The Interaction of Herpes Simplex Virus with Cultures of Peripheral Nervous Tissue: an Electron Microscopic Study

By T. J. HILL AND H. J. FIELD

Department of Bacteriology, The Medical School, University Walk, Bristol BS8 1TD, U.K.

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SUMMARY

Strains of herpes simplex virus types 1 and 2 were used to infect 3-day-old cultures of chick embryo dorsal root ganglia. Evidence of c.p.e. was seen by means of light microscopy at 14 h after infection. Ganglia were removed at intervals after infection and fixed and sectioned for electron microscopy. All stages of virus replication were observed in neurons, while few glial cells showed evidence of infection. Glial cells which did appear to have become infected showed signs of defective virus replication. The significance of the findings are discussed in relation to the mechanism of latency in herpes simplex virus infections.

INTRODUCTION

Underlying the present study is an interest in the different types of interaction between herpes viruses and neural cells. In the case of herpes simplex virus such interactions may range from the more lytic response seen in encephalitis, to the more prolonged state of latency. Evidence for the involvement of the sensory ganglia in the latter state is accumulating for man (Kibrick & Gooding, 1965; Bastian et al. 1972), mouse (Stevens & Cook, 1971b) and rabbit (Stevens, Nesburn & Cook, 1972). However, little is known about the nature of this latent infection at the cellular level.

In the following experiments cultures of chick embryo dorsal root ganglia were infected with herpes simplex virus in vitro and studied by electron microscopy in the hope of gaining information which might be relevant to the state of latency in vivo.

METHODS

Viruses. Two strains of herpes simplex virus (type 1 and 2, isolated from cases of Herpes labialis and Herpes genitalis, respectively) were obtained from the Public Health Laboratory, Bristol.

The viruses were cloned by three successive single plaque isolations in HEp2 cells; each of the two strains was passaged less than ten times in all.

Chick embryo dorsal root ganglia cultures. Dorsal root ganglia of the chick embryo were chosen for two main reasons. Firstly, methods were established whereby it was relatively easy to set up large numbers of replicate cultures which remained viable in vitro and developed in a regular way. Secondly, by using eggs obtained from leukosis-free chickens, it was possible to be reasonably certain that the cells in the culture were free from infection with wild viruses.

Embryonated eggs were obtained from our own flock of leukosis-free hens. The large
ganglia from the lumbar region of 8-day-old chick embryos were carefully removed under a dissecting microscope and placed on 15 mm squares of melinex (Firket, 1966), obtained from ICI Ltd. The melinex had been previously coated with rat tail collagen, prepared according to the method of Bornstein (1958). The collagen-coated melinex formed a convenient substrate to which the explanted ganglia readily adhered. Also the melinex was easily removed from the Araldite-embedded cultures with the minimum damage to the cells. Melinex has the disadvantage, however, that the molecular lattice impairs the phase-contrast image.

Each explanted dorsal root ganglion was transferred, on its Melinex coverslip, into a specially designed culture vessel. The vessels were constructed with a lid made from a large microscope slide and two adjacent glass rings (36 mm in diam.) and a base from a similar slide and two rings 30 mm in diam. A silicone rubber glue, ‘Bath tub caulk’ (Dow Corning) was used to fix the glass rings to the slides. The lid was loose fitting to allow a free gas exchange with the atmosphere of the incubator. The working vol. of each chamber was 0.5 ml medium.

The culture medium was as follows: 199 medium (Wellcome Reagents Ltd.) 70%, foetal calf serum (Flow Laboratories Ltd.) 30%, nerve growth factor (Wellcome Reagents Ltd.) 0.1 unit/ml (Levi-Montalcini, 1964), glucose 6 mg/ml (Murray, 1965); penicillin 100 units/ml, streptomycin 100 units/ml, and aerosporin 50 units/ml. The pH was maintained at 6.9 by means of NaHCO₃ and culture was carried out in a humidified incubator continuously flushed with an atmosphere of 5% CO₂ in air. The incubation temperature was 34 ± 1 °C.

