Herpes Simplex Virus Type 2 Latency in the Footpad of Mice: Effect of Acycloguanosine on the Recovery of Virus

By S. A. AL-SAADI,* P. GROSS AND P. WILDY

Department of Pathology, Division of Virology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QP, U.K.

(Accepted 25 October 1987)

SUMMARY

Herpes simplex virus type 2 has been reactivated from the latent state in the footpad and dorsal root ganglia of acycloguanosine-treated BALB/c mice. Virus was also recovered from the footpad tissue but not from the ganglia of denervated, latently infected mice. Treatment in vitro of explanted footpad cultures with acycloguanosine or phosphonoacetic acid did not affect the rate of virus reactivation. In all the isolates examined the virus was found to be acycloguanosine-sensitive. Recovery of virus from footpad tissue of mice after a long period of acycloguanosine treatment supports the theory that virus had been truly latent in the footpad and not in a state of persistent infection.

Primary exposure of individuals to herpes simplex virus (HSV) occurs early in life most often with HSV-1 (Scott et al., 1941). The incidence of primary genital infection with HSV-2 increases when sexual activity commences, and a significant proportion of the adult population has antibodies to HSV-2 (Nahmias et al., 1974). Although HSV has been associated with a wide range of clinical manifestations the disease is usually self-limiting and subclinical in nature. However, the virus can establish lifelong latent infection and may reactivate periodically to initiate a new cycle of herpes lesions. Experimental studies have shown that the latent virus genome resides in neuronal cells (Cook et al., 1974; McLennan & Darby, 1980; Kennedy et al., 1983) and is found in both the sensory and sympathetic ganglia of man and other animals (Bastian et al., 1972; Plummer, 1973; Price et al., 1975; Warren et al., 1978). Virus can not be detected in cell-free homogenates but can be recovered upon cocultivation of the explanted tissue. In man, the natural host of HSV, attempts to recover latent virus from non-neuronal tissue have proved unsuccessful (Rustigian et al., 1966; Smith & McLaren, 1977). However, in experimental animals, the virus has been recovered from the site of inoculation in guinea-pig footpad (FP) and vaginal tissue (Donnenberg et al., 1980; Scriba, 1981). Unlike reactivation of virus in the dorsal root ganglia (DRG), infectious virus could be recovered from FP tissue within 2 to 4 days of explantation. Frequent episodes of recurrent herpes in guinea-pigs have been noticed and the infection can be eradicated with phosphonoacetic acid (PAA) or acycloguanosine (ACG) chemotherapy (discussed by Wildy, 1985). This implies that the virus has established a persistent infection, not the classical latent infection. Hill et al. (1980) have also reported the isolation of HSV-1 from the ear skin of mice in the absence of observable lesions. Recovered virus was considered to have travelled to the skin following reactivation at the cervical sensory ganglia. Despite the above evidence, the view that the neuron cell body is the principal site of virus latency has become so entrenched that the ability of HSV to establish latency in non-neuronal cells has been largely discounted. Nevertheless the recovery of latent HSV from mouse FP tissue was reported by Al-Saadi et al. (1983) using Biozzi mice infected with wild-type virus or temperature-sensitive mutants and this result was reproduced in other strains of mice (C57BL/6J, BALB/c, Pirbright and A mice) by Subak-Sharpe et al. (1983) and Al-Saadi (1984).
Short communication

Table 1. Effect of neurectomy on recovery of HSV-2 from DRG and FP tissue of BALB/c mice

<table>
<thead>
<tr>
<th>Mouse group</th>
<th>DRG (No. positive mice/total no. mice)</th>
<th>FP (No. positive mice/total no. mice)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Homogenized</td>
<td>Incubated</td>
</tr>
<tr>
<td>Control</td>
<td>9/9 (100)</td>
<td>0/9 (0)</td>
</tr>
<tr>
<td>Neurectomy</td>
<td>0/5 (0)</td>
<td>0/5 (0)</td>
</tr>
</tbody>
</table>

*Values in parenthesis are the percentages of virus recovery.

