

Myelin-associated Glycoprotein and Myelinating Schwann Cell-Axon Interaction in Chronic B,B'-Iminodipropionitrile Neuropathy

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ABSTRACT The myelin-associated glycoprotein (MAG) is a heavily glycosylated integral membrane glycoprotein which is a minor component of isolated rat peripheral nervous system (PNS) myelin. Immunocytochemically MAG has been localized in the periaxonal region of PNS myelin sheaths. The periaxonal localization and biochemical features of MAG are consistent with the hypothesis that MAG plays a role in maintaining the periaxonal space of myelinated fibers. To test this hypothesis, MAG was localized immunocytochemically in 1- μ m sections of the L5 ventral root from rats exposed to B,B'-iminodipropionitrile. In chronic states of B,B'-iminodipropionitrile intoxication, Schwann cell periaxonal membranes and the axolemma invaginate into giant axonal swellings and separate a central zone of normally oriented axoplasm from an outer zone of maloriented neurofilaments. Ultrastructurally, the width of the periaxonal space (12–14 nm) in the ingrowths is identical to that found in normally myelinated fibers. These Schwann cell ingrowths which are separated from compact myelin by several micra are stained intensely by MAG antiserum. Antiserum directed against Po protein, the major structural protein of compact PNS myelin, does not stain the ingrowths unless compact myelin is present. These results demonstrate the periaxonal localization of MAG and support a functional role for MAG in maintaining the periaxonal space of PNS myelinated fibers.

Rats continuously exposed to B,B'-iminodipropionitrile (IDPN)¹ develop giant axonal swellings in proximal portions of large axons (3–5). These swellings contain accumulations of maloriented neurofilaments caused by a deficit in slow axonal transport of neurofilament proteins (9). In chronic states of intoxication these proximal axons undergo segmental demyelination, remyelination, and onion-bulb formation (10, 11, 19). In addition, multiple Schwann cell processes invaginate into the giant axonal swellings and separate the internodal axoplasm into two concentric compartments: a central zone of normally oriented axoplasm and an outer zone containing maloriented neurofilaments (11). These ingrowths

arise regularly from Schmidt-Lanterman incisures, consist of four membranes (axolemma, Schwann cell plasmalemma, Schwann cell plasmalemma, axolemma) with intervening Schwann cell cytoplasm and can result in nearly complete segregation of the maloriented neurofilaments from the central core of normally oriented axoplasm. These ingrowths represent an unusual response of Schwann cells to the neurofilamentous axonal changes produced by IDPN.

The myelin-associated glycoprotein (MAG) is an integral membrane glycoprotein (mol wt \approx 100,000) that is quantitatively a minor component (\approx 0.07%) of purified rat peripheral nervous system (PNS) myelin (8, 16). Within PNS fibers, MAG has been localized to the Schmidt-Lanterman incisures, the paranodal region, the other mesaxon, and to a ring corresponding to the innermost aspect of the myelin sheath adjacent to the axon (22, 25). This latter periaxonal ring has

¹ Abbreviations used in this paper: IDPN, B,B'-iminodipropionitrile; MAG, myelin-associated glycoprotein; PNS, peripheral nervous system.

been interpreted as staining of the adaxonal Schwann cell plasmalemma. Based on biochemical features of MAG, projection of the molecule into the periaxonal space has been proposed, and a role in the maintenance of the periaxonal space of myelinated fibers has been suggested (25). This localization has been challenged by recent studies of central nervous system fibers utilizing an avidin-biotin peroxidase or peroxidase-antiperoxidase technique to stain thin sections (7, 29). These preparations have suggested the localization of MAG within compact central nervous system myelin, with no periaxonal localization.

This report describes the immunocytochemical localization of MAG within the ventral roots of rats continuously exposed to IDPN for periods up to two years. The Schwann cell ingrowths into the giant axonal swellings in these roots provide a unique system for examining MAG localization, because the ingrowths consist of a complex composed of adaxonal Schwann cell plasmalemma and axolemma separated by several micra from the overlying myelin. Such spatial separation of the periaxonal complex from myelin is well suited to localization based on light microscopy and thick-thin comparisons. Using this approach, we found intense staining of the ingrowths using anti-MAG antiserum, but no staining with anti-Po antiserum. The absence of compact myelin in the ingrowths was confirmed by ultrastructural examination of the adjacent thin sections. These results support the presence of MAG in the periaxonal Schwann cell membrane, and are consistent with the hypothesis that MAG plays a role in maintenance of the periaxonal space and/or axon-Schwann relationships in myelinated fibers. Preliminary reports of this work have appeared in abstract form (26, 27).

