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

2. Electron Microscopic Observations of Myelin Degeneration

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ABSTRACT

Organotypic cultures of mouse spinal cord with attached dorsal root ganglia, which contain both central and peripheral myelin in the one unit of tissue, were infected with HSV 1 or HSV 2 and studied using electron microscopy. Intracellular viral nucleocapsids and intracytoplasmic enveloped particles were found in the Schwann cells associated with peripheral myelin and in oligodendroglia associated with central myelin. Degeneration of peripheral myelin most commonly involved an asymmetrical swelling of the myelin lamellae, whereas degeneration of central myelin was characterized by a more generalized swelling resulting in separation of the myelin lamellae. Degeneration of both central and peripheral myelin was found in the presence of intact axons which were indistinguishable from those in controls.

INTRODUCTION

In a previous paper (7) we described a light microscopic study of the effect of herpes simplex virus types 1 and 2 (HSV 1 and HSV 2) on organotypic cultures of mouse spinal cord with attached dorsal root ganglia (DRG), which contain both central and peripheral myelin in one unit of tissue. We presented evidence which suggested that 1) both HSV 1 and HSV 2 cause loss of both central and peripheral myelin, 2) the cytopathic effect of type 2 begins earlier, and is completed sooner, than type 1, 3) the appearance of cytopathic changes in peripheral myelin following HSV 1 infection was different from those following infection with HSV 2, 4) the loss of peripheral myelin was not dependent on neuronal loss or dysfunction, i.e. was not Wallerian degeneration as had been supposed by others (18). Similar evidence, that loss of central

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myelin was independent of neuronal loss, was difficult to assess at the light microscopic level because of the complexity of the spinal cord fragment.

We now present the results of an electron microscopic study of these same spinal cord-DRG cultures, which provides direct evidence of virus replication in the cells responsible for the synthesis and maintenance of peripheral myelin (Schwann cells) and central myelin (oligodendroglia), and which also demonstrates that axons are preserved in the presence of degenerating central or peripheral myelin.

MATERIALS AND METHODS

Mouse spinal cord-dorsal root ganglia cultures (cord-ganglion cultures)

Transverse sections of mouse spinal cord, each with an attached pair of dorsal root ganglia, were prepared from 13-14 day old mouse fetuses as already described (7). Two fragments were placed on each collagen-coated glass coverslip and incubated at 34.5°C in a Maximow slide assembly (1). Cultures were grown in a medium containing 50% Eagle's minimum essential medium (MEM), 33% serum, 10% chick embryo extract and supplemented with 600 mg/ml of glucose. The source of serum for the first 21 days in vitro was human placental cord serum, and thereafter was fetal bovine serum (6) which has no inhibitory effect on the virus. 500 units/ml of nerve growth factor was added at explantation but was not incorporated into the medium afterwards.

Viruses and virus infection of cultures

The macroplaque strain of HSV 1 and the MS strain of HSV 2, used and described previously by Whetsell, et al. (17), were again utilized in this study. Mature, well-myelinated cultures, 26-31 days in vitro, were washed with balanced salt solution and 0.04 ml virus inoculum (1 × 10^4 TCID_50/ml in Eagle's MEM plus 10% fetal bovine serum) adsorbed for 2 hours at 37°C. Cultures were refed, remounted in the Maximow slide assemblies, and incubated at 37°C.

At 24, 36, 48, 65 and 75 hours post infection (p.i.) a culture infected with HSV 1, one infected with HSV 2 and a mock-infected control culture were each fixed for electron microscopy by immersion in cold 2% gluteraldehyde for one hour and postfixed in cold OsO_4 for one hour. Cultures were then dehydrated, embedded in Epon and the two explants were reoriented (4) and sectioned for light microscopy (stained with 1% aqueous toluidine blue), or electron microscopy (stained with uranyl acetate and lead acetate) and viewed, using an Hitachi 12A microscope. It was therefore possible to maintain the orientation of each of the 2 fragments and to section, for example, either the DRG, the dorsal root or the spinal cord, as required. The experiment was repeated two more times.

To relate these experiments to our previous light microscopic study (7), segments of myelin were located in the outgrowth region of a few selected cultures, the cultures were infected, and these same segments of myelin were viewed during the infection. When the myelin showed severe degeneration, the cultures were fixed and stained by the Palmgren silver impregnation technique (14) to determine the state of preservation of the axons in the presence of myelin breakdown.

RESULTS

Light Microscopic Observations

Living and silver-impregnated cultures. Figure 1 summarizes some of our findings as seen with the light microscope; these were further observed using the electron microscope. Panel A shows some segments of myelin in the outgrowth zone of a cord-ganglion culture prior to infection. The Schwann cell
nuclei are flattened along the sheath and nodes of Ranvier are visible. Panel B shows this same piece of myelin in the living culture 36 hours after infection with HSV 1. The Schwann cell nuclei are contracted, and myelin is in an advanced state of degeneration showing asymmetrical focal swellings on one side of the fiber. The culture was fixed at this time and stained by Palmgren's silver impregnation technique to demonstrate the axon profile (Panel C). It can be seen that, in the presence of advanced myelin degeneration, the axon remains smooth and intact.

