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Reactivation of Latent Herpes Simplex Virus from Dissociated Identified Dorsal Root Ganglion Cells in Culture

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SUMMARY

Herpes simplex virus (HSV) types 1 and 2 reactivate from dissociated cultured dorsal root ganglia of latently infected mice. The neurons in culture were identified morphologically and by using the specific anti-neuronal monoclonal antibody A2B5. HSV antigen expression during reactivation was first seen in neurons on day 3 after dissociation, and infectious virus was released subsequently. Approximately 0·4% of neurons released infectious virus. The presence of neutralizing antibody to HSV did not modify the reactivation process. After infection of mouse neuronal cultures *in vitro* with HSV, cytopathic effect and viral antigen expression appeared within 18 h predominantly in fibroblastic cells.

It is well established that herpes simplex virus (HSV) can remain in latent form in sensory ganglia of experimental animals and man (Wildy *et al.*, 1982). Previous studies have generally used ganglion explants to study virus release *in vitro*; the one study (Walz *et al.*, 1976) which has claimed successful reactivation of HSV from dissociated dorsal root ganglia (DRG) of mice latently infected with virus has not been confirmed by others (Wildy *et al.*, 1982). Recent evidence has suggested that neurons within DRG harbour latent HSV (McLennan & Darby, 1980). We have employed indirect immunofluorescence using A2B5 [a neuron-specific marker (Eisenbath *et al.*, 1979; Walsh, 1980)], a polyspecific rabbit antiserum to HSV and a monoclonal antibody to HSV glycoprotein D (gD), to study reactivation in dissociated cell cultures prepared from DRG of mice latently infected with HSV types 1 and 2. As shown below, the latent HSV reactivates in such cultures as early as 3 days after dissociation. Initial viral antigen expression and virus reactivation, producing loss of processes and cell rounding occur in neurons.

Three-week-old Pirbright or Biozzi (high antibody responder) strain mice were infected with wild-type HSV-2 or HSV-1 respectively. As previously described (Al-Saadi *et al.*, 1983; Clements & Subak-Sharpe, 1983), 10^5 p.f.u. of virus were inoculated into the right hind footpad of mice which were then kept for not less than 3 months until sacrificed. No mice showed any clinical evidence of active infection with HSV when sacrificed. Uninfected mice of the same strain were used as controls. The mice were killed with chloroform and the thoracic 12, 13, lumbar 1 to 6 and sacral 1, 2 DRG from each side were removed aseptically and washed in phosphate-buffered saline containing 25 µg/ml gentamicin (PBS). The ganglia from two to five mice were pooled for each experiment, ganglia from the right side forming one pool and those from the left (contralateral to the site of inoculation) a second pool.

Dissociated cell cultures of adult mouse DRG were prepared using modifications of previously described techniques (Kennedy *et al.*, 1980). The pooled ganglia were gently teased apart with fine forceps and transferred to vials in which there was 2 ml of PBS containing 0·25% collagenase (Worthington) and incubated for 2·5 h at 37 °C. The ganglia were then dissociated by repeated trituration through Eppendorf plastic pipette tips and washed twice in CMRL-1066

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Table 1. *Recovery of virus from, and development of c.p.e. in, dorsal root ganglia**

Mouse strain	Input virus	Day on which supernatant fluid first positive on BHK21/C13 cells	Day on which c.p.e. first observed in neural cells	Number of positive cultures/total number of cultures
Pirbright	HSV-2 HG52 wt	5	6	3/6
Pirbright	HSV-2 HG52 wt	3	5	1/5
Pirbright	HSV-2 HG52 wt	4	6	1/5
Pirbright	HSV-2 HG52 wt	4	6	1/5
Biozzi	HSV-1 17 syn ⁺	3	5	3/3
Biozzi†	HSV-1 17 syn ⁺	3	5	3/3
Biozzi†‡	HSV-1 17 syn ⁺	5-7	5	3/3

* No virus ever reactivated from equivalent cultures from the left side or from uninfected mice.

† A single dissociation with and without serum.

‡ Human serum (4%) was added to incubation medium immediately after dissociation.