MATERIALS AND METHODS

Sprague-Dawley rats were intoxicated for 24 mo with a 0.05% solution of IDPN (Eastman Laboratory and Specialty Chemicals, Eastman Kodak Co., Rochester, NY) in their drinking water. All animals were fed a standard laboratory chow diet ad lib.

For tissue fixation, animals were anesthetized, given heparin intravenously and perfused through the aorta with a saline rinse followed by 5% glutaraldehyde in 0.1 M phosphate buffer. After fixation, the fifth lumbar ventral horn, ventral root exit zone, and initial 5-mm segment of the L5 ventral root were dissected, postfixed in 1% osmium tetroxide, dehydrated in a graded series of alcohol, and embedded in Araldite. Serial 1- μ m thick sections were cut with glass knives, mounted on glass slides, and encircled with a diamond scribe. Even numbered sections were stained immunocytochemically with MAG antiserum; the others were stained with Po antiserum. Additional 1- μ m thick sections were cut adjacent to thin sections with silver interference colors. The thin sections were mounted on formvar coated slot grids, stained with uranyl acetate and lead citrate and examined in a Philips 400 electron microscope. The 1- μ m thick sections were stained with MAG or Po antiserum. Areas of MAG or Po staining in these 1- μ m thick sections were photographed, and their negative images were enlarged and compared with the fine structure in electron micrographs from identical areas of the adjacently cut thin sections.

1- μ m thick Araldite sections mounted on glass slides were placed in a 60–80°C oven for 24–48 h. Araldite was removed from the sections by sodium ethoxide as previously described (24). The slides were then placed in 0.2% hydrogen peroxide for 5 min, rinsed in 0.5 M Tris buffer, and stained with a (1:250) dilution of MAG antiserum or a (1:500) dilution of Po antiserum by the peroxidase-antiperoxidase method as previously described (24, 25). Following the immunostaining procedure some sections were counterstained with hematoxylin. All sections were examined microscopically with bright-field illumination.

MAG and Po antisera were prepared in rabbits. The purity of the MAG and Po proteins used in preparing the antisera and the immunological and immunocytochemical specificity of these antisera have been described (16, 17, 22–25).