Hence virus recovered from peripheral sites where no neuron cell body is known to exist could (i) be reactivating from the corresponding ganglia and travelling down the nerve fibre to the peripheral site where it can be recovered, (ii) have established a persistent infection with a low level of virus replication or (iii) be existing in a latent state in a non-neuronal cell. To address these possibilities, further studies have been carried out and the ability of ACG to eradicate persistent or reactivating virus both in the DRG and FP tissue has been investigated. The results show that HSV could be reactivated from denervated FP tissue of ACG-treated and untreated mice.

HSV-2 strain HG52 was used to inoculate 3- to 6-week-old female BALB/c mice subcutaneously via the right rear FP. Mice were inoculated with $5 \times 10^6$ p.f.u. in 30 μl of virus suspension. All mice were numbered by ear notch for individual identification. In some experiments the right sciatic and femoral nerves of 3-week-old mice were removed under general anaesthesia (Valium/Hypnorm). Mice were then allowed to recover for 3 weeks before the initiation of infection. Apart from flaccid paralysis of the hind limb the mice appeared normal. Inoculation of mice with $5 \times 10^6$ p.f.u. of virus produced death in 24% (12 of 50) of the control group, whereas there were no deaths (none of 36) in the infected group with the denervated FP. The surviving animals were divided into several groups and supplied with ACG by intraperitoneal (i.p.) injection or in drinking water (see below). Two to 3 months after the primary infection, the animals were killed by an overdose of chloroform anaesthesia and the inoculated FP and corresponding ganglia were explanted and cultured as described previously (Al-Saadi et al., 1983). These cultures were screened for the release of reactivating virus by inoculating the supernatant medium on to a semiconfluent BHK cell layer grown in microtitre plates (Falcon 96-well tissue culture plates). Cell cultures were checked for the presence of characteristic cytopathic effect and virus stock was prepared from each positive culture.

The results in Table 1 show the rate of virus recovery from the DRG and FP tissue of the control and experimental (neurectomy of the sciatic and femoral nerve fibre) groups of mice. As expected, no virus was detected in the DRG of the experimental group but all mice in the control group were found to harbour latent virus in their DRG. HSV was recovered in 40% of denervated FPs and in the 11% of FPs explanted from the control mice. The results suggest that HSV can be recovered from FP tissue in the absence of any virus detectable in the corresponding sensory ganglia. It should be stressed that in these experiments FP tissue was divided into two; one half was homogenized immediately after explantation while the other half was cultured and screened for reactivable virus. Virus was never detected by direct assay of the homogenized footpad tissue.

It is well documented that, unlike the persistent infection which can be eradicated by treatment with ACG or PAA, established latent infection is not sensitive to such drugs (Field et al., 1979; Blyth et al., 1980; Klein et al., 1981). To investigate this further, experiments were carried out on ACG-treated mice. Following infection as described above, mice were divided into three groups (see Table 2). All mice were supplied with ACG in drinking water at a concentration of 1.5 mg/ml; treatment commenced 4 weeks after the initial infection and continued until 8 to 10 weeks later. Fresh ACG-containing water was supplied twice weekly. In addition, mice in groups 2 and 3 received an extra dose of ACG (60 mg/kg/day) administered intraperitoneally every 8 h. The latter schedule of chemotherapy started 10 days before the
Effect of ACG on replication of HSV-2 (HG52) in the footpad of BALB/c mice. Infected mice were supplied with ACG either in the drinking water at a dose of 1.5 mg/ml (△), or by i.p. injection of 60 mg/kg/day administered every 8 h (■). Mice of the control group received no ACG (□). Each point represents the mean of at least four mice at a given time post-infection.

