Trauma to the Skin Causes Recurrence of Herpes Simplex in the Mouse

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SUMMARY

Mild trauma was induced in the skin of mice latently infected with herpes simplex virus type I by stripping the originally infected ear with cellophane tape. Recurrent herpes simplex developed at this site 2 to 5 days later. It was detected clinically by the development of erythema and vesicles and by the appearance of virus in the skin. On any one occasion about 30% of mice showed reactivated disease and increasing the severity of trauma did not increase this proportion. However the majority of animals developed reactivated disease on some occasions when stripping was repeated at monthly intervals. The results are discussed in relation to the skin trigger theory of reactivation of herpes simplex.

INTRODUCTION

Progress towards an understanding of the mechanisms underlying reactivation of herpes simplex has been hampered by lack of an animal model analogous to recurrent disease in man. A serious limitation of the mouse footpad model (Stevens & Cook, 1971) is that latently infected animals do not suffer recurrent clinical disease either spontaneously or after stimulation (Stevens & Cook, 1973). This difficulty was overcome by use of the guinea pig footpad (Scriba, 1975) and the mouse ear (Hill et al. 1975) in both of which clinical disease recurs spontaneously. We have since used ultraviolet (u.v.) light or prostaglandin E2 (PGE2) to reactivate the disease (Blyth et al. 1976). However, u.v. light is an unsatisfactory stimulus since the erythema produced by the irradiation masks that which might result from reactivated infection. Consequently, definition of reactivation on clinical grounds was not possible.

We now report the use of mild trauma as a stimulus to reactivation. This allows the detection of reactivated disease by observation of clinical signs.

METHODS

Infection of the mouse ear. Four-week-old female Swiss white mice were injected subcutaneously in the right pinna with $6 \times 10^4$ p.f.u. of HSV-1 strain SC16 (Hill et al. 1975). Only mice that showed definite erythema or paralysis of the ear were used for experiments on reactivation.

Cellophane tape stripping. The adhesive side of cellophane tape was applied to the ear and gently removed. This was repeated as required, a fresh area of tape being used each time (Hennings & Elgjo, 1970).

Isolation of virus from the ear. Mice were killed by cervical dislocation. The skin was
Isolation of virus from cervical ganglia. Mice were killed by injecting sodium pentobarbital onto intraperitoneally and the second, third and fourth cervical ganglia were removed as quickly as possible. They were washed in growth medium (Hill et al. 1975) and were cultured with Vero cells in multi-dishes (Linbro Chemical Co. Inc.).

Electron and light microscopy. Pieces of skin were fixed in 2.5% gluteraldehyde in cacodylate buffer at 4 °C, postfixed in 1% osmium tetroxide, and embedded in Araldite. Thin sections for electron microscopy were stained with lead citrate and uranyl acetate and thick sections (approx. 1 μm) for light microscopy were stained with methylene blue.

RESULTS

Effects of stripping the ears of normal mice with cellophane tape

When cellophane tape was lightly pressed on to the upper surface of the pinna and peeled away a layer of hairs and keratinized epithelial cells adhered to the tape; this was repeated with each application and removal. The degree of damage to the skin could thus be varied, one application produced little if any visible alteration but after twelve applications tissue exudate appeared on the surface and the skin took more than 6 days to heal. In most experiments the tape was applied six times to the upper surface of the ear. This removed most of the hairs and left the skin smooth, and shiny, but unbroken. Within a few minutes of
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Fig. 2. Mitotic figures in the epidermis after stripping the ear with cellophane tape. ○—○, Stripped surface; ●—●, unstripped surface of stripped ear. Each point represents the mean of counts in a 2 mm length of epidermis from each of 4 ears. ▲—▲, Mean of 4 counts from each side of 4 unstripped ears.

stripping, erythema developed in all animals but within 10 h it disappeared from 60% of them. After 24 h only 20% showed erythema. By the second day this proportion was 6% and by the third it was 2% (Fig. 1).

Sections of the ear taken immediately after stripping showed that almost all the fully keratinized cells had been removed but no other tissue damage was apparent. Sections taken 2 to 4 days after stripping showed epidermal hyperplasia and replacement of the keratinized cells but no infiltration by leucocytes.