Virus infection. Cultures of 3 days in vitro were used in the experiments. The medium was removed and 0.1 ml virus suspension in growth medium was added. The virus suspension contained 1 × 10⁷ p.f.u./ml which gave a theoretical input multiplicity of greater than 1 p.f.u./cell. In practice the multiplicity was probably much less than this since an unknown proportion of the virus is adsorbed onto the collagen (Feldman, Sheppard & Bornstein, 1968) or is not locally available to all the cells in the culture. After 1 h at 34 °C the ganglia were washed by adding, then removing, 0.5 ml medium. Finally 0.5 ml of fresh growth medium was added. Incubation was continued at 34 °C and cultures were examined regularly using phase-contrast microscopy. At intervals during the period 7 to 72 h post-infection cultures were removed (4 to 6 on each occasion), washed in phosphate-buffered saline, and fixed for electron microscopy.

Electron microscopy. Cultures were fixed in 2.5% glutaraldehyde in cacodylate buffer, post-fixed in 1% OsO₄, dehydrated in acetone and propylene oxide and embedded in araldite. Sections were cut with a diamond knife and stained with lead citrate and uranyl acetate, then were examined in a Philips 201 electron microscope.

RESULTS

Light microscopy

In uninfected control cultures, after 3 days in vitro, the formerly spherical ganglia had become flattened on the substrate. There was a dense halo of outgrowing neurites and migrating cells surrounding the central mass of neurons and glia (Fig. 1). The centres of the cultures were sufficiently flattened for a few neuron cell bodies to be discerned by phase-contrast microscopy, and Schwann cells were observed to be associated with the radiating bundles of neurites.

In virus-infected cultures c.p.e. was first observed at 14 h post-infection with the appearance of small groups of rounded cells at the outer margin of the explant. By 18 to 24 h
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Fig. 1. Uninfected dorsal root ganglion culture after 3 days in vitro.
Fig. 2. Dorsal root ganglion culture 4 days in vitro 18 h post-infection. (A) Central area containing neurons, (B) radiating neurites, (C) refractile infected cells. Magnification × 70.

Post-infection these areas of c.p.e. were more prominent and extensive and present in all parts of the peripheral outgrowth (Fig. 2). Rounding of cells was also thought to occur at least on the surface of the central area, though this was difficult to discern owing to the dense nature of the central part of the culture. Small syncytia (each seemed to be composed of only 2 to 3 cells) were associated with the groups of rounded cells in the type 1-infected cultures. The type 2 strain produced syncytia of a much more extensive nature. The first cells to show c.p.e. were thought to be the glial cells associated with the neurite outgrowth. By 24 to 48 h post-infection the neurites in the periphery became clumped, irregular and often retracted. The rounded cells in the periphery of the culture formed a contracted mass thereby increasing the opacity of the culture and making impossible any further observation of the central area.

Electron microscopy

After 3 days in vitro the control cultures, cut in transverse section (Fig. 3), revealed a layer of neurons sandwiched between two thin layers comprising mainly Schwann and fibroblastic cells. Neurites were present at all levels of the cultures. Each neuron cell body was seen to be associated with a satellite cell which appeared to envelop it. Schwann cells, associated with unmyelinated nerve fibres, were present in small numbers in the central area of the culture, but were more numerous in the cellular outgrowth around the periphery. The fine structure of the neurons and glial cells within the uninfected control cultures was similar to that described by Pannese (1969) and Weis (1971).
In virus-infected cultures no significant differences were noted between the ultrastructure of cultures infected with type 1 or type 2 herpes simplex virus. The Figs. show cultures infected with type 1 virus.

The first signs of virus replication were seen in the most superficial layer of fibroblasts at 24 h post-infection. Infected fibroblasts showed the normal stages of herpes simplex virus replication with the production of mature enveloped particles.

By 48 h after infection most neurons throughout the depth of the cultures showed many of the following characteristic changes associated with herpes simplex virus infection. There was margination of chromatin and disruption of the nucleoli (Figs. 4, 5). Virus capsids, either hollow or containing dense cores, were scattered throughout the nucleus or aligned along the nuclear membrane (Figs. 4, 5). Areas of nuclear membrane were reduplicated (Figs. 5, 6), and there was evidence of envelopment of virus capsids both at the inner nuclear membrane (Fig. 6) and at membrane sites within the cytoplasm (Fig. 7). Enveloped particles were present in the cytoplasm (Fig. 8), in some cases aligned within intracytoplasmic channels (Fig. 9). Accumulations of extracellular mature enveloped particles were observed around the periphery of many neurons (Fig. 9).
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Fig. 4. Nuclei of two neurons (N), showing scattered intranuclear virus capsids (arrows). Ch, clumped and margined chromatin; adjacent satellite cell (S) appears normal. 72 h post-infection.