(Gibco) medium containing 2 mM-glutamine, 6 g/l glucose, 25 mM-KCl, 25 µg/ml gentamicin and 50% foetal calf serum. The resulting cell suspension was passed through nylon mesh, centrifuged at 500 *g* for 10 min, and the cell pellet resuspended in CMRL-1066. Cells were plated on 13 mm collagen-coated glass coverslips (approx. 5×10^5 cells in 1 ml CMRL per coverslip) in Linbro multiwell plates (Flow Laboratories) and incubated at 37 °C in a humidified atmosphere of 95% air-5% CO₂. The cell yield was approximately 1.5×10^6 viable cells per mouse (including both left and right sides).

Cultures were screened daily for the presence of released HSV by plating 0.1 ml of culture supernatant onto BHK21/C13 cells (Macpherson & Stoker, 1962) which were then observed for cytopathic effect (c.p.e.): the characteristic rounding-up of cells in discrete plaques within the cultures. DRG culture supernatant from right-sided (latently infected) ganglia first produced c.p.e. in BHK21/C13 cells between 3 and 5 days after dissociation (Table 1). The plaques formed were morphologically typical of wild-type HSV. HSV was never recovered from left-sided ganglia, contralateral to the site of inoculation, or from uninfected mice.

Discrete foci of c.p.e. were first seen in right-sided DRG neural cell cultures from HSV-1- and HSV-2-infected mice 5 or 6 days after dissociation (Table 1). Such areas were initially sparse, there being 1 or 2 foci per coverslip; within 24 h of the first appearance of c.p.e., approximately 10% of the total cell population showed a c.p.e. and within 48 h more than 50% did so. Reactivation also occurred in the presence of medium containing 4% human serum which had a 50% neutralization titre of 1/64 by plaque assay using HSV-1 17 syn⁺. Reconstruction experiments in which 5% human serum was added to monolayers of BHK21/C13 cells just before infection with 100 p.f.u. of HSV-1 resulted in a 90% reduction in plaque number and clearly restricted the spread of virus. A cytopathic effect appeared in the dissociated neural cultures of latently infected mice on day 5 after dissociation, as was the case in the absence of HSV antiserum. Detection of virus in the supernatant was delayed until 5 to 7 days after dissociation; this was presumably due to the neutralization of the majority of virus released from cells (Table 1).

In order to ascertain the identity of the neural cell types supporting HSV reactivation, indirect immunofluorescence experiments were performed using the mouse monoclonal antibody A2B5 (a gift from Dr F. Walsh), which reacts only with neurons and a polyclonal antiserum directed against HSV (rabbit anti-HSV, a gift from Dr H. Marsden). Additional experiments were done using a mouse monoclonal antibody directed against gD (a gift from Dr J. Palfreyman). The two fluorochrome immunofluorescence technique was employed as previously described (Raff *et al.*, 1979; Kennedy *et al.*, 1980, 1983).