RESULTS

Virtually all large spinal motor neurons from animals chron-

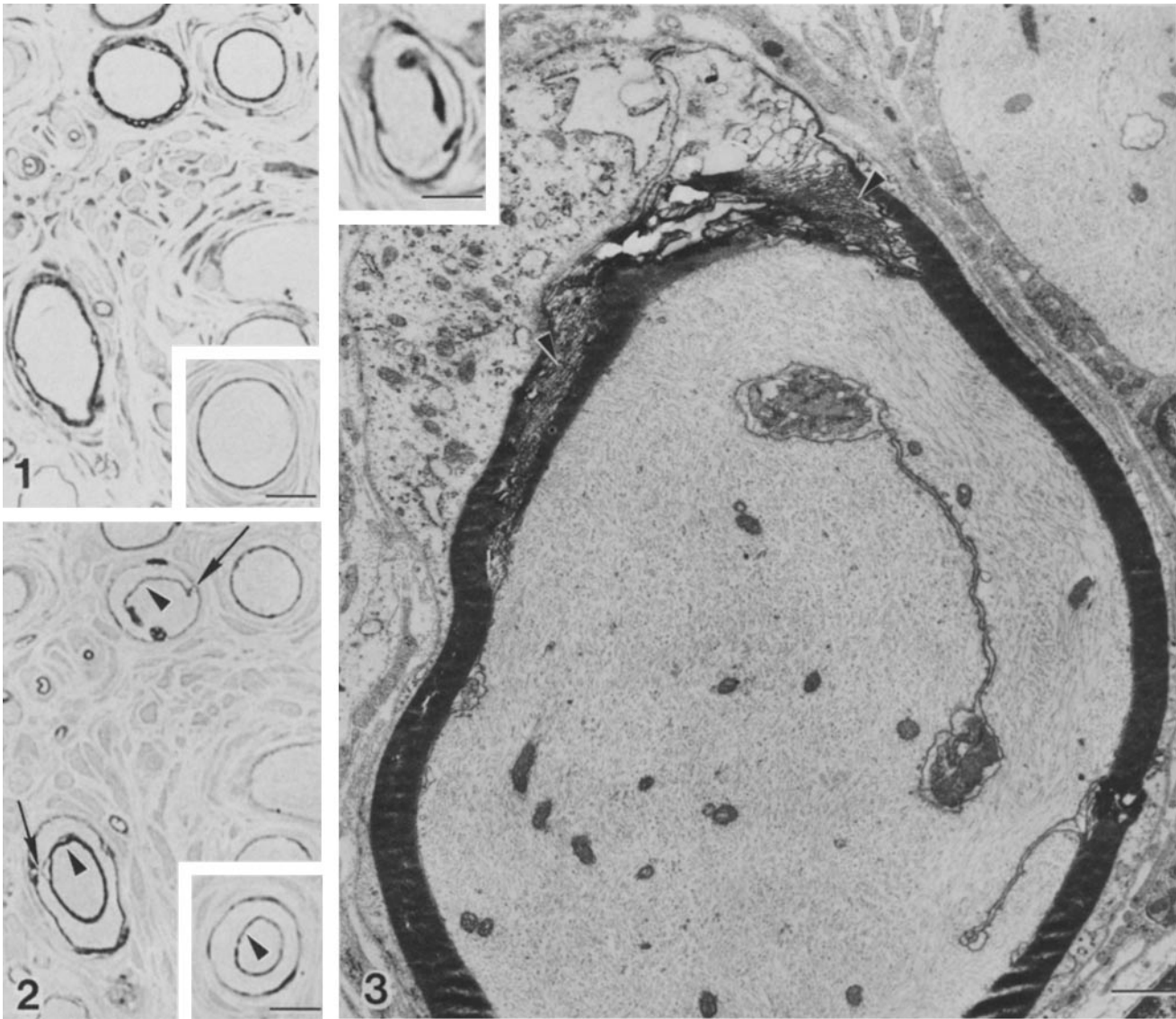
ically exposed to IDPN developed swellings of their proximal axons. Multiple, deep invaginations of these axons by Schwann cell processes occurred following demyelination, remyelination, and onion-bulb formation. Ultrastructurally, these changes were identical to those described in detail previously (11). In 1- μ m thick sections of the L5 spinal root from animals chronically treated with IDPN, the giant axonal swellings were surrounded by a ring of Po staining (Fig. 1). This staining represents compact myelin in these remyelinated fibers. When sections cut adjacent to the Po-stained sections were treated with MAG antiserum, a ring of staining surrounded the giant axonal swellings (Fig. 2). This ring of staining was generally narrower than the ring of Po staining. Occasional dark patches or bands of MAG staining partially surrounded these axons. This staining corresponded to the location of Schmidt-Lanterman incisures (see Figs. 3 and 4). In addition, MAG positive structures were present within the giant axonal swellings (Fig. 2, arrowheads). These structures were not stained by Po antiserum (Fig. 1) and were often continuous with the outer ring of MAG staining (Fig. 2, arrows). They formed a partial or complete ring of MAG staining within the axon.

These MAG positive invaginations were definitively identified by comparing the immunocytochemical distribution of MAG in 1- μ m thick sections to the fine structure in adjacently cut thin sections. All Schwann cell ingrowths identified in electron micrographs were stained intensely by MAG antiserum in the adjacent 1- μ m thick sections (Figs. 3–5). Most ingrowths arose from the base of Schmidt-Lanterman incisures (Fig. 3) and partially (Figs. 3 and 4) or totally (Fig. 5) separated the outer zone of maloriented neurofilaments from the central core of normally oriented axoplasm. These Schwann cell ingrowths were usually stained with a greater intensity than the periaxonal ring surrounding the axonal swellings. This increased staining intensity is probably due to the presence of two periaxonal membranes in each invagination (Fig. 6). The distance between the Schwann cell plasmalemma and axolemma (periaxonal space) in the ingrowths was 12–14 nm which is identical to the periaxonal space in myelinated fibers from normal peripheral nerve.