Table 2. Effect of ACG on the reactivation of HSV-2 from FP and sensory ganglia of denervated BALB/c mice

<table>
<thead>
<tr>
<th>Group no. and status</th>
<th>Type of treatment</th>
<th>DRG</th>
<th>FP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (Normal)†</td>
<td>ACG (oral)</td>
<td>6/6 (100)</td>
<td>1/6 (16)</td>
</tr>
<tr>
<td>2 (Normal)</td>
<td>ACG (oral and i.p.)</td>
<td>11/11 (100)</td>
<td>2/11 (18)</td>
</tr>
<tr>
<td>3 (Neurectomy)</td>
<td>ACG (oral and i.p.)</td>
<td>0/10 (0)</td>
<td>3/10 (30)</td>
</tr>
</tbody>
</table>

* Figures in parentheses are the percentages of virus recovery.
† Normal, sciatic and femoral nerves were not removed.

Animals were killed. As shown in Table 2, administration of ACG in drinking water over a long period of time, or in drinking water with an i.p. injection, to the normal (non-neurectomized) groups did not prevent reactivation of latent virus in the DRG or FP. Similarly such treatment had no effect on virus recovery from denervated FPs. Footpad and DRG isolates were sampled and tested for their ACG sensitivity either by plaque reduction assay in the presence of various concentrations of ACG or by infecting BHK cell monolayers, which were incubated for 24 h in the presence of various concentrations of ACG, before they were harvested, sonicated and titrated as usual. For the control experiment virus isolates were similarly treated except no ACG was added. To date all examined isolates were found to be ACG-sensitive.

The efficacy of ACG in eradicating infectious HSV in acutely infected mice has been widely reported by other workers (Kerren et al., 1982; Field et al., 1984). However to ascertain that ACG is also effective in inhibiting HSV-2 replication in the mouse FP, acutely infected mice were treated with ACG either in the drinking water or by an i.p. injection as above. Treatment commenced immediately after the initial infection. The control group of mice received no ACG. At different times after infection a group of mice was killed and the virus titre in the FP was determined. Fig. 1 shows that treatment with ACG administered intraperitoneally reduced virus titre in the FP by 10- to 1000-fold. No p.f.u. were detected in FP homogenates on the 10th day of treatment. Administration of ACG in drinking water was less effective. However no virus was detectable on day 12 (or thereafter) of the chemotherapy period in this group of mice,
whereas virus was detectable in the FPs of some of the control mice 16 days or later after the initial infection. Both methods of ACG dosing were found to be equally effective in eradicating infectious virus at the DRG (data not shown).

It has also been shown that pretreatment in vitro of explanted ganglia with ACG or PAA failed to eliminate the latent virus (Wohlenberg et al., 1979; Klein et al., 1981). Further experiments were therefore carried out to test the effect of ACG and PAA on virus reactivation in vitro. After removal of the sciatic and femoral nerves, mice were infected with the virus then treated with ACG (drinking water/i.p. injection) as described. The explanted piece of FP tissue (roughly 6 × 2 mm in size) was further divided into three and incubated in the presence of 20 μg/ml of ACG or 250 μg/ml of PAA in the supernatant medium. Explanted DRG were incubated with ACG-containing medium only. All cultures were screened daily and the supernatants were replaced with fresh ACG- or PAA-containing medium. The drugs were then removed after 8 days of culture and the tissue was rinsed several times before incubation in drug-free medium. Screening for reactivating virus was then continued for a further 5 weeks and the results were recorded.

Table 3 shows further evidence for the inability of ACG and PAA to prevent virus recovery from the explanted FP tissue. Three of the five FP cultures released virus in the supernatant medium. Virus was first detected 7 days after removal of ACG from the FP culture of mouse number 3. The other two positive cultures (2 and 4) released virus in the supernatant at days 7 and 11 after removal of PAA. The virus in these cases was found to be sensitive to both ACG and PAA. A persistent infection would be eradicated by treatment with ACG or PAA or result in selection of drug-resistant virus. As expected, no virus was detected in the DRG of this group of mice. In general HSV was recovered more frequently from the denervated FP than from the FP of control mice. In total, eight of 20 (40%) of the denervated FPs released virus in the supernatants while only four of 26 (15%) of the non-neurectomized mice showed virus in their FP. Removal of nerve fibres clearly prevented virus flow to the sensory ganglia, which presumably led to higher virus titre in the FP and the subsequent establishment of a larger number of latent foci.

