Isolation of Herpes Simplex Virus from the Skin of Clinically Normal Mice During Latent Infection

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SUMMARY

Herpes simplex virus (HSV) was isolated from the ears of clinically normal, latently infected mice by culturing the skin in vitro. The results are discussed with reference to current theories of HSV latency.

Herpes simplex virus (HSV) can establish latent infection in the sensory nerve ganglia in man or animals following peripheral infection (Baringer, 1975; Stevens, 1975). Periodically, clinical disease recurs, but the mechanism by which it develops is not known. Two theories have been promoted: in the first (Cook & Stevens, 1973; Merigan, 1974; Klein, 1976) a stimulus to the ganglion causes release of virus, which travels via the nerve to the skin to produce clinical disease. This was called the 'ganglion-trigger' theory by Hill & Blyth (1976) to contrast it from their 'skin-trigger' theory. They suggested that virus is frequently released from the ganglion and produces microfoci of infection in the skin which are usually eliminated. Stimuli acting on the skin can produce conditions favourable for virus growth and clinical disease recurs.

The 'skin-trigger' theory implies that virus can be present at peripheral sites, for example the skin, in clinically normal individuals. Until recently, attempts to demonstrate such virus, either by isolation from homogenized tissues or by organ culture, have been unsuccessful (Findlay & MacCallum, 1940; Rustigian et al. 1966; Wohlenberg et al. 1976). However, Nesburn et al. (1976) reported that, following mechanical stimulation of the trigeminal ganglion, 83% of latently infected rabbits shed virus in the tears, apparently in the absence of clinical lesions, and Scriba (1977) isolated HSV type 2 from organ cultures of clinically normal footpads of latently infected guinea-pigs. Moreover, Hoyt & Billing (1976) reported recurrent herpes simplex in patients with 'blow-out' fractures which had severed the nerve supply to the area of skin involved, so that the lesions must have arisen from virus already present in the skin.

We therefore attempted to isolate HSV from the skin of latently infected mice in the absence of clinical lesions by an explantation technique. Four-week-old female Swiss white mice were injected subcutaneously in the ear with $6 \times 10^4$ p.f.u. HSV-1 strain SC.16. The primary infection resolved within 2 weeks and virus could not thereafter be isolated from disrupted skin (Hill et al. 1975). Four to twelve weeks after primary infection, mice were examined for persisting or recurrent lesions (Hill et al. 1975) and rejected if any were found. The mice were killed by cervical dislocation and the skin of the ear was cultured as follows. The two surfaces of the pinna were separated by a subcutaneous injection of 50 to 100 td Leibowitz L-15 medium and the edge of the pinna (a strip about 1 mm wide) was cut off and discarded. The upper and lower layers of skin could then be peeled apart and were treated separately. The interval between death of the mouse and removal of the ear was about 2 min. The tissue was cut into 2 to 3 mm² fragments and embedded, epidermis outwards, on the surface of drops of molten 1% Noble agar (Difco) at 42 °C in 5 cm diam. Petri dishes. Before addition of the agar, half of the dish was roughened by scratching with a scalpel blade to stop the agar pellet sliding off the surface. After the agar had set, the dish
Table 1. Isolation of HSV from the ears of latently infected but clinically normal mice by culture of skin fragments in vitro

<table>
<thead>
<tr>
<th>Treatment to ear</th>
<th>Virus isolated/no. ears cultured (%)</th>
<th>Mean day ± s.d. on which plaques first seen</th>
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<tbody>
<tr>
<td>None</td>
<td>7/88 (8)</td>
<td>11.8 ± 4.4</td>
</tr>
<tr>
<td>Stripped with cellophane tape not more than 30 min before explantation*</td>
<td>8/85 (9.4)</td>
<td>10.0 ± 4.1</td>
</tr>
<tr>
<td>Stripped with cellophane tape 4 days before explantation</td>
<td>9/31 (29)</td>
<td>5.8 ± 3.8†</td>
</tr>
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</table>