Occasional Schwann cell ingrowths were stained by both Po and MAG antisera (Figs. 7 and 8). Analysis of thin sections cut adjacent to 1- μ m thick Po stained sections demonstrated that these Po positive ingrowths were internal compact myelin sheaths that separated the outer zone of maloriented neurofilaments from the central core of normally oriented axoplasm (Fig. 9). These internal myelin sheaths have been described in detail (11).

DISCUSSION

The internodal periaxonal membrane of myelinating Schwann cells is separated from the axolemma by a 12–14-nm gap or periaxonal space (25). A collar of Schwann cell cytoplasm separates the Schwann cell periaxonal membrane from compact myelin. The periaxonal space and cytoplasmic collar are maintained in all myelinated fibers from normal peripheral nerve. Even in pathological conditions that result in axonal swelling (14, 21), axonal shrinkage (14), or swelling of the inner collar of Schwann cell cytoplasm (13, 14) dilation or compression of the periaxonal space is rare. Thus, the periaxonal space, which is the major interface between myelinating Schwann cell and axon, demonstrates a remarkable resistance to change (15).



FIGURES 1-3 Fig. 1: Composite of two 1- μm thick sections from the L5 ventral root of a rat chronically intoxicated with IDPN. These sections are stained with Po antiserum. Myelin sheaths surrounding axons are immunostained. Bright field. Bar, 10 μm . \times 750. Fig. 2: 1- μm thick sections cut serial to those in Fig. 1 and stained with MAG antiserum. A thin ring of MAG staining surrounds the outer perimeter of myelinated axons. MAG positive structures are present within three of the swollen axons (arrowheads) and totally or partially form an intra-axonal ring of MAG staining. Two of these structures are continuous with the periaxonal ring of MAG staining (arrows). The cells, scattered among the myelinated nerve fibers, include many free Schwann cells: they are unstained. Bright field. Bar, 10 μm . \times 750. Fig. 3: Electron micrograph of a myelinated fiber from the L5 ventral root of a rat chronically intoxicated with IDPN. The inset shows the same fiber in an adjacent 1- μm thick section that was stained by MAG antiserum. Two Schwann cell ingrowths are present in the electron micrograph and they are intensely stained by MAG antiserum in the adjacent 1- μm thick section. A periaxonal ring of MAG staining surrounds the outer circumference of the axon. Schmidt-Lanterman incisures (arrowheads) are intensely stained. Bar, 1 μm . \times 10,000. (Inset) Bar, 10 μm . \times 900.

Previous reports have suggested that MAG could be involved in maintaining the periaxonal space (22, 25-28) and in separation of the external surfaces of Schwann cell plasmalemmae within Schmidt-Lanterman incisures, lateral loops, and the outer mesaxon (25-28). These suggestions have been based on the immunocytochemical localization of MAG in normal and pathological PNS fibers, and on the fact that MAG is a large integral membrane glycoprotein (18). Rat brain MAG is 30% carbohydrate by weight and contains multiple complex carbohydrate moieties (18). The molecule is highly negatively charged because of an excess of acidic over basic amino acids, and because of sialic acid and sulfate residues. It is likely that rat PNS MAG has similar properties

since peptide maps are very similar to those obtained from central nervous system MAG, although the PNS MAG has a slightly higher apparent molecular weight (8). The bulk and polarity of the glycosylated part of the MAG molecule may prevent the close apposition of the membrane surfaces containing it, and by this means MAG may function in stabilizing the 12-14-nm space.

A potential limitation of previous studies of MAG localization is the limited resolution of the light microscope. Even with thick-thin comparisons, the possibility that MAG might be localized in the inner compact myelin sheath, and not in periaxonal membranes, could not be definitively excluded in previous studies; recent immunocytochemical studies have