The majority of HSV-2 reactivants from explanted ganglia first appeared between 8 and 14 days after explantation. (Virus was never detected earlier than the 7th day after explantation.) In FP cultures, virus was not observed before the 12th day after explantation and the majority shed virus between days 14 and 21. (On two occasions virus was first detected on days 26 and 28.) The reason for the difference in time of virus detection from the DRG and FP is unknown. It probably reflects the differences in the actual process of reactivation in two different types of tissue. Other factors may also contribute to these differences, for example the number of latent foci at either site of infection, differences in yield of virus from each latently infected cell, or not all cells initiating reactivation synchronously. On the basis of present evidence it is not fruitful to speculate further on these possibilities. However, it should be stressed that the interval required before HSV could be detected in these tissues is evidence for latent and not persistent virus.

Table 3. Reactivation of HSV from FP cultures of denervated mice treated with ACG or PAA

<table>
<thead>
<tr>
<th>Mouse no.</th>
<th>Sacral</th>
<th>Lumbar</th>
<th>Thoracic</th>
<th>FP*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Explanted FP tissue was divided into three pieces and pretreated in vitro either with PAA or with ACG as described. All mice received ACG therapy in drinking water as well as by i.p. injection.
It is important to identify the type(s) of cell that may harbour the latent virus in the FP. Candidates include epithelial cells, fibroblasts, keratocytes and melanocytes. It is interesting that melanocytes and sensory neurons are closely related ontogenetically, both being derivatives of the neural crest. In one elegant study, Cook & Brown (1987) presented evidence for the ability of HSV-1 to establish latent infection in vitro using rabbit corneal cell cultures. Virus was recovered from epithelial cells, keratocytes and endothelial cells that had been treated with ACG at a supraoptimal temperature. Virus has also been recovered from clinically normal rabbit corneas (Cook et al., 1987). The task of identification of the cells supporting latency at the periphery may be achieved by using techniques of in situ hybridization and immunofluorescence. Initial results from our laboratory show that virus can be recovered from the dermis and possibly from the underlying tissue whereas no virus or viral antigen was detectable in epidermal cell cultures established from the skin of latently infected mice.

Equally important is the characterization of the state of the virus genome in the latently infected cell. Recent reports have suggested that latent HSV genomes residing in neurons lack the terminal fragments (Rock & Fraser, 1983; Efstathiou et al., 1986) and that two copies of the joint region exist, suggesting a circularized form of the latent virus DNA (Rock & Fraser, 1985; Mellerick & Fraser, 1987). Whether the virus genome latent in the FP exhibits a form similar to or different from that latent in the DRG remains to be seen. One problem which can be envisaged is that the number of latent foci in the FP is much less than those in the DRG and less viral DNA can be extracted. The overall value of virus recovery from the DRG of normal infected mice was 100% but only 10% to 20% of the examined FPs released virus in the supernatants. Inoculation of mice with a high dose of virus, particularly with an avirulent or temperature-sensitive mutant virus, should help to overcome this difficulty.

The evidence presented in this paper confirms our earlier report (Al-Saadi et al., 1983) that HSV can establish latent infection both in neuronal and non-neuronal cells. It is possible then to postulate that stimuli causing virus reactivation may act on latently infected cells at the periphery and/or at the ganglia. Experiments investigating the effect of external stimuli on the rate of virus recovery from denervated FPs of mice are under way.

In man, herpes lesions have been observed in accidently denervated areas of skin (Hoyt & Billson, 1976) and virus has been isolated from human cornea explanted in cultures, suggesting the possibility of latency in these peripheral tissues (Shimeld et al., 1982; Tullo et al., 1985). The observation that HSV can establish classical latent infection in neuronal and non-neuronal cells opens a new area of research and could be of clinical significance.

This work is supported by a grant from the Medical Research Council, U.K.

REFERENCES


(Received 5 August 1987)