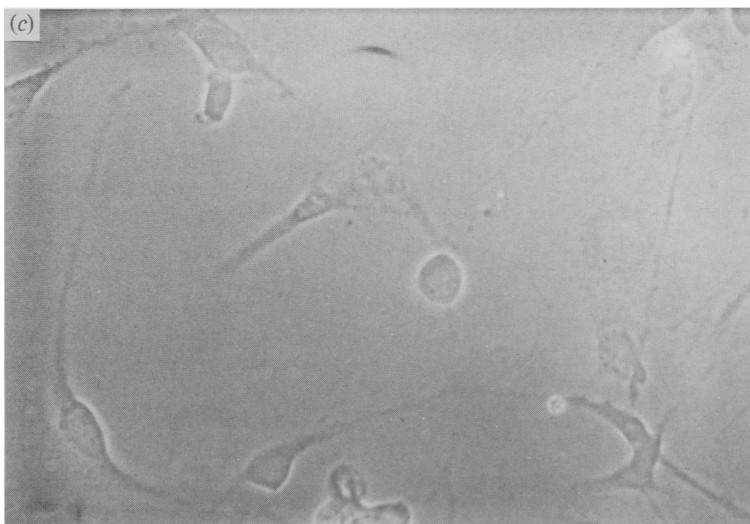
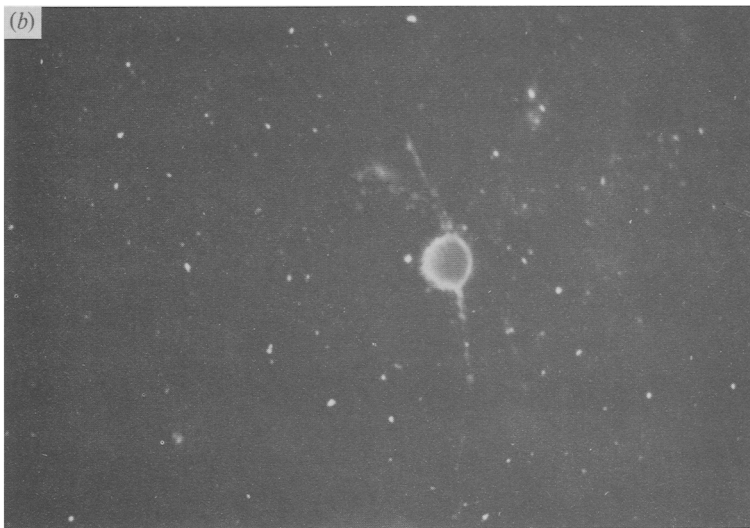
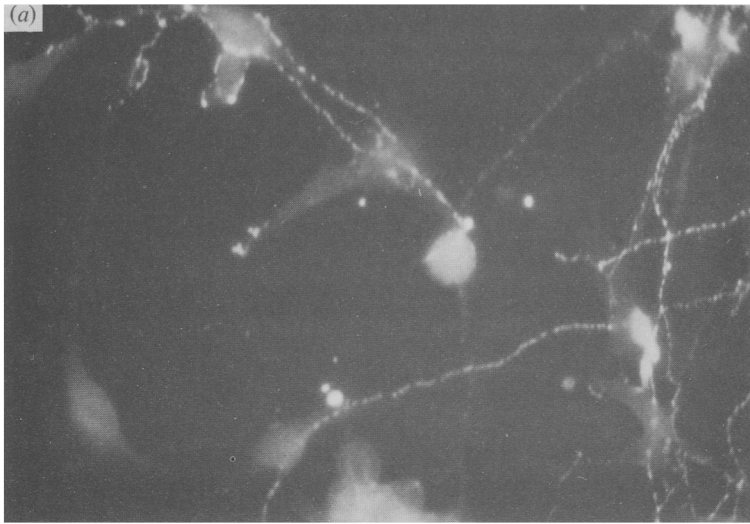
Cells on glass coverslips were removed from Linbro wells, rinsed in fresh complete medium and placed on the top of small plastic pedestals in humidified staining boxes. A2B5 was diluted 1/20 in fresh complete medium and 40 µl added to each coverslip. Incubation was carried out at room temperature for 30 min. Subsequently, the coverslips were washed thoroughly in fresh complete medium and then incubated with 40 µl rhodamine-conjugated goat anti-mouse

immunoglobulin (Cappel Laboratories, Cochranville, Pa., U.S.A.) diluted 1/20 in complete medium for 30 min at room temperature. The coverslips were then washed in PBS and fixed in 95% ethanol/5% glacial acetic acid for 10 min at -20°C . They were then incubated for 30 min with 40 μl rabbit anti-HSV antiserum (1/20) at room temperature. The cells were then washed in PBS and exposed to 40 μl sheep anti-rabbit IgG conjugated to fluorescein (Miles-Yeda, Rehovot, Israel) diluted 1/20 for 30 min. In some experiments single label fluorescence was performed using rabbit anti-HSV and the monoclonal antibody to gD. In these experiments, the staining was carried out as described except that the incubations with the A2B5 and the rhodamine conjugate were omitted. After washing in PBS, the coverslips were mounted in glycerol on glass slides, sealed with nail varnish and viewed under a $\times 40$ or $\times 50$ objective lens on a Leitz Ortholux fluorescence microscope equipped with epi-illumination and phase-contrast optics. The cells were then viewed with either phase-contrast microscopy or by immunofluorescence with the appropriate filters.

