Can Oligodendrocytes Attached to Myelin Proliferate?

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The uptake of thymidine by oligodendrocytes in the brains of adult mice was examined after the induction of cortical traumatic lesions in an attempt to determine whether mature oligodendrocytes actively attached to myelin sheaths were capable of proliferating. In view of the great difficulty in visualizing the connection between a given oligodendrocyte and a myelin sheath even in the normal adult animal, the neuropil was made edematous in the traumatized animals in order to separate out the components and to study the oligodendrocyte processes. Uptake of tritiated thymidine was found in oligodendrocytes, as well as in endothelial cells, astrocytes, and microglia. The percentage of labeled oligodendrocytes appeared low in relation to the total number of the oligodendrocytes. In addition, in a few labeled cells, cytoplasmic processes could be seen extending to and apparently forming the myelin sheath. The possibility that a differentiated cell still attached to myelin may at the same time be able to proliferate is of great significance in understanding the potential for remyelination and recovery, in the adult, because it would imply that any oligodendrocyte is a candidate for proliferation, rather than only uncommitted or immature glial cells, which may be limited in the mature brain.

The importance of regeneration of oligodendrocytes and their role in remyelination has been receiving increasing attention recently in view of the possible potential recovery from demyelinating disease. The relationship between oligodendrocyte proliferation and remyelination has been shown to be of significance (Debbage, 1985; Manuelidis and Manuelidis, 1985; Raine and Traugott, 1985; Ludwin, 1987). Although it was felt for a long time that oligodendrocytes in adult animals were not capable of regeneration or proliferation, this has been shown in oligodendrocytes following demyelination induced by Cuprizone (Ludwin, 1979) and JHM hepatitis virus (Herndon et al., 1977). In the former case, it appeared that the cells were derived from less mature forms that matured. The ability of mature oligodendrocytes to take up thymidine was recently shown in response to trauma (Ludwin, 1984, 1985) and to lysolecithin injection (Arenella and Herndon, 1984). This finding was confirmed *in vitro* by Wood and Bunge (1986b) who showed that

oligodendrocytes from adult animals proliferated in response to exposure to axons. The critical question raised in all these experiments, however, was whether proliferation occurred in oligodendrocytes still attached to myelin sheaths or whether it could occur only in differentiated oligodendrocytes "uncommitted" to myelin formation or in less mature cells. This is of great significance in understanding the reserve capacity of oligodendrocytes to regenerate myelin. The demonstration of continuity between the cytoplasmic membrane of a given oligodendrocyte and a myelin sheath is extraordinarily difficult in the adult animal due to the tight packing of the neuropil and the very slender oligodendrocyte processes; indeed, it has been demonstrated only very infrequently in the literature (Hirano, 1968). In the latter case, this was possible only because the neuropil had been spread apart by edema. It was therefore decided to look for connections between myelin sheaths and mature oligodendrocytes that had taken up thymidine following trauma in the adult animal, in whom visualization of the connections was facilitated by the induction of significant edema.

Materials and Methods

Adult white mice, between 3 and 4 months of age, were anesthetized with methoxyfluorane, and after a craniotomy was performed over the dorsal surface of the right parieto-occipital region, a small piece of cortex, measuring about 1 mm in diameter, was removed from the cortex as has been described previously (Ludwin, 1984, 1985). A small amount of underlying white matter was often removed as well. In addition, an attempt was made to increase the edema around the wound by rubbing the adjacent cortex and abrading the tissue with a blunt forceps. The wound was packed with gelfoam and the skin sutured. Forty animals were operated upon. Either 2 or 3 d after craniotomy, the animals were injected with tritiated thymidine (6 μ Ci/gm body weight) and either 12 or 24 hr later were perfused with Karnovsky's solution. The brains were removed, and extensive samples of tissues from around the wound in both the gray and white matter were taken. The specimens were embedded in Epon and prepared for electron microscopic autoradiography as has been described previously (Ludwin, 1979). In contrast to previous experiments, however, where intact tissue was preferentially examined, in the present experiments in selecting material for ultrastructural examination from 1- μ m-thick sections an attempt was made to look at areas where large amounts of edema separated the elements of the neuropil. This allowed for better visualization of connections between the oligodendrocytes and the myelin sheaths.

Results

In both the gray and white matter, widespread edema had separated the myelinated axons, cells, and cellular processes. The findings 3 d after surgery were very similar to those seen after 4 d, except that in the latter there appeared to be slightly more debris in the macrophages. Some of the myelin sheaths appeared distorted with separation of the lamellae, and many axons were disrupted. Nuclear silver grains indicating uptake of tritiated thymidine were well seen in endothelial cells, astrocytes, and