* The ears of 70 mice were removed within 2 min of stripping. Those of 15 mice were removed 30 min after stripping. HSV was isolated from 2 of the 15.
† Compared with 'no treatment', $P < 0.05$; compared with 'stripped within 30 min of explantation', $P > 0.05$ (t-test).

was seeded with about $10^6$ Vero cells suspended in Leibowitz L-15 medium containing 10% foetal calf serum, glutamine (300 mg/ml), penicillin (100 units/ml), streptomycin (100 µg/ml), aerosporin (50 units/ml) and nystatin (25 units/ml). Sufficient medium was added so that the skin fragments were in the surface of the liquid. The cultures were incubated at 35 °C on a rocker oscillating 12 times/h through an arc of 12°. The Vero cells formed a thin monolayer in 24 h. The medium was changed after 1 day and on alternate days thereafter. In histological sections, the skin cultures appeared healthy and showed features similar to those described by Reaven & Cox (1968). In particular, there was evidence of growth and differentiation of the epidermis throughout the 3 weeks of culture. In some cases there was extensive outgrowth of epidermal cells from the margins over the underlying dermis. The Vero cells were examined daily for 2 to 3 weeks for c.p.e. characteristic of HSV. When plaque morphology was not characteristic, cells were subcultured to confirm the result and in two instances the isolated virus was characterized by neutralization with anti-HSV serum.

In some experiments, the ears were stripped with cellophane tape (Hill et al. 1978) six or twelve times on the upper surface, or six times on both surfaces, not more than 30 min before death; in others the ears were not stripped. The ears of a further group of mice were stripped with cellophane tape six times on the upper surface 4 days before the mice were killed (Table 1).

It seems certain that there would be insufficient time between death of the mouse and removal of the ear for virus to reach the skin from the ganglia. Therefore, in the 8% of animals from which virus was isolated without stripping it was already present in the skin. Stripping the skin immediately before death did not significantly increase this amount. If, however, the ears were stripped 4 days before explantation, virus was recovered from 29% of mice, a figure comparable to that found when ear tissue was ground and put on to Vero cell monolayers (Hill et al. 1978).

From disrupted tissue we, like others (Findlay & MacCallum, 1940; Rustigian et al. 1966) have failed to isolate virus from clinically normal skin during latent infection (0/54 mice, Hill et al. 1978; 0/27, our unpublished results). Therefore, either culturing the skin is a more efficient method of isolation, or the virus is present in vivo in a non-infectious state and becomes infectious during culture.

Plaques were first seen on average 4 to 6 days earlier in cultures made 4 days after stripping the skin, when compared with those from unstripped mice or mice stripped immediately before death. This suggests that more virus was present in the skin, or that it multiplied more quickly or spread through the tissue more quickly.
Since latently infected mice occasionally develop recurrent clinical disease spontaneously (Hill et al. 1975), it might be argued that the 8 to 9% from which virus was isolated represent animals about to develop such a lesion. However, observations on 2286 mice indicate that, at most, the incidence of spontaneous recurrences at any one time is approx. 3.5% (Hill et al. 1978) so that it seems likely that of the 8 to 9% of mice with virus in the skin, most would not develop clinical lesions.

Stripping the skin with cellophane tape during latent infection induces recurrent disease in about 30% of animals (Hill et al. 1978). Of these, half show erythema continuously after stripping and it is therefore possible that virus was present in the skin before stripping. In remaining animals, where erythema develops 2 to 5 days after stripping, the virus presumably arrives in the skin during this period. Whether the trauma to the skin affects the rate of supply of the virus, as well as conditions in the skin, has yet to be determined.

The results reported and those of Scriba (1977) show that virus can be present in the skin without associated disease. The ‘skin-trigger’ theory of reactivation (Hill & Blyth, 1976) provides one explanation, but latency in the skin cannot be excluded. However, the skin is unlikely to be the most important site in view of the substantial evidence that the ganglion is essential for continued latent infection (Klein, 1976).

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**REFERENCES**


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