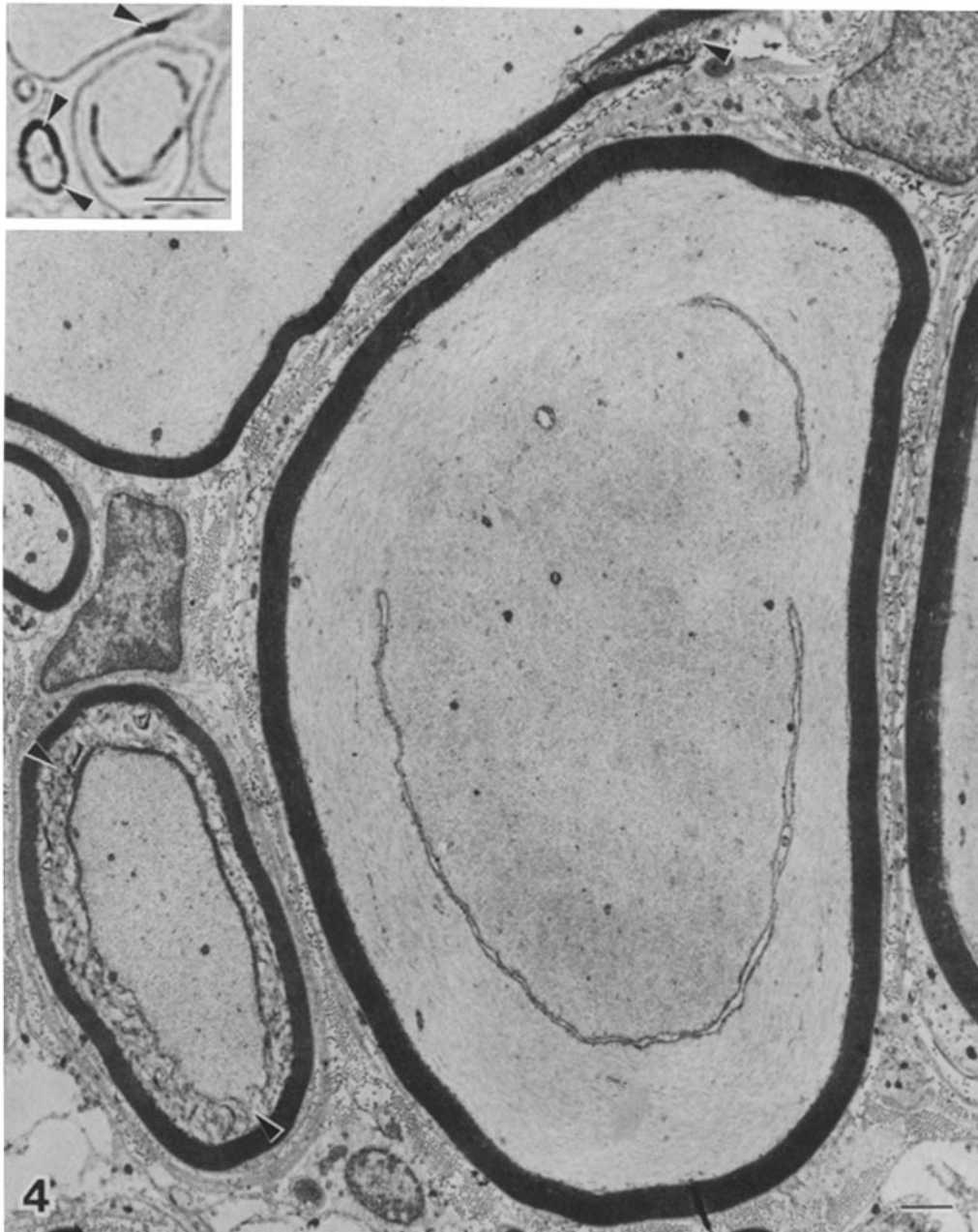
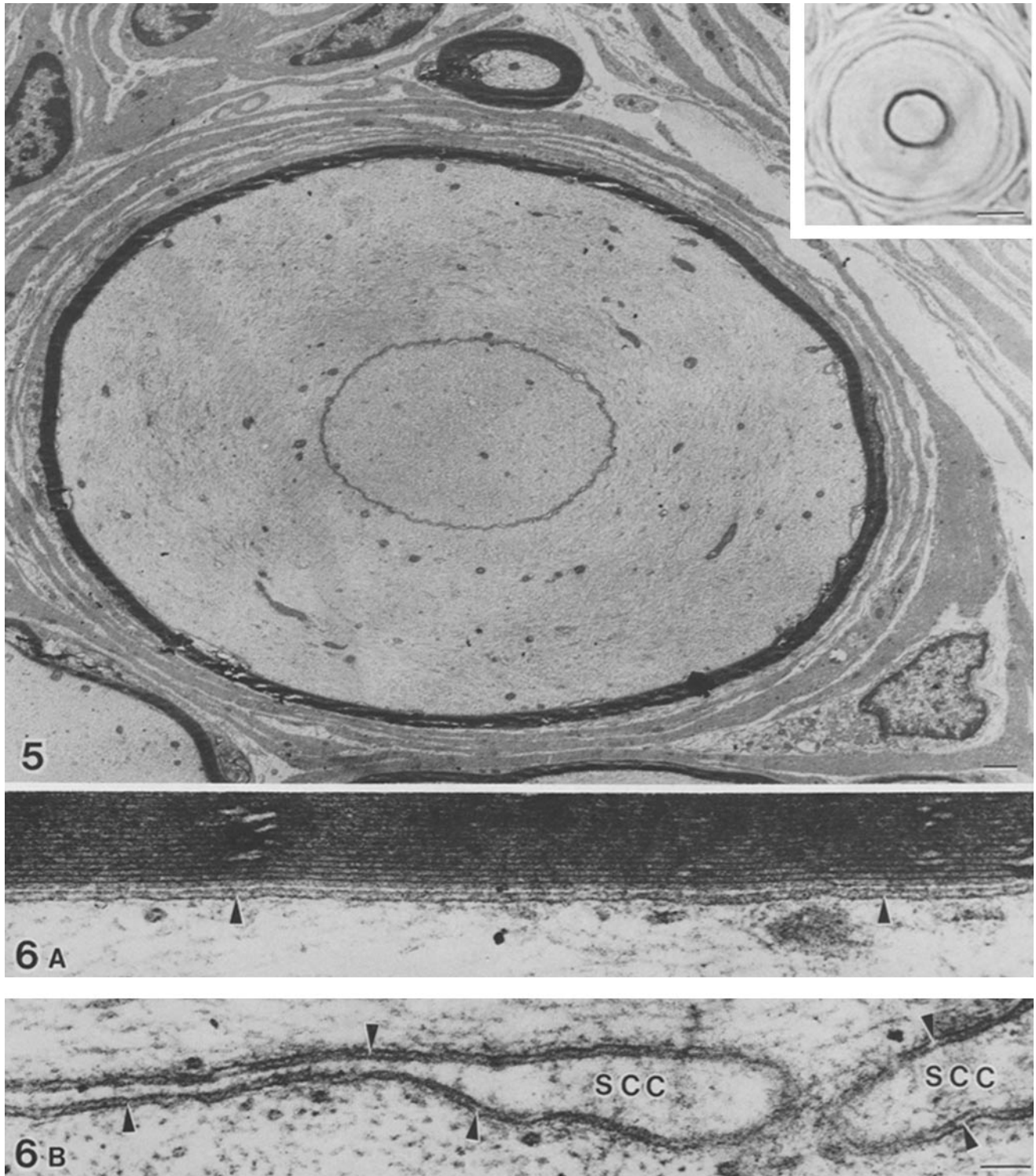