In uninfected DRG cultures, and infected cultures prior to HSV reactivation, 20% of cells approximately were A2B5-positive and had characteristic neuronal morphology, 30% were bipolar Schwann cells and 50% were fibroblastic cells, the latter two cell types being distinguished morphologically. A total of 150 cells per coverslip were counted. HSV antigens were first detected 3 days after dissociation in cultures from right-sided DRG using both the polyclonal rabbit anti-HSV serum and the monoclonal antibody to gD, but HSV immunoreactivity was never detected in cultures from left-sided DRG. In all 3 day-old cultures examined, the HSV-specific immunofluorescence was always restricted to neurons, which could be distinguished both by their characteristic morphology and by staining specifically with A2B5. In some cases such neurons had well developed processes and in others loss of processes and cell rounding were seen. HSV antigen expression in such cells was weak compared to that seen subsequently in cells within foci of c.p.e. (Fig. 1). Both large (e.g. 100 μm diam.) and small (e.g. 30 μm diam.) neurons were positive for HSV antigens, indicating that that population of neurons in which HSV can successfully establish latency and from which it reactivates is anatomically heterogeneous. The neurotransmitters used by the sensory neurons of the DRG are unknown. However, there is evidence to suggest pharmacological heterogeneity, and a recent report suggests that a minor subpopulation of rat DRG neurons are catecholaminergic (Price & Mudge, 1983). It is therefore not possible to determine the pharmacological characteristics of the neurons from which HSV reactivates.

By the fourth day after explantation, both neurons and occasional adjacent fibroblasts and Schwann cells were expressing HSV-specific antigens. By the fifth day, foci of c.p.e. were clearly visible and cells within these foci were labelled with rabbit anti-HSV serum. There were few intact neurons within such areas and more than 90% scored positive with this serum. Within Foci, <5% of intact Schwann cells and fibroblastic cells were labelled with rabbit anti-HSV (Fig. 1 *d, e*). More than 90% of the rabbit anti-HSV-positive cells within the foci of c.p.e. were A2B5-positive, whereas outwith the foci in the rest of the culture only about 5% of A2B5-positive neurons were labelled with rabbit anti-HSV. Within 48 h of the first appearance of c.p.e., a large proportion (>50%) of all cell types showed c.p.e., due to spread of virus from the original foci of reactivation.

We directly infected some left-sided DRG cultures with wild-type HSV-2 (at 1 p.f.u./cell) after 11 days *in vitro*. (These cultures had been monitored during this time and at no time found to be positive.) Twenty-four h after infection, 30% of the total cell population showed c.p.e., while 60 to 70% of all the cells were positive with rabbit anti-HSV serum. In contrast to the pattern seen in the foci of c.p.e. within reactivating right-sided ganglion cultures, only 10% of neurons were positive with rabbit anti-HSV serum. The relative resistance of neurons within cultures of dissociated mouse DRG cells has been observed 4 to 6 days after dissociation, using either HSV-1 or HSV-2. Mouse neurons thus appear to be more resistant than other cell types to lytic HSV infection, which is consistent with results with human foetal brain tissue (Kennedy *et al.*, 1983). These apparent differences in the neural cell susceptibility to HSV during reactivation of latent virus or lytic infection are intriguing and suggest that the underlying mechanisms in these two situations differ.



