HERPES SIMPLEX VIRUS TYPES 1 AND 2 IN ORGANOTYPIC CULTURES OF MOUSE CENTRAL AND PERIPHERAL NERVOUS SYSTEM

I. LIGHT MICROSCOPIC OBSERVATIONS OF MYELIN DEGENERATION

MARION S. ECOB-JOHNSTON*, Ph.D.
JEROME SCHWARTZ, Ph.D.
TERISITA S. ELIZAN, M.D.

AND

WILLIAM O. WHETSELL, Jr., M.D.

(New York, New York)

ABSTRACT

Mature mouse spinal cord-ganglion cultures, which contain both peripheral and central nervous system as one unit, were infected with herpes simplex virus type 1 (HSV 1) or type 2 (HSV 2) and observed by bright field microscopy for up to 72 hours. There was degeneration of both central and peripheral myelin in cultures infected with either virus, but the pattern of peripheral myelin degeneration associated with HSV 1-infected cultures was different from that in HSV 2-infected cultures. Type 1 was characterized by focal dilatations; type 2 by “sausage-shaped” swellings, and the cytopathic effect of HSV 2 both began (6 hours p.i.) and was completed (36 hours p.i.) earlier than in cultures infected with HSV 1 (12 hours and 48 hours p.i. respectively).

In central nervous tissue, the appearance of degenerating myelin after infection with HSV 1 was indistinguishable from that in HSV 2-infected cultures, but the rate of myelin loss was greater in cultures infected with the type 2 virus.

Evidence is presented which suggests that, at least in the peripheral nervous system, myelin degeneration did not appear to be dependent on neuronal or axonal dysfunction or death, but was a direct result of virus infection.

INTRODUCTION

There is a paucity of experimental data on the role of herpes simplex virus (HSV) in myelin degeneration. Herpes has been considered as a possible viral agent involved in the etiology of multiple sclerosis (11) and a recent report suggests that HSV may be associated with the demyelinating process in man (22). Organotypic nerve tissue cultures have been used to study the cytopathic effect of either HSV 1 (3, 10, 12, 13) or HSV 2 (8, 12, 13, 24) but the effects of

The Department of Neurology, Nerve Cell Tissue Culture Laboratory and Neurovirology Laboratory, The Mount Sinai School of Medicine of The City University of New York, Fifth Avenue and 100th Street, New York, New York 10029

* Dr. E cob-Johnston is a Visiting Fellow in the Nerve Cell Tissue Culture Laboratory, Department of Neurology, Mount Sinai Hospital Medical Center.
these viruses on myelin has not been clarified. The breakdown of peripheral myelin in organotypic cultures of rat dorsal root ganglia (DRG) infected with HSV 1 was first noted by Feldman et al. (10), but more extensive data was not available until recently when Whetsell et al. (23) described the effects of both HSV 1 and HSV 2 on cultures of mouse DRG. Among their findings they showed that although both viruses caused degeneration of peripheral myelin, the pattern of myelin breakdown characteristic for HSV 1 differed from that of HSV 2.

It is the purpose of the present study to extend the observations of Whetsell et al. (23) by focusing on the specific effects of the two types of HSV on both central and peripheral myelin, using organotypic nerve tissue cultures of mouse spinal cord with attached dorsal root ganglia. Such spinal cord-ganglion cultures, described by Peterson et al. in 1965, contain both peripheral and central nervous system components explanted and maintained as a unit of tissue in which the ganglion, its proximal root and spinal cord elements remain in continuity as in vivo (7) and are all available for infection by a single inoculum of virus. This model has been used in physiological studies (16, 17, 18) and toxicity studies (21).

Mature, well-myelinated mouse spinal cord-ganglion cultures were infected with either HSV 1 or HSV 2, and observed by direct light microscopy over the course of the infection. Production of infectious virus was detected in the supernatant fluid of the cultures by routine assays. Light microscopic findings are reported here; electron microscopic observations in these cultures at various stages of infection will be reported separately.