The number of mitotic figures in the epithelium was measured to follow the course of healing. For this experiment the upper surface of one ear of each of a group of mice was stripped six times. At daily intervals four mice were infected intraperitoneally with 0.15 mg of colchicine. They were killed 4 h later and a strip of skin was taken from the centre of each ear. Mitotic figures were counted in a 2 mm length of the epithelium of each side of the ear (Fig. 2). In the stripped tissue the number was increased 2 days after stripping and returned approximately to normal after 7 days.

Effects of stripping the ears of latently infected mice

At least 4 weeks elapsed between infection and stripping the ears and mice whose right ears were then erythematous were excluded from the experiment. Of 2286 mice examined in this respect, 81 (3.5%) were discarded which gives an estimate of the incidence of spontaneous reactivation of clinical disease on any occasion between 5 and 8 weeks after primary infection (Hill et al. 1975). In preliminary experiments the upper surfaces of the originally infected ears of groups, usually of 15 mice, were stripped six times and the mice were examined daily for signs of reactivated disease. In some animals the reaction to stripping was the same as that in uninfected mice but in about 15% of latently infected animals the erythema lasted for much longer (on average for 5.5 days) than in control groups (Fig. 1). In addition, in a further proportion of animals (again on average 15%) new
Fig. 3. Epidermal/dermal border from recurrent lesion seen 4 days after stripping. N, nucleus of keratinized epidermal cell showing margination of chromatin and virus capsids; P, polymorphonuclear leucocyte adjacent to basement membrane of epidermis; M, macrophage; arrow, enveloped virions between epidermal cells, shown at higher magnification in inset.

Erythema developed after the skin had regained a normal appearance following stripping, a reaction that was never seen in control animals (Fig. 1). This new erythema was often first seen 2 to 4 days after stripping as one or more discrete red patches; later these sometimes merged to affect the whole ear. This erythema lasted on average 3.5 days. At a 6× magnification, large raised vesicles, either singly or in clumps, were frequently seen in the area of erythema. In some instances areas of exudate or scabs were seen. Sections of ear tissue containing vesicles or ulcerated areas showed changes similar to those of spontaneously recurring lesions (Hill et al. 1975) namely, infiltration of the dermis and epidermis with neutrophils, lymphocytes and macrophages. In epidermal cells there was morphological evidence of productive virus replication (Fig. 3).

For use in later experiments the following criteria were drawn up to define clinical reactivation. If erythema after stripping was continuous, it should last for longer than 3 days (a time by which more than 98% of uninfected animals were normal). Alternatively, if erythema appeared after a period of normality, it should remain for at least 2 consecutive days. Erythema rarely developed more than 7 days after stripping and since it might not then have arisen as a result of stripping, such observations were excluded from experimental results. By these criteria, in a series of experiments, 28 of 128 animals (22%) showed reactivated disease after the ears were stripped. In individual groups the incidence of reactivation varied from 2 of 30 animals (7%) to 10 of 31 (32%). In most experiments
Skin trauma and recurrent herpes simpex

Table 1. Incidence of recurrent clinical disease in 63 mice stripped on 4 occasions at monthly intervals

<table>
<thead>
<tr>
<th>Frequency of recurrence</th>
<th>Never</th>
<th>Once</th>
<th>Twice</th>
<th>3 times</th>
<th>4 times</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observed number of mice</td>
<td>21</td>
<td>24</td>
<td>11</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Expected number*</td>
<td>20</td>
<td>29</td>
<td>16</td>
<td>4</td>
<td>0.5</td>
</tr>
</tbody>
</table>

* Calculated from the binomial expansion of \((p + q)^4\) where \(p\) is the probability of recurrent clinical disease.

Table 2. Isolation of HSV from stripped ears

<table>
<thead>
<tr>
<th>Days after stripping</th>
<th>Not stripped</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus isolated*</td>
<td>0</td>
<td>0</td>
<td>7</td>
<td>3</td>
<td>16</td>
<td>9</td>
</tr>
<tr>
<td>Total</td>
<td>54</td>
<td>30</td>
<td>59</td>
<td>61</td>
<td>60</td>
<td>32</td>
</tr>
<tr>
<td>%</td>
<td>0</td>
<td>0</td>
<td>12</td>
<td>5</td>
<td>27</td>
<td>28</td>
</tr>
</tbody>
</table>

* Accumulated results from several experiments.

stimulation was 5 to 8 weeks after infection but in one where the interval was 14 weeks, 5 of 30 mice (17%) showed reactivation.