Fig. 5. Neuron showing intranuclear capsids with dense core (D) and hollow core (H); Ch, clumped chromatin. 72 h post-infection.

At 48 h post-infection many of the satellite cells appeared normal (Fig. 4) with only about 30% showing morphological evidence of virus replication (Table 1). Even at 72 h post-infection there was little increase in the number of affected satellite cells. The cells which did appear to be infected showed most of the following features: there was margination of chromatin (Fig. 10), and either scattered intranuclear virus capsids or crystalline arrays of capsids (Figs. 10, 12). In all cases the capsids were hollow, having no dense core. Some cells
Fig. 6. Envelopment of virus particle (arrow) at inner nuclear membrane of neuron. N, nucleus; C, cytoplasm. 48 h post-infection.

Fig. 7. Envelopment of virus particles at membrane sites (arrows) within cytoplasm of neuron. 48 h post-infection.

Fig. 8. Enveloped virus particles (arrows) in cytoplasm of neuron. 48 h post-infection.

Fig. 9. Enveloped virus particles within membrane-bound channel (fine arrow) in cytoplasm (C) of neuron. Broad arrows, extracellular enveloped particles. SC, cytoplasm of satellite cell. 48 h post-infection.
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Fig. 10. Satellite cell with intranuclear crystalline array of hollow virus particles. Ch, clumped chromatin; arrows, reduplication of nuclear membrane. 48 h post-infection.

Fig. 11. Intracytoplasmic aggregate of virus particles in satellite cell. Nucleus (N) appears normal. 48 h post-infection.

Fig. 12. Satellite cell with hollow intranuclear particles (fine arrow), reduplication of nuclear membrane (broad arrow) and intracytoplasmic aggregate of virus particles (V) surrounded by dark staining material. 48 h post-infection.
Table I. Occurrence of the main morphological features in the replication of herpes simplex virus in neurons and satellite cells; 48 h post-infection

<table>
<thead>
<tr>
<th>Morphological features*</th>
<th>Neurons</th>
<th>Satellite cells</th>
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<tbody>
<tr>
<td>FC HC Mr Rd Ev</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ + + + +</td>
<td>41</td>
<td>0</td>
</tr>
<tr>
<td>+ + + - -</td>
<td>3</td>
<td>0</td>
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<td>- + + + -</td>
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<td>4</td>
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<td>- - + + -</td>
<td>0</td>
<td>16</td>
</tr>
<tr>
<td>- - - - -</td>
<td>1</td>
<td>51</td>
</tr>
<tr>
<td>Total no. cells examined</td>
<td>45</td>
<td>74</td>
</tr>
</tbody>
</table>

* FC = cells containing full capsids (with dense cores), HC = cells containing hollow capsids (without dense cores), Mr = cells with margination of chromatin, Rd = cells with reduplication of nuclear membrane, Ev = cells containing enveloped particles.

showed reduplication of the nuclear membrane (Figs. 10, 12). A few cells had intracytoplasmic aggregates of hollow virus capsids surrounded by dark staining material (Fig. 12). In one such case an aggregate was present in the absence of any nuclear changes (Fig. 11). Some of the infected satellite cells had a few scattered hollow capsids within the cytoplasm but in all cases there was a complete absence of enveloped particles. A representative sample of the frequency of the main morphological events in neurons and satellite cells is shown in Table I.

Similar changes were also observed in a small proportion of the Schwann cells, but unlike satellite cells these often contained a few intranuclear capsids with dense cores. However, there was no evidence of envelopment and large numbers of Schwann cells appeared to remain uninfected.

DISCUSSION

Our present in vitro studies represent an attempt at a critical morphological evaluation of the susceptibility to herpes simplex virus of different cell types in the dorsal root ganglion; a comparatively simple region of the peripheral nervous system from a histological point of view. The results indicate that in short-term cultures of avian dorsal root ganglia there is a clear-cut difference between the response of neurons and glial cells to infection with herpes simplex virus.

The morphological evidence suggests that the neurons of the ganglia cultures were capable of supporting the complete replication of herpes simplex virus with the production and release of mature enveloped particles (Figs. 4 to 9 and Table I). Envelopment of particles was seen both at the more usually described site of the inner nuclear membrane (Darlington & Moss, 1968; Nii, Morgan & Rose, 1968a) and also at membranes within the cytoplasm (Figs. 6, 7). The latter has also been described for herpes simplex virus by Nii et al. (1968a) and Leestma et al. (1969), and for infectious bovine rhinotracheitis virus by Zee & Talens (1972).