MATERIALS AND METHODS

Embryonic Mouse Spinal Cord-Ganglion Cultures

Cultures of mouse spinal cord with attached DRG were established as follows. The entire spinal cord with attached DRG was removed from 13–14 day old mouse fetuses. The lower thoracic and lumbar regions of the cord were cut into sections one vertebral segment long (0.5–1 mm) each of which had a pair of attached DRG. A small nick was made from the central canal to the dorsal aspect of the cord to allow the hemisections to open out. Two explants were placed on each collagen-coated coverslip (14) in a Maximow double-coverslip assembly (1) and incubated at 34.5°C in growth medium of 33% human placental cord serum, 50% Eagle’s minimum essential medium (MEM) and 10% chick embryo extract supplemented with 600 mg.% glucose. 500 unita/ml. of nerve growth factor was added to the medium at explantation but was omitted thereafter (6). (An explant is optimally placed with the cut surface on the collagen film so that meningeal growth does not obscure the spinal cord and the spinal cord does not obscure the DRG and dorsal roots.) Cultures were washed in balanced salts solution and fed a drop of fresh medium twice a week until maturity.

A few cultures of DRG alone or spinal cord alone were prepared from 15–17 day mouse fetuses using similar techniques (1).

Virus Stocks and Assay

The macroplaque strain (Mp) of HSV 1 and the MS strain of HSV 2 used in this study were previously described by Whetsell et al. (23). Virus samples were assayed in HeLa cells and the 50% tissue culture infectious dose (TCID50) was calculated using standard procedures.
Experimental Procedure

Spinal cord-ganglion cultures and cultures of either DRG or spinal cord alone were established and fed with growth medium (GM), which contained human placental cord serum for 18–21 days in vitro. When profuse amounts of both central and peripheral myelin were present, GM was replaced (9) by maintenance medium (MM) which was identical in constituents except that it contained 33% horse serum instead of 33% human placental cord serum.*

Mature cultures, 26–31 days old, were washed once in balanced salts solution and $4 \times 10^4$ TCID$_{50}$ of virus contained in 0.04 ml MEM plus 10% fetal bovine serum was adsorbed to each coverslip for two hours at 37°C in an atmosphere of 5% carbon dioxide. Control cultures were mock-infected under the same conditions. The inocula were drained off and the cultures refed with MM before incubation at 37°C.

 Cultures were studied by direct bright field light microscopic observation throughout the course of the infection, and individual neurons and segments of myelin were photographed sequentially using High Contrast Copy film (Kodak).

 In order to determine the state of preservation of axons during myelin degeneration, experimental and control cultures were fixed in unbuffered formal-saline at different times after infection (6, 12, 18, 24, 30, 36, 48 hrs.) and then stained using the Palmgren's silver impregnation technique (15).

RESULTS

Observations on Living Uninfected Cultures

At explantation of spinal cord-ganglion fragments, spinal cord tissue was contiguous to the DRG but within 72 hours the ganglia began to move away leaving sturdy roots. As differentiation proceeded, the explant flattened, and within a week cellular detail became visible by bright field microscopy. Individual cell types were easily identified in the DRG (neurons, satellite cells, Schwann cells, fibroblasts and macrophages) but, although corresponding cell types could be found in the spinal cord, the thickness of the fragment made a detailed study in the light microscope more difficult. Figure 1 illustrates the appearance of the spinal cord-ganglion unit after three days in vitro. By 16 days (Fig. 2) the ganglia had migrated away but were still connected to the spinal cord by the dorsal roots.

Neurons had large round cell bodies with a centrally placed nucleus and a single prominent nucleolus. In the DRG, neuron cell bodies were surrounded by three or four satellite cells which were so closely applied that only small oval nuclei could be distinguished around the cell body.

Both peripheral and central myelin were present in the tissue cultures. Peripheral myelin appeared at 14–16 days in vitro, and was easily recognized by the flattened Schwann cell nuclei which lay in slight depressions on the outer surface of the sheath. Nodes of Ranvier were seen between myelin segments, and Schmitt-Lantermann clefts were visible along the sheath. Cen-

* Samples of placental serum had neutralizing activity against herpes virus which was not found in horse serum and the procedure of changing to MM was adopted to avoid the neutralizing effects on infected cultures. Although the presence of specific antibody does not prevent the replication and spread of intracellular HSV 1 in organotypic cultures, there may be a selective advantage for those viruses capable of syncytial formation. By using horse serum prior to and after infection we imposed no such advantage.
Fig. 1. Mouse spinal cord with attached dorsal root ganglia, three days in vitro. Living culture photographed using bright field microscopy (×120). The dorsal root ganglia (DRG) are beginning to migrate away from the spinal cord; a ventral root-like projection can be seen at the ventral aspect of the spinal cord explant (arrow).

Fig. 2. Mouse spinal cord with attached dorsal root ganglion (DRG), 16 days in vitro. Living culture photographed using bright field microscopy (×300). Three fascicles of myelinated fibers are present in the dorsal root and individual neurons can be seen in the DRG.