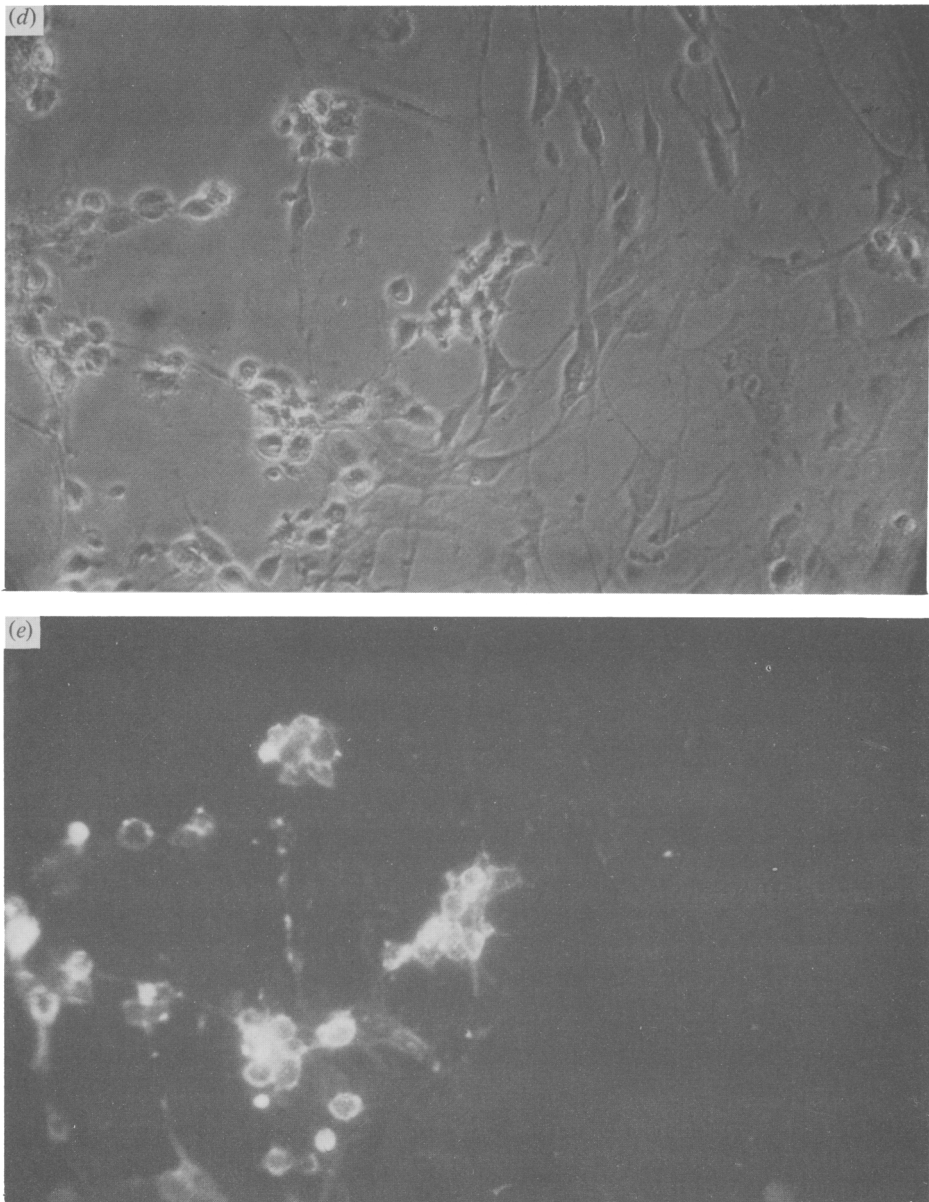


Fig. 1. Reactivating HSV in collagenase-dissociated adult mouse DRG cells after culture for 3 days. (*a*, *b*, *c*) Stained by two fluorochrome immunofluorescence for (*a*) neuron specificity with A2B5 (rhodamine), and (*b*) for HSV antigen with rabbit anti-HSV (fluorescein); magnification $\times 180$. In the centre of the field is a neuron showing an early c.p.e. adjacent to fibroblastic cells. The neuron and processes of other neuronal cell bodies not in the field stain characteristically with A2B5 (*a*). No other cells stain specifically. This neuron is the only cell in the field to stain with the rabbit anti-HSV polyclonal antiserum (*b*) and the pattern of staining representing the HSV antigen distribution is clearly different from that with A2B5 (*a*). (*c*) The same field photographed with phase-contrast illumination showing the distribution and morphology of cells within the culture. (*d*, *e*) An equivalent culture on day 5, 18 h after the c.p.e. was first observed, viewed either (*d*) by phase contrast or (*e*) after staining with rabbit anti-HSV (fluorescein); magnification $\times 80$. The fibroblastic cells to the right of the picture are unlabelled, in contrast to the HSV-positive cells to the left, where there is a focal c.p.e.