FIGURE 4 Electron micrograph from the L5 ventral root of a rat chronically intoxicated with IDPN. The inset shows the same area in an adjacent 1- μ m thick section that was stained by MAG antiserum. MAG staining is restricted to a periaxonal ring of staining, Schmidt-Lanterman incisures (arrowheads) and Schwann cell ingrowths that separate the outer maloriented neurofilaments from the central core of normally oriented axoplasm. Bar, 1 μ m. \times 7,000. (Inset) Bar, 10 μ m. \times 1,100.

raised this possibility (7, 29). The results of the present study clearly demonstrate MAG within the Schwann cell ingrowths which protrude into giant axonal swellings. In cross-sections, these ingrowths are separated from the myelin sheath by distances up to 4–5 μ m (Fig. 5). Only a few ingrowths contain compact myelin and these ingrowths can readily be recognized by thick-thin comparison and by the presence of Po staining (Fig. 9). The majority of the ingrowths, MAG-positive and Po-negative, provide unequivocal evidence that MAG does not have an obligatory localization near (or in) compact myelin. Rather, the present study supports previous interpretations of MAG's localization in the periaxonal membrane. The ingrowths can be regarded as severely distorted periaxonal regions in which two axolemma-Schwann cell plasmalemma

complexes are separated from each other by a very thin finger of Schwann cell cytoplasm (Fig. 6B). The intensity of staining of the ingrowths (Fig. 3–5) probably results from the presence of two closely spaced Schwann cell periaxonal plasmalemmae.