Central myelin was smaller in diameter than peripheral myelin, slightly less refractile and appeared at 12–14 days in vitro. Although myelinated fibers radiated out from the DRG itself, peripheral myelin was most extensive in the root where it formed a thick band. Three such bands are visible in Figure 2.
Where the dorsal root crossed the meningeal mat and entered the spinal cord, myelin abruptly changed from the peripheral Schwannian type to the central glial type (4, 5) and formed a thick band of central myelin around the periphery of the spinal cord similar to the position of white matter \textit{in vivo}. The specific localization of both central and peripheral myelin in this model facilitated serial observations of virus-specific effects on myelin. The time of development of myelin in either spinal cord or DRG explants alone was comparable to that of spinal cord-ganglion cultures, but myelin formation was not as extensive even when cultures were fed with GM followed by MM.

The virus-specific changes to be described are based on results from three sets of experiments using groups of spinal cord-ganglia cultures and two sets using groups of spinal cord cultures or DRG cultures alone. The changes described were not seen in mock-infected cultures.

\textit{Observations on Living Infected Cultures}

\textit{Peripheral Myelin:}

HSV 1 and HSV 2 both caused segmental degeneration of peripheral myelin but the cytopathic effect of HSV 1 differed from that of HSV 2 both in the appearance of the changes and the time at which these occurred. The earliest change in a segment of peripheral myelin in cultures infected with HSV 1 (Fig. 3) was seen about 12 hours p.i. and was centered around the Schwann cell nucleus which became rounded and prominent. Nuclear swelling and increased granularity progressed until, by 42–48 hours p.i., Schwann cell nuclei could no longer be found. In those segments showing Schwann cell nuclear changes, there was an increased refractility of the myelin sheath at about 12 hours p.i. and, by 20 hours p.i., swellings appeared in the region of the nucleus. These swellings in the myelin spread progressively so that by about 36 hours p.i., multiple focal swellings were found along the sheath. The swellings were maximal about 40 hours p.i. and by 48 hours little trace of myelin remained.

In cultures infected with HSV 2 (Fig. 4) swelling of the Schwann cell nucleus, which began at 6–12 hours p.i., was less marked, but changes in the myelin sheath began earlier than in cultures infected with HSV 1. These early changes at 12 hours p.i. occurred in the region of the Schwann cell nucleus and appeared as asymmetrical swellings in the myelin which increased in size and number to form "sausage-shaped" swellings involving most of the segment by 24 hours p.i. Although these swellings were characteristic of HSV 2 infection, focal dilations similar to those described for HSV 1 could also be found occasionally. The "sausage-shaped" swellings appeared to collapse by about 30 hours p.i. and disintegrated leaving only traces of membranes by 36 hours p.i. At this time Schwann cell nuclei could not be found.

It is important to note that the changes just described were occurring in individual myelin segments, and that all segments along a given nerve fiber degenerated at independent times and at different rates. Some Wallerian degeneration was seen, but only late in infection (after 40 hours p.i.) when cellular necrosis was well advanced.
Fig. 3. The same segment of peripheral myelin in a living culture before and after infection with HSV 1; photographed using bright field microscopy at different hours (h) post infection (×750). A. Before infection, the Schwann cell nucleus (curved arrow) lies flattened against a smooth myelin sheath. B. By 12 hours p.i. the Schwann cell nucleus is contracted and more prominent, and the myelin sheath shows increased refractility. The positions of some Schmitt-Lantermann clefts are indicated by the small arrows. The swellings that develop appear to do so at these points. C. By 24 hours p.i., focal dilatations of the myelin sheath in the region of the nucleus are seen. D. By 36 hours p.i., these focal dilatations are seen along the entire length of the segment of myelin. E. By 42 hours p.i., the Schwann cell nucleus cannot be found and myelin begins to disintegrate. F. By 48 hours p.i., only fragments of membranes remain.

Central Myelin

In central myelin there was no qualitative difference between the effects of HSV 1 and HSV 2 infections. The earliest changes at about 14 hours p.i. consisted of the appearance of small knob-like projections on the sides of otherwise smooth pieces of myelin. Similar projections were also found in control cultures but were not as numerous as in infected cultures. By 20–24 hours p.i., segments of myelin appeared as chains of bubbles which grew larger and then disappeared by 30–40 hours p.i. (Fig. 4).