Alterations in the number of applications of the cellophane tape altered the results very little. Three applications (which caused virtually no recognizable trauma) induced reactivation in 10 of 64 mice (16%). After 12 applications healing took too long for reactivation to be recognized clinically (see below for results of virus isolation). Since the effect of six applications did not interfere with observation, this treatment was used for the next series of experiments.

The fact that only a proportion of mice suffered recurrent disease after stripping might suggest that this group was especially susceptible to recurrence. This possibility was investigated by stripping the right ears of a group of mice on four occasions at monthly intervals. After each stripping the ears were observed for erythema. Of the 63 mice, 21 (33%) never showed recurrent disease, whereas 2 mice showed erythema on every occasion of stripping (Table 1). When the frequency of reactivation was compared with that expected for a random incidence, no difference could be detected statistically between the two series (by \(\chi^2\) test \(P > 0.05\)). Thus there was no evidence that some mice were particularly prone to reactivation.

Isolation of HSV from ears after stripping

The right ears of groups of latently infected mice were stripped. At daily intervals, groups, usually of 15 mice, were killed, and attempts were made to isolate HSV from the skin of the stripped ear. Without stripping, or on the first day after stripping, virus was not found but it was isolated from a proportion of the animals on subsequent days (Table 2). Since this proportion reached a peak 4 and 5 days after stripping, the incidence of virus isolation at these times was compared with that of clinical disease. Clinical reactivation occurred in 55 of the 179 mice (31%). HSV was isolated from 40 of these 55 (73%) but from only 3 of the 124 mice (2.5%) that did not show reactivated disease.

In these experiments mice often showed erythema some days before they were due to be tested so that the probability of virus isolation might have been decreased. Indeed the design
Table 3. *Isolation of HSV from the ears of mice which showed prolonged or recurrent erythema after being stripped with cellophane tape*

<table>
<thead>
<tr>
<th></th>
<th>Number of mice</th>
<th>Virus isolated of mice tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>First stripping</td>
<td>55</td>
<td>7/13</td>
</tr>
<tr>
<td>2nd stripping</td>
<td>49</td>
<td>7/11</td>
</tr>
<tr>
<td>3rd stripping</td>
<td>23</td>
<td>2/4</td>
</tr>
<tr>
<td>4th stripping</td>
<td>9</td>
<td>1/1</td>
</tr>
<tr>
<td>Total (percentage)</td>
<td>17/29</td>
<td>(59)</td>
</tr>
</tbody>
</table>

of the experiments precluded comparison of the time of onset of erythema with that of isolation of virus. In an experiment to test this, a group of latently infected mice was stripped repeatedly at monthly intervals. Either 3 days after stripping (when erythema was present continuously) or when erythema developed after a period of normality, mice were killed and attempts were made to isolate virus from the ear (Table 3). Of the 55 mice at the start of the experiment, 29 showed clinical signs of reactivated disease and HSV was isolated from 17 (59%) of them.

In all experiments described so far, only the upper surface of the pinna was stripped. However, in primary HSV infection the virus could be isolated from both sides of the ear when these were harvested separately. Indeed no differences were found in titres of virus in either side of the ear when titrations were done 3 days after infecting the ear with $6 \times 10^4$ p.f.u. of virus. The possibility therefore existed that more reactivations would be induced by stripping both surfaces of the ear but this treatment caused too much trauma for recurrent disease to be diagnosed clinically. Therefore virus isolation was attempted 4 days after stripping the top surface, the bottom surface or both. HSV was isolated from only 3 of 30 animals when the bottom surface was stripped compared with 8 of 30 after stripping the top surface and 9 of 30 after stripping both surfaces. Similarly, the incidence of virus isolation was not increased by stripping the upper surface of the ear 12 times; after such treatment virus was isolated from 8 of 31 mice (21%). In a further experiment stripping the bottom surface alone did not induce clinical reactivated disease in a group of 27 mice.

*Isolation of HSV from cultured ganglia*

Of 14 mice that had suffered clinical disease during primary infection, 7 were shown to harbour virus in the 2nd, 3rd or 4th cervical ganglia 5 to 6 weeks after infection. By contrast, HSV was isolated from only one of 20 animals of a matched group that had not originally had clinical disease. For this reason only mice that had shown clinical signs during primary infection were used in experiments on reactivation.