To demonstrate that the HSV recovered from these cultures was identical with that originally inoculated into the mice, we studied the DNA restriction enzyme profiles of recovered HSV using *Bam*HI, *Hind*III and *Kpn*I (Lonsdale, 1979). As expected, virus isolated from DRG cultures was indistinguishable from the input virus.

An estimate of the number of neurons from which HSV reactivated, on the basis of the observed number of cells positive for HSV antigens by immunofluorescence at 3 days, is 15 to 30 cells per mouse. This is likely to be an underestimate since neurons from which virus reactivated spontaneously at later times could not be distinguished from the neurons that had become infected in culture by virus released from neurons reactivating more rapidly. Cultures older than 3 days, therefore, could not be scored. To obtain a more accurate estimate, a focal assay was performed. Dissociation of ganglia from latently infected mice was carried out as described previously except that only half of the dissociated cell suspension was plated onto coverslips in Linbro wells. The remainder was made up to 30 ml in complete medium to which 2% pooled human serum was added. Volumes of 3 ml of this cell suspension were inoculated to each of 10 semiconfluent monolayers of BHK21/C13 cells in 30 mm Falcon vented Petri dishes. After 5 days incubation at 31 °C the cells were fixed and stained. (The lower temperature was used to slow cell replication and so prevent the monolayers becoming overconfluent.) Reactivation took place in the presence of the human serum which was added to reduce the spread of virus from productively infected cells and to allow foci to be scored. The foci seen were of differing sizes, some being large, indicating release of virus several days previously, others being small and thus either of more recent origin or perhaps due to a slower cell-to-cell spread. In two separate experiments 74 and 93 foci were scored per mouse (each being the average from two mice); this is three- to sixfold higher than the figure derived directly from the 3-day immunofluorescence observations above.

An estimation of the plating efficiency of infectious centres is not available because accurate counting of neurons within the disaggregated cell suspension is precluded by the presence of large amounts of myelin debris. However, on the basis of estimates derived by Duchon & Scaravilli (1977) there are approximately 20000 to 25000 neurons in ten dorsal root ganglia. Thus, making no allowance for loss of neurons during plating, approximately 0.4% of neurons release virus by the fifth day after explantation. Additional neurons may potentially be capable of reactivating virus at longer times after explantation. On the basis of our previous work involving the screening of individual intact ganglia, 30 to 40% of lumbar ganglia would be expected to shed virus after infection with 10^5 p.f.u. of virus per mouse (Al-Saadi *et al.*, 1983; Clements & Subak-Sharpe, 1983).

The estimate that 0.1% of dissociated DRG cells (5 to 10% of which were neurons) from mice latently infected with HSV-1 release virus (Walz *et al.*, 1976) is in good agreement with our results. However, these authors' technique had a number of shortcomings compared with that described here. For example, the cells (including neurons) were not positively identified with markers, and no attempt was made to classify the cultured ganglionic cells into different cell types. Secondly, the method of assessing the number of cells from which HSV reactivated was indirect and no correlation could be made between morphological type and virus production.

Our observations are consistent with those of McLennan & Darby (1980) who have also provided direct evidence that the neuronal cell body is the site of latent infection. However, interpretation of these findings must take into account the recent report by Warren *et al.* (1982) that HSV could be isolated from the trigeminal nerve roots of some human cadavers, suggesting that the virus may remain latent in cell types other than ganglionic neurons. There is also evidence that HSV may be capable of establishing latency in non-neural sites (Scriba, 1977, 1981; Al-Saadi *et al.*, 1983).

In summary, we have been able to reactivate HSV from dissociated DRG of Pirbright and Biozzi strain mice. The c.p.e. and HSV antigens arose first in neurons, both large and small, during the process of reactivation. Both the time course and the cell types affected during reactivation differed from those during *de novo* infection of left-sided ganglion controls *in vitro*. Reactivation took place in the presence of neutralizing antibody, a finding which is not consistent with the conclusions of Stevens & Cook (1974). Sekizawa *et al.* (1980) provide

evidence on the basis of passive immunization experiments that serum neutralizing antibody is not needed to maintain the latent state *in vivo*. Moreover, the ability to reactivate virus from dissociated cells clearly indicates that the retention of pre-existing relationships between neurons and supporting cells is not a requirement for reactivation of latent HSV.

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