There was, however, a quantitative difference between the cytopathic effect
Fig. 4. The same segment of peripheral myelin in a living culture before and after infection with HSV 2; photographed using bright field microscopy at different hours (h) post infection (×750). A. Before infection, the Schwann cell nucleus (curved arrows) lies flattened against a smooth myelin sheath. B. By 12 hours p.i. asymmetrical swellings appear in the myelin sheath in the region of the Schwann cell nucleus which is slightly contracted. C. By 24 hours p.i., these swellings have increased in size and become “sausage-shaped”, involving the entire segment of myelin. D. By 30 hours p.i., the swollen myelin appears to begin breaking down. E. By 36 hours p.i., the segment of myelin has completely disappeared but its location can be judged by the vertical pieces of myelin which are still visible (straight arrows in A and E).

of HSV 1 and HSV 2 on central myelin. Absolute measurements of the quantity of myelin being broken down at any one time were difficult, but the areas of the “transition zone” (where the roots of nerve fibers invested with peripheral myelin entered the spinal cord and became invested with central myelin) was useful for assessments as it represented an easily located region of abundant myelin in all cultures where both central and peripheral myelin of the same group of nerve fibers could be seen. Although the cytopathic effect of both viruses began about the same time (14 hours p.i.), by 24 hours most of the central myelin (about 70%) was involved in cultures infected with HSV 2,
whereas in cultures infected with HSV 1, central myelin was much less affected (about 40%) when compared to uninfected controls. Complete loss of central myelin occurred about 36 hours p.i. with HSV 2 infection, and at 40-44 hours p.i. with HSV 1. At both times, some segments of peripheral myelin were still present in the dorsal root as it entered the spinal cord.

Neurons and Associated Cells

Neurons in the spinal cord explant appeared to undergo changes similar to those in the DRG. Because serial photographic observation of the spinal cord was much more difficult, only observations of the neurons in the DRG are presented here (Fig. 5). The cytopathic changes in nerve cells infected by HSV 1 appeared similar to those in cells infected by HSV 2, as previously observed (23).

Although satellite cell nuclei became prominent by about 12 hours p.i., earliest neuronal changes were not observed until 24 hours p.i. when the nucleus became eccentric and the cytoplasm more granular. The nucleus then began to swell and by 36 hours p.i., appeared like a bag of fluid. The cytoplasm then became heterogenous, the nucleus continued to swell, the nucleolus became irregular and fragmented (44 hours p.i.), and the whole cell finally disintegrated after 48 hours p.i.

Formation of Syncytia

Syncytia were observed in HSV 1 infected cultures but not in cultures infected with HSV 2. Syncytia were observed in about half the spinal cord.

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**Fig. 5.** The same region of central myelin in a living culture before and after infection with HSV 2; photographed using bright field microscopy at different hours (h) post infection (×750). The appearance of the changes in central myelin after infection with HSV 1 is very similar, but progresses more slowly. A. Before infection, the myelin is smooth except for a few knob-like projections. B. By 24 hours p.i. the myelin appears as chains of bubbles. C. By 36 hours p.i. only a few myelin blebs remain.
ganglion cultures infected with HSV 1 and, when present, numbered at least 20. They had two or three nuclei at about 14 hours p.i. and increased in size to an average of 18 nuclei by 40 hours p.i.

To determine whether the spinal cord, the DRG, or both were involved in the syncytial formation, cultures of spinal cord or DRG alone were infected with HSV 1 or HSV 2. The results are presented in Table 1, from which it may be concluded that syncytia are formed when spinal cord tissue was present and not when cultures of DRG alone were infected.

Observations on Fixed Cultures after Silver Impregnation (Palmgren Technique)

The appearance of axons in spinal cord-ganglion cultures at 6, 12, 18, 24, 30, 36 and 48 hours after infection with either HSV 1 or HSV 2 was very similar, and was indistinguishable from that seen in simultaneously fixed, uninfected control cultures. In cultures in which at least 80% of the peripheral myelin had undergone degeneration (36 hours p.i. with HSV 1; 30 hours p.i. with HSV 2), neuron cell bodies with their emergent smooth intact axons could be found in the fascicles of the dorsal roots in either control (Fig. 6A) or infected cultures (Fig. 6B).