Mice from the experiments where clinical examination for recurrent disease was made after stripping on four occasions (Table 1) were also tested for virus in the cervical ganglia. HSV was recovered from 8 of 14 animals (57%) that had never shown reactivated disease and from 16 of 19 animals (84%) that had shown reactivation more than once.

**DISCUSSION**

The present work provides a simple, reliable method for producing reactivation of clinical herpes simplex in the mouse. A major advantage of the model is the association of easily observable clinical signs, similar to those that occur in man, with the appearance of virus in
the skin. Although only about 30% of animals show recurrent lesions after stripping with cellophane tape, this figure is about tenfold greater than that for spontaneous reactivation. Moreover, the great majority of the lesions develop within 2 to 5 days of stripping so that it is likely that the recurrent disease is induced by the stimulus. Relatively minor stimuli can induce recurrent herpes in humans and in this respect the mild trauma produced by stripping provides a stimulus closer to the situation in humans than do more complex techniques such as immunosuppression (Underwood & Weed, 1974; Hurd & Robinson, 1976) or surgical manipulation of the trigeminal ganglion (Nesburn, et al. 1976).

The proportion of mice which show reactivated disease after stripping is similar to that observed after u.v. irradiation or injection of PGE$_2$ (Blyth et al. 1976), and cannot be increased by inducing more severe trauma. The present experiments show that this 20 to 30% of animals does not represent a special group of mice which is particularly prone to reactivated disease after a stimulus. Rather, the results suggest that recurrent disease can occur in all mice but that in addition to the stimulus, a chance phenomenon is involved in determining whether it develops. This would be explained best by the skin trigger theory (Hill & Blyth, 1976) in which it is suggested that production of recurrent disease depends on the coincidence of virus in the skin with conditions which favour virus multiplication. Support for the theory can also be inferred from two recent reports which indicate that production of a recurrent lesion does not depend only on the presence of virus in the peripheral tissues. By mechanical manipulation of the trigeminal ganglion Nesburn et al. (1976) induced virus shedding in the eyes of 83% of latently infected rabbits even though clinical ocular lesions apparently did not develop. Also, Scriba (1977) found that HSV was present in the footpads of 75 to 93% of latently infected guinea pigs in the absence of clinical lesions.

Furthermore, Hoyt & Billson (1976) recently reported recurrent herpes simplex in man 7 to 10 days after 'blow-out' fractures which severed the nerve supply to the area in which the lesions subsequently developed. This suggests that the lesions arose from virus already present in the skin.

The skin trigger theory also suggests that the conditions which favour virus multiplication in the peripheral tissues are produced by changes induced there by a reactivating stimulus such as u.v. light or trauma. Both of these induce mild inflammation in the skin and many of their physiological effects are common to both, for instance vasodilation, increased vascular permeability, epidermal hyperplasia and the release of various chemical mediators of inflammation. We are now investigating which of these changes are most likely to be involved in affecting virus growth in the skin.

Results of experiments in vitro suggest that PGE$_2$, a potent mediator of inflammation in the skin (Goldyne, 1975), significantly increases the size of HSV plaques in Vero cells (our unpublished results). Preliminary results also suggest that PGE$_2$ can induce reactivation in mice when injected into the site of previous infection (Blyth et al. 1976).

After stripping with cellophane tape, about 10 to 15% of latently infected mice showed erythema in the stripped ear continuously for 4 days or longer. Others appeared normal for 1 or more days before erythema and vesicles developed. HSV was isolated from a proportion of mice 2 to 5 days after stripping but least often on the third day. The early recurrences with continuous erythema might well result from subclinical foci of infection present in the ear tissue before stripping. This interpretation is supported by the fact that without stripping HSV can be isolated from 10% of latently infected mice if the ear tissue is cultivated in vitro (our unpublished results).

The development of later recurrences raises the possibility that, in these animals, virus
arrived in the ear tissue after stripping at a time when conditions still favoured virus multiplication.

In conclusion, the frequency of recurrent herpes simplex is likely to be controlled by conditions in the skin as well as the frequency of virus release from the sensory nerves into the skin. Production of recurrent disease by trauma in the mouse ear appears very closely analogous to the situation in humans and should be useful in studying further the mechanisms of reactivation of the disease.

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REFERENCES


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