In contrast to the productive response of the neurons, the glial cells, and in particular the satellite cells, either appeared uninfected or exhibited signs of abortive infection with no production of enveloped particles or capsids with dense cores (Figs. 10, 12, and Table I). These findings agree closely with the in vivo observations on herpes simplex virus infection in mice ganglia (Dillard, Cheatham & Moses, 1972) and pseudorabies virus infection in
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rabbit (Becker, 1968) and calf ganglia (McCracken, 1973). In all cases a productive and abortive infection was shown by neurons and satellite cells, respectively.

From the purely morphological observations made here it is difficult to determine the defective stage in the infection of satellite cells with herpes simplex virus. The absence of intranuclear capsids with dense cores in satellite cells may indicate a defect in virus DNA production as was described by Nii et al. (1968b) following inhibition of herpes simplex virus DNA synthesis with hydroxyurea. A similarly defective replication cycle was also described by Stevens & Cook (1971a) following herpes simplex infection in macrophages from mature mice. The occurrence of intranuclear virus crystals in satellite cells may also be indicative of some restriction in virus replication. The observation by Schwartz & Roizman (1969) of similar crystals in continuous cell lines infected with unadapted strains of herpes simplex virus was also interpreted by them as evidence of some defect or restriction in virus replication. Spring, Roizman & Schwartz (1968) have also described failure of virus envelopment as a possible cause of abortive herpes simplex virus infection. The morphological evidence of this defect was a lack of nuclear membrane reduplication. We have observed that satellite cells exhibiting signs of infection often show evidence of nuclear membrane reduplication (Figs. 10, 12), therefore the primary defect in virus replication in these cells may not involve failure of envelopment. The lack of enveloped intracytoplasmic virus could be explained on the basis of the observations by Nii et al. (1968a) that only particles with dense cores are selected for envelopment.

The intracytoplasmic aggregates of naked virus capsids seen in some satellite cells (Fig. 12) probably represent particles undergoing lysosomal degradation. Similar intracytoplasmic inclusion bodies have been described in cytomegalovirus-infected cells (Luse & Smith, 1958; Ruebner et al. 1964; Ruebner et al. 1965) and in satellite cells from ganglia of rabbits infected with pseudorabies virus (Becker, 1968).

The intracytoplasmic inclusion shown in Fig. 11 may represent phagocytosed virus material (possibly an effete nucleus) from another cell.

It is of interest to consider the possible significance of our observations to the postulated latency of herpes simplex virus in the ganglia of man (reviewed by Kibrick & Gooding, 1965) and experimentally infected mice (Stevens & Cook, 1971b) and rabbits (Stevens et al. 1972). It could be argued that the state of latent herpes simplex virus might resemble the state of DNA viruses in transformed cells. In the latter situation it is generally infection of non-permissive cells which leads to transformation and integration of the virus genome (Schlesinger, 1969; Sambrook, 1972). Hence by analogy it would seem that the non-permissive satellite cell might be the most likely cell to harbour herpes simplex virus in the latent state.

However, it seems reasonable to suppose that the recurrence of clinical herpes simplex is associated with production of infectious virus at the site of latency (probably the Gasserian ganglion in man). Hence, if the satellite cells of human ganglia show a similar abortive response to infection with herpes simplex virus, it seems unlikely that the latent virus is carried in these cells. It therefore seems probable, as suggested by McCarthy (1972) and Stevens & Cook (1973) that the ganglion neurons carry the latent herpes infection. However, recurrence of clinical herpes simplex is not associated with any apparent loss of neuron function. This circumstantial evidence suggests that if the neurons are involved, the production of infectious virus by ganglion neurons must be so controlled in vivo that it does not lead to the eventual death of the cells.

The cultures used in the present study were rather short term, and hence less differentiated in vitro than those used by other workers, (e.g. Leestma et al. 1969). However, it has been
shown with other systems, that when organ cultures are maintained in vitro for long periods, they change the pattern of their in vivo susceptibility to virus infection (Hoorn & Tyrrell, 1969). Since our in vitro observations are very similar to the in vivo findings of Dillard et al. (1972) with herpes simplex virus and of Becker (1968) and McCracken (1973) with pseudorabies virus, we believe that our in vitro system represents a valid model for further studies of herpes virus infections of the nervous system.

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REFERENCES


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