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Figure 1. Typical mature (dark) differentiated oligodendrocytes from the cortex (*a*) and white matter (*b*) around the wound of an adult mouse 3 d after craniotomy and trauma and injected with tritiated thymidine 12 hr before. In *a* there are many nuclear grains, whereas in *b* only a single grain is seen. In *b* the cytoplasm is intimately associated with surrounding myelinated axons, although it cannot be proved that this is the actual

debris-filled macrophages. In addition, many cells interpreted as microglia, with characteristic morphology as described before (Ludwin, 1985) had taken up thymidine. Macrophages and microglia were the most numerous labeled cells. As was found previously, oligodendrocytes were also found with nuclear grains. It should be pointed out that, although this was a readily observed phenomenon, the great majority of oligodendrocytes did not take up label. The morphology of oligodendrocytes and their differentiation from microglia have been discussed before (Ludwin, 1985; Peters et al., 1976). Silver grains were seen both in typical dark mature oligodendrocytes (Fig. 1, *a, b*), with a dark cytoplasm, a high microtubular content, and prominent Golgi apparatus, as well as medium oligodendrocytes (Fig. 1*c*). There was also a variation in the number of grains seen: These were numerous in some cells (Fig. 1*c*) and moderate in others (Fig. 1*a*); at times, only a single grain was seen (Fig. 1*b*). It appeared in many cases that cells with small numbers of grains were more mature cells. In many cases, labeled oligodendrocytes were associated with myelinated axons, whose outer or inner tongues appeared similar to the cytoplasm. These axons were often arrayed around the cell soma (Fig. 1*b*) or adjacent to cell processes.

The widespread edema separating the various constituents of the neuropil allowed a much greater visualization of the cytoplasmic processes of the oligodendrocytes which could be seen extending at a distance away from the perikaryon. These processes were often very slender (Figs. 1, *d, e*; 2, *a, b*) but could also be broader (Fig. 2, *c, d*). In a very small number of cells, (± 10) the cell membrane of cytoplasmic processes of oligodendrocytes in whose nuclei silver grains were present appeared to be in continuity with the outer cytoplasmic tongue and to form the myelin sheath (Figs. 1, *d, e*; 2, *a-d*). In other cells, although there was a strong suggestion of continuity, this could not be proven (Fig. 2, *e, f*). This method of ultrastructural autoradiography displays the nuclear grains directly on the same section where the cytoplasmic processes are being studied and is advantageous in proving that the identical cell had taken up thymidine; it has, however, the disadvantage of not allowing for easy serial sectioning, and this decreased the number of connections that could be visualized.

Discussion

The results of this experiment strongly suggest that in response to trauma differentiated oligodendrocytes attached to myelin sheaths take up tritiated thymidine. Although it is possible that uptake of tritiated thymidine may represent a process other than mitosis, such as DNA repair, this is most unlikely, especially in the heavily labeled nuclei and in the absence of any apparent reason for DNA damage. Uptake of tritiated thymidine has always been the hallmark of proliferation of CNS cells during development (Altman, 1966; Dalton et al., 1968; Skoff, 1975; Imamoto et al., 1978; Paterson, 1983) and as a response to pathological injury (Mori, 1972; Skoff, 1975; Willis et al., 1976; Adrian et al., 1978; Kitamura et al., 1978; Gall et al., 1979;

Latov et al., 1979; Ludwin, 1979, 1984, 1985; Marabe et al., 1982; Skoff et al., 1982; Arenella and Herndon, 1984). This is especially so *in vivo*, where often the degree of proliferation is too low to allow for meaningful cell-counting techniques, whereas in culture, cell counting is much more feasible. The significance of the observation that a myelinating oligodendrocyte in adults may be capable of proliferation is of profound importance in remyelination. It has been suggested that the capacity for remyelination following demyelinating diseases is strongly associated with the number of oligodendrocytes (Ludwin, 1978, 1987; Raine et al., 1981; Raine and Traugott, 1985), and the source for the regeneration of those cells has been under investigation in recent years. Oligodendrocytes involved in remyelination were first shown to have regenerated following JHM hepatitis virus demyelination by Herndon et al. (1977), and their origin from less mature forms was demonstrated in the Cuprizone model of demyelination (Ludwin, 1979). It had always been held that mature oligodendrocytes in adult animals were incapable of proliferation and that the reason for poor CNS remyelination in adults was a paucity of reserve immature glia to provide for oligodendrocyte regeneration. However, recently it has been shown that mature oligodendrocytes take up thymidine in response to pathological insults induced by trauma (Ludwin, 1984, 1985) and lyssolecithin (Arenella and Herndon, 1984). In addition, Wood and Bunge (1986a) showed not only that cells taken from adult rats could myelinate dorsal root ganglion neurons in culture, but also that proliferation of these adult oligodendrocytes *in vitro* occurred in response to exposure to axons (Wood and Bunge, 1986b). These authors questioned whether this proliferation took place in cells already involved in myelination, as they were unable to show MBP positivity in the proliferating galactocerebroside-positive oligodendrocytes. They also wondered whether there was a subpopulation of differentiated oligodendrocytes in the mature brain not attached to myelin, which could have served as a reserve source of proliferating cells (Wood and Bunge, 1984, 1986b). The present experiments suggest that even if this attractive hypothesis is correct, at least some myelinating oligodendrocytes attached to myelin sheaths may be able to take up thymidine.

