythema sufficient to be defined as recurrent disease correlated well with the presence of specific lesions. However, the incidence of recurrent disease was defined most accurately when both specific lesions and erythema were taken into account.

In the experiments on the effects of treatment on existing disease, mice were first taken into treatment groups on the day that new erythema first appeared, but even in animals that received ACV more had vesicles on the 2nd day of treatment than at the beginning. This suggests that under these experimental conditions ACV cannot immediately prevent formation of vesicles. Perhaps more frequent application of drug would be more effective, but if a certain level of tissue damage occurs as a result of virus replication, vesicle development might be inevitable. When the effect of 2% ACV cream was tested, the treated ears became normal by microscopical examination almost 2 days before untreated mice or mice treated with placebo. In addition, erythema lasted for a shorter time. Mice treated with 5% ACV cream did not develop vesicles on the 5th or 6th day of treatment, whereas vesicles did appear at this time in a few mice treated with placebo.

When the ganglia of mice treated with 5% ACV or placebo for 6 days were tested for latent virus 11 weeks after treatment, there was no difference between the two groups in the proportion of mice from which virus was isolated. This result is similar to those obtained with systemic treatment with ACV [3, 6, 10, 16]. This might argue that between recurrences of clinical disease the virus is in a non-replicating state [6], but it could equally mean that only a part of the pool of latent virus induces lytic infection at any one time.

Thus, in summary, when treatment is begun before the induction of recurrent lesions, ACV can decrease the incidence of disease. If ACV therapy is started when vesicles have developed, new lesions rarely appear after the 2nd day of treatment. Thus, healing times were shortened, which confirms data reported by others [1, 9, 11, 15] who used ACV cream in primary disease.

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