**1μ sections.** Sections were cut at right angles to the plane of the culture and axons, with a sheath of peripheral myelin, were seen in a bundle in the dorsal root, or interspersed between neurons in the ganglia. Central myelin was located in a crescent-shaped band around the edge of the spinal cord. When sections of infected cultures were compared to those of mock-infected control cultures, it was seen that at later times following infection there was a progressive decrease in the amount of myelin and a concomitant increase in cellular necrosis. This was more marked in sections from cultures infected with HSV 2 than in those from HSV 1-infected cultures. By 65 hours p.i. (HSV 2) or 75 hours p.i. (HSV 1) myelin could not be found and necrotic cells remained.

**Electron Microscopic Observations**

**Peripheral Myelin.** Degenerating peripheral myelin was found as early as 24 hours p.i. in cultures infected with either HSV 1 or HSV 2, although it was more frequent in sections of HSV 2-infected cultures. The proportion of myelin
showing cytopathic changes increased at later periods following infection so that by 36 hours p.i. (HSV 2) or 48 hours p.i. (HSV 1) approximately 80% of the myelin was involved. Thereafter, the amount of myelin seen in the sections decreased dramatically.

The degenerating myelin in cultures infected with HSV 1 was indistinguishable in appearance from that in HSV 2-infected cultures. The earliest stage of degeneration appeared to be an asymmetrical swelling of the outermost lamellae of the myelin sheath (Fig. 2A) and this may or may not precede the appearance of degenerating myelin seen in Figures 2B and 2C. In Figure 2B, the asymmetrical swelling of the outermost lamellae appears to have become so extreme that they have “burst” although the plasma membrane and basal lamina of the Schwann cell are intact. The appearance of the myelin in Figure 2C may be the result of swelling of the innermost tongue of Schwann cell cytoplasm so that the myelin sheath is no longer in close proximity to the axon. Focal disruptions of the myelin sheath are also seen. However, in Figures 2A, 2B and 2C, the axons appear normal and intact.

Virus replication in Schwann cells engaged in myelin formation was found in cultures infected with either HSV 1 or HSV 2. Several nucleocapsids, both hollow and dense-cored, can be seen in the nucleus of a Schwann cell infected with HSV 1 (Fig. 3A). The Schwann cell infected with HSV 2 (Fig. 3B) has both nucleocapsids and tubular structures, 16-18 nm in diameter, in the nucleus. Once again, the axons do not show any abnormalities.

Schwann cells not involved in myelination also showed evidence of infection with HSV 1 or HSV 2. There was margination of chromatin, reduplication and thickening of the nuclear membrane, and the appearance of intranuclear nucleocapsids which were enveloped at the outer nuclear membrane.

Central Myelin

There was little change in the appearance of central myelin in sections from cultures 24 hours p.i. with either HSV 1 or 2. The abnormal changes were pronounced however, by 48 hours p.i. and had the same appearance in cultures to those infected with type 1 as those infected with type 2. There was a general swelling, resulting in separation of the myelin lamellae (Fig. 4A). Few “intermediate” stages were found; myelin was either very swollen in appearance or was similar to that of controls.

Both intranuclear nucleocapsids and enveloped virus particles were found in oligodendroglia infected with either HSV 1 or HSV 2. An oligodendroglium, which appears to have a process extending to form the myelin sheath of a nearby axon, is shown in Figure 4B. At this time, 36 hours p.i. with HSV 1, intranuclear viral nucleocapsids and intracytoplasmic enveloped virus particles are seen. Enveloped virus particles were also occasionally seen in the inner tongue of the oligodendroglial cytoplasm and more rarely between the outer myelin lamellae.

DISCUSSION

This study supports certain conclusions drawn from our light microscopic
Fig. 2. Electron micrographs of cross-sections of peripheral myelin in mouse spinal cord-DRG cultures showing signs of myelin degeneration in the presence of intact axons (uranyl acetate-lead acetate stain). A. 24 hours p.i. with HSV 1. The outer lamellae have become separated from the myelin sheath (×12,000). B. 24 hours p.i. with HSV 2. The separated outer lamellae appear to have "burst" although the Schwann cell plasma membrane is still intact (×12,000). C. 48 hours p.i. with HSV 1. The myelin sheath has undergone severe degeneration although the axon remains intact (×14,400).

observations upon living spinal-cord-DRG cultures infected with HSV 1 or HSV 2 (7, 17): that both viruses cause loss of central and peripheral myelin, that the cytopathic effect of type 2 begins earlier than that of type 1, and that, in the presence of degeneration of peripheral myelin, the axons may show no
Fig. 3. Electron micrographs of the nuclei of Schwann cells involved in myelin formation in cultures of mouse spinal cord and DRG (uranyl acetate and lead acetate stain). A. 24 hours p.i. with HSV 1. Numerous viral nucleocapsids are present in the Schwann cell nucleus; the myelin sheath shows separation of outer lamellae while the axon within the myelin sheath appears intact (×12,000). B. 24 hours p.i. with HSV 2. There are viral nucleocapsids in the nucleus, as well as the tubular structures frequently observed in HSV 2 infection. Bundles of these can be seen in longitudinal and cross-sectional orientation (arrows) (×10,800). The area indicated by the right hand arrow is represented at a higher magnification in the inset (×36,000).

