Retention of herpes simplex virus DNA sequences in the nuclei of mouse footpad keratinocytes after recovery from primary infection

A. Simmons, R. Bowden and B. Slobedman

Infectious Diseases Laboratories, Institute of Medical and Veterinary Science, Frome Road, Adelaide, SA 5000, Australia

Persistence of herpes simplex virus (HSV) DNA in mouse footpad keratinocytes was studied by non-isotopic in situ hybridization. HSV DNA was retained in keratinocyte nuclei for more than 2 weeks after disappearance of infectious virus and viral antigens. The anatomical location of viral DNA became more superficial with increasing time post-infection, reflecting the migration of cells from the basal layer of the epidermis towards the stratum granulosum. Latency-associated transcripts (LATs) were not detected in footpad cells at any of the times studied. In contrast, LATs were detected readily in the nuclei of lumbar ganglionic neurons innervating HSV DNA positive footpads. It was concluded that, after termination of productive infection in the skin, HSV DNA persists transiently in keratinocyte nuclei, in the absence of abundant latency-associated transcription. An implication of these data is that detection of HSV DNA in the skin may reflect recent, but not necessarily current, cutaneous virus replication.

Between recurrent episodes of herpes simplex virus (HSV) infection, viral DNA is sequestered in a latent, non-replicating state, in the nuclei of primary sensory neurons (reviewed by Stevens, 1989). During latency, viral RNA transcription is restricted to the repeated (diploid) region of the HSV genome and, despite intensive study, the functions of latency-associated transcripts (LATs) are currently unknown.

In addition to classic latency in neurons, there is experimental evidence suggesting that HSV can persist outside the nervous system, notably in the eyes and skin of experimentally infected animals (Scriba, 1977; Hill et al., 1980; Openshaw, 1983; Cook et al., 1987; Clements & Subak-Sharpe, 1988; Claué et al., 1990). However, at the present time, the existence and biological significance of peripheral latency remain objects of debate, partly because this phenomenon is poorly characterized in molecular terms. A pertinent question is, what cell-types could harbour HSV genomes in the skin? Keratinocytes, which are the predominant epidermal cell-type, are the main cutaneous site of productive HSV infection but, as sites of classic latency, are not ideal candidates owing to their rapid turnover rate. Nonetheless, non-productive HSV infection has been described in three-dimensional keratinocyte cultures (Syrjänen et al., 1996), in which persistence of viral DNA was restricted to cells infected after induction of terminal differentiation. It is reasonable, therefore, that HSV DNA might persist transiently, in a non-replicating state, in epidermal keratinocytes in vivo, following an episode of productive infection.

In the current study, this hypothesis was addressed by testing mouse footpad cells for the presence, in situ, of HSV antigens and HSV DNA, at various times after virus inoculation. Viral DNA was retained in keratinocyte nuclei for approximately 2 weeks after recovery from productive infection. Initially, cells containing HSV DNA were distributed throughout all structural layers of the epidermis but, at later times [24 days post-infection (p.i.)], viral DNA sequences were confined to the stratum granulosum, the most superficial layer of nucleated cells. LATs were not detected in HSV antigen negative–DNA positive footpad cells but, in contrast, in lumbar ganglia innervating the same footpads, LATs were detected readily in neuronal nuclei.

Initially, the model system was characterized by testing footpads for the presence of infectious virus at daily intervals, from 5 to 10 days p.i. A well characterized, virulent isolate of HSV type 1, strain SC16 (Hill et al., 1975), was used throughout. Virus was grown and titrated in Vero cells and stored at $-70 \degree C$ until required. Adult female BALB/c mice (Specific pathogen free facility, Animal Resource Centre, Perth, Western Australia) were lightly anaesthetized and left hind footpads were scratched 15 times with a 27 gauge needle, through a 20 µl drop of virus suspension containing $1\times10^{6}$ p.f.u. To quantify infectious virus in footpads, tissues were homogenized in 1 ml cell culture maintenance medium and tenfold dilutions were tested for the presence of infectious virus, using

Author for correspondence: Tony Simmons.
Fax +61 8 222 3538. e-mail tsimmons@immuno.imvs.sa.gov.au

0001-4436 © 1997 SGM
Fig. 1. Photomicrographs of PLP fixed spine/left hindlimb tissue sections (5 µm), stained with haematoxylin and eosin (H & E), showing: (a) a heavily infiltrated, acutely infected footpad (arrowed) and lumbar ganglia (e.g. L3 ringed) innervating the site of
a standard plaque assay (Russell, 1962). At 5 and 6 days p.i. the mean levels of virus recovered from groups of 3 mice were $8 \times 10^6$ and $1 \times 10^4$ p.f.u. per footpad, respectively. Infectious virus was not detected ($< 10$ p.f.u. per footpad) on or after day 7 and consequently it was concluded that productive infection is terminated between 6 and 7 days after inoculation of $10^6$ p.f.u. SC16 into the footpads of BALB/c mice.