DISCUSSION

Although organotypic nerve cell tissue cultures have been used in studies involving herpes simplex virus types 1 or 2 (3, 8, 10, 12, 13, 24), there has been no systematic comparison of the effects of these viruses in the cultures until recently (23). However, in that report, only the effects on peripheral nervous tissue were considered. The present study was therefore undertaken to compare the cytopathic effects of HSV 1 with that of HSV 2 in cultures of both central and peripheral nervous system, with special regard to their effects on myelin.

Cultures of mouse spinal cord with attached DRG are an excellent model for such studies because they are composed of a unit of tissue containing both central and peripheral nervous system elements which can be simultaneously infected with a single inoculum of virus. The extensive myelin formation in well-defined, easily-located regions, makes this model especially useful for any study where the interaction of chemical or biological agents with myelin is an

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MM = maintenance medium, containing horse serum.
GM = growth medium, containing human placental cord serum.
important issue. A particular advantage of organotypic cultures is that individual cells or segments of myelin can be studied sequentially during infection without disturbing the living culture and without the necessity of attempting to interpret dynamic phenomena from the appearance of cells fixed and stained at different times after infection.

In spinal cord-ganglion cultures infected with either HSV 1 or HSV 2 there was degeneration of both central and peripheral myelin. In peripheral nervous tissue, the pattern of myelin degeneration associated with HSV 1-infected cultures was different from that in HSV 2-infected cultures. Type 1 was characterized by focal dilatations; type 2, by "sausage-shaped" swellings, and the cytopathic effects of HSV 2 began earlier than those of HSV 1. This confirmed the previous findings of Whetsell et al. (23). In the case of both viruses, the earliest changes were centered around the Schwann cell nucleus with subsequent spread along the myelin sheath, so that the distortion of the sheath was a relatively earlier phenomenon. The peripheral myelin appeared to degenerate in a segmental fashion.
In central nervous tissue, the appearance of degenerating myelin due to HSV 1 was indistinguishable from that due to HSV 2. Although cytopathic effects of both viruses began about 14 hours p.i., the rate of degeneration of central myelin in cultures infected with HSV 2 was faster than in cultures infected with HSV 1. The changes in the central myelin appeared similar to those reported by Bornstein (2) following application of serum from animals with experimental allergic encephalomyelitis or from patients with multiple sclerosis or organotypic cultures of mouse cerebellum.

Changes in peripheral myelin were seen before changes in central myelin with either virus type. This could reflect either a true difference in susceptibility to virus infection or a difference in the accessibility of the myelin types. However, once cytopathic effects were noted in central myelin, they appeared to progress more rapidly and to involve more myelinated fibers in a given area than did degeneration of peripheral myelin. The faster rate of myelin degeneration in the central nervous tissue may be because infection of one oligodendroglial cell could result in the disruption of several pieces of myelin, whereas infection of one Schwann cell in the peripheral nervous tissue would be followed by disruption of only one myelin segment.

Degeneration of neurons was similar in cultures infected with either HSV 1 or HSV 2, and this corroborated previous studies of herpes virus infection of organotypic cultures (8, 10, 23, 24). Cytopathic effects began at 20–24 hours p.i., and led to degeneration of cells by 48 hours p.i. The question arose as to whether the degeneration of myelin was independent of neuronal degeneration or whether it was secondary to dysfunction or death of the neuron or axon. Data were difficult to assess for central myelin, but in peripheral myelin, the earliest cytopathic changes began 8–18 hours before pathological changes were demonstrable in neurons by light microscopy. In the presence of morphologically intact neurons, it is unlikely that neuron dysfunction could explain the degree of myelin degeneration observed in our experiments. Individual segments of myelin along a single axon did not degenerate in a sequential fashion along the axon as they would have if degeneration were dependent on neuron death. Cytopathic changes began with alterations in the Schwann cell nuclei and resulted in their loss; if myelin were disrupted as a consequence of neuron death, one would expect the Schwann cell nuclei to remain intact (20). Furthermore the preservation of smooth, continuous axons in the presence of extensive myelin degeneration in infected cultures was demonstrated by the Palmgren silver impregnation technique. Our observations, therefore, strongly suggested that the myelin degeneration occurred as a direct viral effect.

In conclusion, we found that in spinal cord-ganglion cultures which contained both peripheral and central nervous system elements, infection with either HSV 1 or HSV 2 led to breakdown of myelin. The myelin degeneration, at least in the peripheral nervous tissue in this system, did not appear to be dependent on neuronal dysfunction or death, but was a direct result of virus infection. Our results also confirm the greater neurovirulence of HSV 2 compared to HSV 1 in organotypic nerve cell cultures (23).
REFERENCES


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