morphological changes. Electron microscopic observation has permitted extension of this last observation to the central nervous system where marked myelin degeneration was seen to occur without evidence of pathological changes in axons. This study has also demonstrated that both HSV 1 and HSV 2 replication can be supported by the cells responsible for synthesis and maintenance of peripheral myelin (Schwann cells) or central myelin (oligodendroglia).

The myelin associated with the infected cells appeared to swell and degenerate. In the previous light microscopic study of living cultures, degenerating peripheral myelin was characterized by either focal dilatations (HSV 1) or more generalized “sausage-shaped” swellings (HSV 2) (7, 17). As seen by electron microscopy, however, it was not possible to distinguish appearances of myelin breakdown that were characteristic for a virus type from the cross-sections of myelinated fibers. We suggest that the differences in degeneration, observed in the living cultures, probably reflect the greater virulence of HSV 2 infection rather than any inherent difference in the way the viruses infected myelinating cells.

The changes observed in central myelin probably reflected a non-specific pathological change resulting from the limited way in which oligodendroglia,
Fig. 4. Electron micrographs of cells in the spinal cord portion on mouse spinal cord-DRG cultures, 36 hours p.i. with HSV 1 (uranyl acetate and lead acetate stain). A. Separation of myelin lamellae appears to occur at the intraperiod line. The same appearance is observed in central myelin after HSV 2 infection, and is indistinguishable from HSV 1 infection (×52,000). B. An oligodendrocyte, with a process probably forming the myelin around the axon seen in the lower right corner, contains a clump of nucleocapsids (nc) and enveloped virus particles (arrows) (×10,000). Similar evidence of virus replication was seen in oligodendroglia infected with HSV 2.

and their associated myelin, can react to noxious stimuli. Similar changes have been described when organotypic spinal cord cultures have been incubated with serum from patients with multiple sclerosis or from animals with experimental allergic encephalomyelitis (3, 15, 16).

Organotypic nerve cell tissue cultures have been used to observe the cytopathic effect of HSV 1 or HSV 2 in several studies (2, 5, 8, 9, 10, 13), but the effects of these viruses on myelin had not been considered until recently when Whetsell, et al (17) compared the effects of HSV 1 and HSV 2 on peripheral myelin. Subsequently, Ecob-Johnston, et al. (7) extended these observations, at the light microscopic level, to the central as well as the peripheral nervous system. Prior to the present report no systematic electron microscopic study of myelin degeneration following HSV infection was available, although several authors had made passing comments to this phenomenon. Ziegler & Pozos (1976), in an electron microscopic study of HSV 2 infection of rat DRG, described the degeneration of peripheral myelin as Wallerian and stated that although satellite and Schwann cells produced enveloped virus, “few changes were seen in myelinating Schwann cells.” We have shown that both HSV 1 and HSV 2 can infect myelinating Schwann cells. (This observation was probably made easier in our culture system where there is extensive myelin formation in easily located areas, such as the dorsal root, as compared to cultures of DRG alone). Our experiments have also demonstrated (7, and this
paper) that the demyelination following herpes virus infection in cultures of mouse peripheral nervous system, and probably that of central nervous system, as well, cannot be regarded as Wallerian degeneration alone. Dubois-Dalcq, *et al.* (5), in an electron microscopic study of HSV 2 in mouse DRG and spinal cord described a disruption of infected Schwann cell plasma membranes and their subsequent loss of association with axons. (There was no mention of effects on peripheral myelin sheaths). We did not find that disruption of the Schwann cell plasma membrane was an initial response to virus infection, and in fact this appeared intact even when the myelin lamellae had split (Fig. 2B).

In electron microscopic studies of organotypic cultures of hamster or rat cerebellum infected with HSV 1 (13) or HSV 2 (9), and of immature 3 day chick DRG infected with either virus (10) there was no mention of viral effects on myelin.

In an *in vivo* study, Kristensson *et al.* (11) reported inoculation of HSV 2 into the left eye of rabbits and the observation of spread of virus into the axons and into the glial cells along the optic tract. Virus was also found in the nuclei and cytoplasm of oligodendroglial cells, as well as into the inner loop of the myelin sheath and between the outer myelin lamellae. Subsequently, using this same system, Kristensson and Wisniewski (12) described an arrest of myelination followed by demyelination after herpes virus inoculation, much as we have described it in our *in vitro* model.

In conclusion, we have demonstrated direct evidence for HSV 1 and HSV 2 replication in myelin-producing Schwann cells and oligodendroglia, and the breakdown of both peripheral and central myelin in the presence of intact axons.

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