There is a suggestion that a similar process may be occurring in the PNS, where Griffin et al. (1987) have shown that in response to paranodal demyelination with IDPN, localized Schwann cells adjacent to the axon and within the basement lamina proliferate. Although these authors could not show continuity of the Schwann cell membrane with the myelin sheath, the other features strongly suggested that Schwann cells still attached to myelin were proliferating.

The relationship between differentiation and proliferation is an interesting one. In normal development, proliferation of oligodendrocytes usually stops before myelination starts (Skoff et al., 1976), and even though myelinating proteins are expressed in oligodendrocytes before the onset of myelination (Sternberger et al., 1978, 1979), they also only appear after proliferation

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myelinating, cell. Ultrastructural autoradiography. *a*, $\times 10,590$; *b*, $\times 16,500$. *c*, Oligodendrocyte from the white matter of an animal, similar to that in *a* and *b*, displays many nuclear grains in this autoradiograph. Although the cell is a typical oligodendrocyte, it resembles more the medium type. Note the myelinated axons widely separated by edema. Ultrastructural autoradiography, $\times 22,000$. *d*, Heavily labeled oligodendrocyte from the white matter of an animal similar to that in *a* and *b* is surrounded by extensive edema formation. Many of the axons appear to be damaged. Arrow marks an apparent cytoplasmic connection between the cell and a myelinated axon. *e*, High magnification of the cytoplasmic connection demonstrates the fusion of the cytoplasmic membrane to form the major dense line of the myelin sheath. *d*, $\times 8600$; *e*, $\times 96,000$.

Figure 2. *a*, Oligodendrocyte from the white matter of an animal similar to that shown in Figure 1*b*. The nuclear grains in this cell resemble those seen in Figure 1*b*. From one end of the cell, a cytoplasmic tail extends toward a myelinated axon (*arrow*). *b*, High-magnification view of the area depicted by the arrow in *a* shows the cytoplasmic tail in continuity with the outer tongue, which apparently forms the myelin sheath. *a*, $\times 6750$; *b*, $\times 69,750$. *c*, Heavily labeled oligodendrocyte lying among myelinated fibers spread apart by edema fluid. The preparation is as has been described before. There is some dilatation of the cisternae of the rough endoplasmic reticulum. The cytoplasmic extension to the myelinated axon (*arrow*) is shown at higher magnification in *d*, where it apparently is in continuity with the myelin sheath. *c*, $\times 18,000$; *d*, $\times 55,000$. *e*, Labeled oligodendrocyte

ceases (Friedrich and Sternberger, 1984). Although Sturrock and McRae (1980) claimed to show mitosis in a cell attached to a myelinating axon, this could not be confirmed by Meinecke and Webster (1984). In dissociated cell culture, differentiation and proliferation may occur together (Pettmann et al., 1980; Bologna et al., 1982; Sensenbrenner et al., 1982a, b), whereas in organotypic culture (Manuelidis and Manuelidis, 1971; Munoz-Garcia and Ludwin, 1986a, b), the situation resembles that *in vivo*. The situation following pathological injury may, however, not be the same as in the normal animal. Indeed, both normal and reactive astrocytes continue to proliferate even when they display features of differentiation, such as glial filaments and GFAP (Latov et al., 1979; Ludwin, 1979; Munoz-Garcia and Ludwin, 1986a, b).

As has been discussed previously (Ludwin, 1985), glia of many kinds proliferate in response to injury. Microglia take up thymidine (Adrian et al., 1978; Kitamura et al., 1978; Marabe, 1982) in response to trauma and toxic injury, as do astrocytes (Latov et al., 1979) and subependymal cells (Willis et al., 1976), and glia can proliferate after deafferentiation (Gall et al., 1979). In young rats labeled oligodendrocytes have been found after stab wounds (Mori, 1972). It is not known what specific factors may be stimulating this glial reaction, but it has recently been shown that macroglial proliferation, including oligodendrocytes, may be stimulated by either lymphokines (Merrill et al., 1984) or by regenerating nervous system extracts (Giulian, 1984; Giulian et al., 1985).

Finally, it should be pointed out that the very small number of cells seen with connections to the myelin sheaths is not surprising. Although these connections are relatively easily found in developing animals (Peters, 1964), they are virtually impossible to demonstrate in normal adults because of the tightly packed neuropil and the attenuation of the processes. Indeed, the continuity of an oligodendrocyte membrane with the myelin sheath has only been convincingly shown in the adult in a single illustration (Hirano, 1968), where the tissue had been separated by edema. In the present study, many of the labeled oligodendrocytes were typical and mature (Peters et al., 1976), but most did not show these connections.

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from an animal similar to those shown previously. *f*, High-magnification view of the upper-left end of the cell. Microtubules (arrows) can be seen in the electron-dense cytoplasm. A cytoplasmic extension reaches a small myelinated axon after passing between 2 other axons. Although suggestive, there is no firm evidence that this cell process is actually connected to the myelin sheath. *e*, $\times 10,000$; *f*, $\times 30,000$.

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