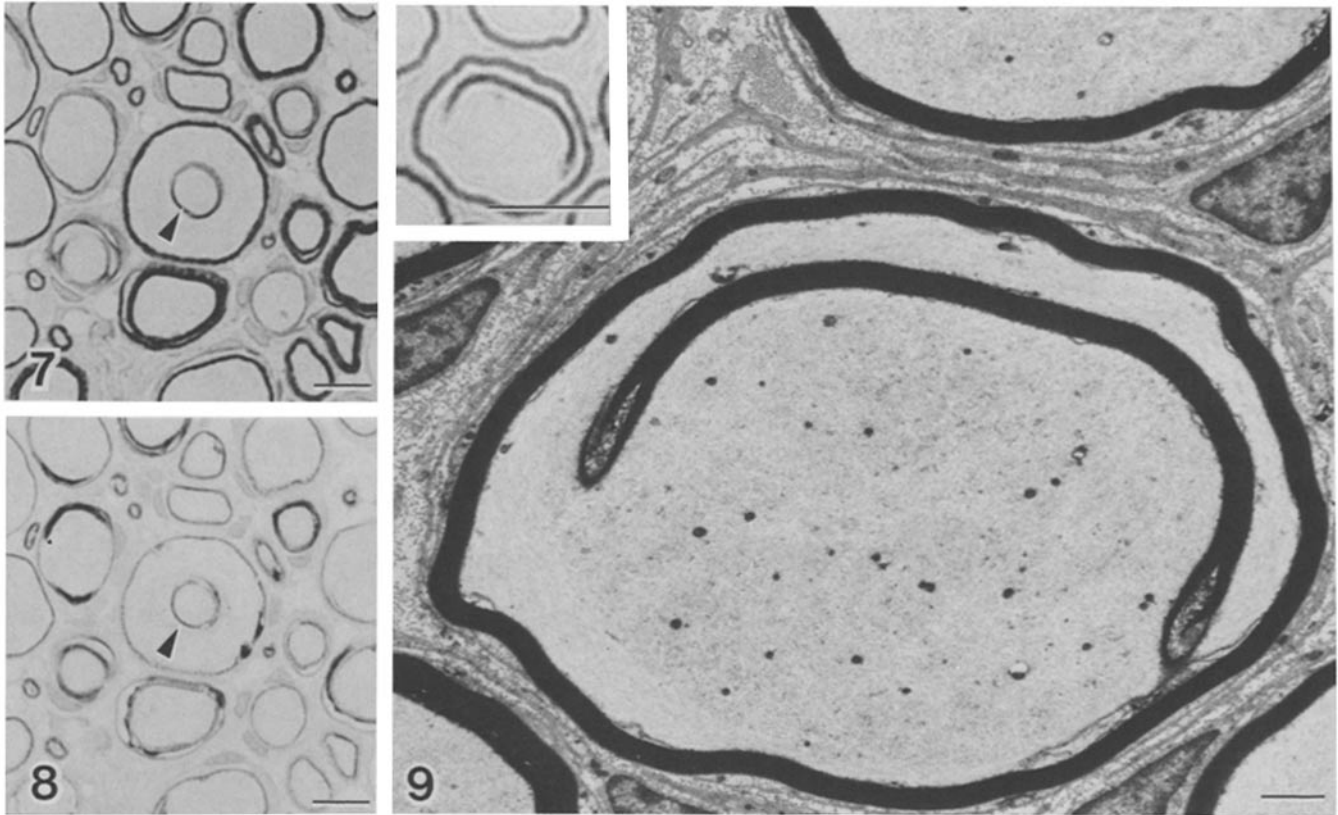
The present results provide no further evidence for why or how the ingrowths form. In the IDPN model they appear to be a late secondary response of the Schwann cell to the masses of maloriented neurofilaments (11). The ingrowths separate these maloriented neurofilaments from the central core of normally oriented axoplasm. Recent results have demonstrated that fast axonal transport is preferentially associated with the central core of normally oriented axoplasm (12). Since the Schwann cell is dependent on the axon for the formation and maintenance of the myelin sheath (1, 2, 6),