Immunohistochemistry and in situ hybridization (ISH) were used to determine the anatomical and cellular locations of viral antigen and nucleic acids, respectively, at various times p.i. (Fig. 1a–i). Groups of 3 mice were killed at daily intervals from 2 to 10 days p.i. (day 0) and further groups were killed on days 24 and 36. To facilitate simultaneous analysis of peripheral and neural tissues from each animal, a block of tissue containing the thoraco-lumbar spine and left hindlimb was fixed in paraformaldehyde–lysine–periodate (PLP) for 16–24 h, decalcified for 3–6 weeks in phosphate-buffered EDTA solution (70 g/l, pH 7.4) and embedded in paraffin wax. Coronal sections (5µm) were collected onto glutaraldehyde activated 3-aminopropyltriethoxysilane (Sigma) coated slides.

To detect HSV antigens, tissue sections were sequentially reacted with rabbit anti-HSV infected cells, swine anti-rabbit Ig and rabbit peroxidase–anti-peroxidase complex (all reagents from Dakopatts). All reactions were allowed to proceed for 30 min at 37 °C with a 10 min wash in Tris–HCl (pH 7.4) between steps. Bound antibody was detected with 3,3’-diaminobenzidine (0.5 mg/ml containing 0.1% H2O2), and sections were lightly counterstained with haematoxylin. Viral antigens were detected in epidermal cells up to and including 6 days p.i. (Fig. 1d, day 5) and in lumbar dorsal root ganglia from days 2 to 6 inclusively (not illustrated). In line with clearance of infectious virus, HSV antigens were not detected on or after day 7 (Fig. 1e, day 9). Acute inflammation was a prominent clinical and histological feature of infected footpads (Fig. 1a, arrowed).

To detect HSV nucleic acids in situ, strand-specific riboprobes complementary to the HSV-I LAT region were generated from pSLAT2, which comprises a 1.6 kb PstH–HpaI subfragment of SC16 BamHI B, cloned into Bluescribe M13 expression vector (Stratagene). Transcription from the T7 promoter generated probes complementary to LATs. Non-isotopic (digoxigenin-labelled) ISH was done as described previously (Arthur et al., 1993) with the following modifications: (i) when required, sections were treated for 1 h at 37 °C with RNase (500 µg/ml) or RNase followed by DNase (1 U/ml in 40 mM Tris–HCl pH 7.5, 6 mM MgCl2); (ii) DNA targets were denatured, after sealing the hybridization mixture in place (50 µl/section), by heating slides for 5 min on a metal tray floating in boiling water. In regions of zosteriform spread, HSV nucleic acid sequences were shown, consistently, to persist in keratinocyte nuclei (Fig. 1f, day 9) as well as in areas adjacent to the site into which virus was scarified (Fig. 1g, day 9). Probes did not hybridize to uninfected tissue (not illustrated). pSLAT2 probes generated from the T7 and T3 promoters stained keratinocytes with equal intensity, from which it was concluded that HSV nucleic acid detected in keratinocytes was primarily DNA rather than LATs. This conclusion was confirmed in tissue removed 9 and 24 days p.i. by treating sections with nuclease prior to hybridization; staining of sections by T7 transcripts of pSLAT2, which are anti-sense to LATs, was not significantly reduced in intensity by RNase but staining was obliterated by RNase plus DNase (not illustrated).

On days 9 and 10, cells containing HSV DNA were abundant in all layers of the epidermis (Fig. 1f, day 9). In contrast, on day 24, HSV DNA was confined, in all mice examined, to keratinocyte nuclei in the stratum granulosum (Fig. 1h), which is the most superficial layer of nucleated cells (Fig. 1c); HSV DNA was not detected in any mice killed on day 36. Although LATs could not be detected in footpads 24 days p.i., LATs were detected readily in the same animals, in lumbar dorsal root ganglia (Fig. 1i).

The conclusion that HSV persists in the skin in a non-replicating state for up to 18 days after recovery from acute infection is based on the observation that digoxigenin-labelled riboprobes detected HSV DNA in the nuclei of epidermal cells 24 days p.i., whereas viral antigens and infectious virus were detected only up to 6 days p.i. Only one cell-type, the keratinocyte, has a distribution and abundance compatible with the DNA staining pattern observed in the footpads of infected animals. In particular, on day 24, HSV DNA was confined to cells of the superficial stratum granulosum, which comprises exclusively terminally differentiated keratinocytes, from which it was concluded that HSV DNA persists in keratinocyte inoculation; (b) H & E stained uninfected footpad (× 100) demonstrating the overall structure of the skin, in particular the boundary between the epidermis (E) and dermis (D) (arrowed); (c) H & E stained uninfected footpad epidermis (× 400) illustrating the densely packed nuclei of cells in the basal layer (B) and the flattened nuclei of superficial keratinocytes in the stratum granulosum (S). Occasional melanocytes (M) can be distinguished morphologically in the basal layer; (d) footpad skin (× 100), 5 days p.i., stained for HSV antigens [black areas throughout epidermis; epidermal–dermal boundary (arrowed)]; (e) footpad skin (× 100), 9 days p.i., stained for HSV antigens [not detected; epidermal–dermal boundary (arrowed)]; (f) footpad skin (× 100), from the same mouse as panel (e), 9 days p.i., stained by non-isotopic ISH for HSV DNA [black areas, corresponding to keratinocyte nuclei throughout epidermis; epidermal–dermal boundary (arrowed)]; (g) footpad epidermis (× 400), 9 days p.i., stained by ISH for HSV DNA, showing a cluster of DNA positive keratinocytes nuclei (open arrow), adjacent to a scab overlying the healed inoculation site (closed arrow); (h) footpad epidermis (× 400), 24 days p.i., stained by ISH for HSV DNA [black keratinocyte nuclei in stratum granulosum are HSV DNA positive; epidermal–dermal boundary (arrowed)]; (i) lumbar dorsal root ganglion (L3, × 400), stained by ISH for HSV LATs [black neuronal nuclei (arrowhead)]. Sections shown in panels (d)–(i) were lightly counterstained with haematoxylin.
nuclei. The change in the anatomical distribution of staining between days 9 and 24, and the lack of staining on day 36, suggested progression of DNA sequences from the basal epidermis towards the stratum granulosum over a 2–3 week period. This most probably reflects the migration of keratinocytes from the basal layer of the epidermis towards the surface of the stratum granulosum, which occurs over a similar timeframe during the formation of keratinized epithelium (Halprin, 1972). No evidence of virus persistence in melanocytes of the basal layer was found, despite their longevity and neural crest origin.

There are profound differences between the persistence of HSV in footpad keratinocytes, as detailed here, and neuronal latency. Firstly, detection of HSV DNA in latently infected neurons by current in situ hybridization protocols has been problematic, whereas persistence of viral DNA in keratinocyte nuclei was detected readily. Secondly, major LATs are abundant in latently infected neurons, but transcripts from the LAT locus were not detected, by in situ hybridization, in keratinocytes. However, it is noted that LATs may have been found in murine corneas in the absence of detectable virus replication (Abghari et al., 1992). In addition, major LATs have been detected in non-productively infected keratinocyte raft cultures by in situ hybridization but, in stark contrast with latently infected neurons in vivo, keratinocyte LATs were located in the cytoplasms rather than nuclei of infected cells (Syrjänä et al., 1996). The significance of the latter observation is unknown.

In recent years there have been significant advances in our understanding of the natural history of herpes simplex virus, and prominent among these is the discovery that, after recovery from primary infection, virus is shed frequently from peripheral sites, such as the genitalic tract, in the absence of symptoms (Koutsy et al., 1990). Asymptomatic virus shedding is thought to be an important mode of transmission of HSV, prompting the application of sensitive molecular biological techniques, such as PCR, to the further study and diagnosis of HSV infection (Cone et al., 1991). However, while the presence of infectious virus is an unambiguous marker of recurrence, the significance of finding HSV DNA sequences in peripheral tissues is less clear. For instance, an obvious but important implication of our data is that the presence of HSV DNA sequences in peripheral tissues is less clear. For instance, an obvious but important implication of our data is that the presence of HSV DNA sequences in peripheral tissues is less clear. For instance, an obvious but important implication of our data is that the presence of HSV DNA sequences in peripheral tissues is less clear. For instance, an obvious but important implication of our data is that the presence of HSV DNA sequences in peripheral tissues is less clear. For instance, an obvious but important implication of our data is that the presence of HSV DNA sequences in peripheral tissues is less clear. For instance, an obvious but important implication of our data is that the presence of HSV DNA sequences in peripheral tissues is less clear. For instance, an obvious but important implication of our data is that the presence of HSV DNA sequences in peripheral tissues is less clear. For instance, an obvious but important implication of our data is that the presence of HSV DNA sequences in peripheral tissues is less clear.


Received 27 August 1996; Accepted 4 December 1996