FIGURES 5 and 6 Fig. 5: Electron micrograph from the L5 ventral root of a rat chronically intoxicated with IDPN. The inset shows the same area in an adjacent $1\ \mu\text{m}$ thick section that was stained by MAG antiserum. A Schwann cell ingrowth, which totally separates the maloriented neurofilaments from the central core of normally oriented axoplasm, is intensely stained by MAG antiserum in the adjacent $1\text{-}\mu\text{m}$ section. Bar, $1\ \mu\text{m}$. $\times 5,500$. (Inset) Bar, $10\ \mu\text{m}$. $\times 750$. Fig. 6: Electron micrographs comparing the normally positioned periaxonal space (A) with a Schwann cell ingrowth (B). Each Schwann cell ingrowth contains two periaxonal spaces. The $12\text{--}14\text{-nm}$ periaxonal space (arrowheads) and Schwann cell cytoplasm (SCC) are maintained in these ingrowths. Bar, $0.1\ \mu\text{m}$. $\times 81,000$.

these ingrowths may be a means of bringing the Schwann cell periaxonal membrane into close association with functional portions of the axons. Spencer and Thomas have suggested that ingrowths may provide a mechanism for removal of

degenerating axonal organelles (20). In any event, the ingrowths provide a system in which the periaxonal complex is markedly distorted, but in which the $12\text{--}14\text{-nm}$ periaxonal space is maintained. This unequivocal demonstration of



FIGURES 7-9 Fig. 7: 1- μ m thick section from the L5 ventral root of a rat chronically intoxicated with IDPN. This section is stained with Po antiserum. Myelin sheaths are intensely stained by Po antiserum. One fiber has an intra-axonal ring of Po staining (arrowhead). Bar, 10 μ m. \times 750. Fig. 8: This section was cut adjacent to that in Fig. 7 and stained with MAG antiserum. The intra-axonal ring stained by Po antiserum in Fig. 7 is stained by MAG antiserum (arrowhead). Bar, 10 μ m. \times 750. Fig. 9: Electron micrographs of a myelinated fiber from the L5 ventral root of a rat chronically intoxicated with IDPN. The inset shows the same fiber in an adjacent 1- μ m section that was stained with Po antiserum. Compact myelin lamellae present at the site of the periaxonal ingrowth is stained intensely by Po antiserum. Bar, 1 μ m. \times 8,200. (inset) Bar, 10 μ m. \times 1,600.

MAG in periaxonal membranes is consistent with a functional role for MAG in maintaining the periaxonal space of myelinated fibers.

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REFERENCES

1. Aguayo, A. J., L. Charron, and G. M. Bray. 1976. Potential of Schwann cells from unmyelinated nerves to produce myelin: a quantitative ultrastructural and radiographic study. *J. Neurocytol.* 5:565-573.
2. Bunge, M. B., A. K. Williams, and P. M. Wood. 1982. Neuron-Schwann cell interaction in basal lamina formation. *Dev. Biol.* 92:449-460.
3. Chou, S. M., and H. A. Hartman. 1964. Axonal lesions and waltzing syndrome after IDPN administration in rats. *Acta Neuropathol.* 3:428-450.
4. Chou, S. M., and H. A. Hartman. 1965. Electron microscopy of focal neuroaxonal lesions produced by B,B'-iminodipropionitrile (IDPN) in rats. *Acta Neuropathol.* 39:590-603.
5. Clark, A. W., J. W. Griffin, and D. L. Price. 1980. The axonal pathology in chronic IDPN intoxication. *J. Neurocytol.* 9:42-55.
6. Dubois-Dalcq, M., B. Rentier, A. Baron-Van Evercooren, and B. W. Burge. 1981. Structure and behavior of rat primary and secondary Schwann cells in vitro. *Exp. Cell Res.* 131:283-297.
7. Favilla, J. T., D. E. Frail, C. G. Palkovits, G. L. Stoner, P. E. Braun, and H. deF Webster. 1983. Immunocytochemical observations on myelin-associated glycoprotein (MAG) localization using polyclonal and monoclonal antibodies. *Peripheral Nerve Study Group, Fontevraud, France. 24a.* (Abstr.)
8. Figlewicz, D. A., R. H. Quarles, D. Johnson, G. R. Barbarash, and N. H. Sternberger. 1981. Biochemical demonstration of the myelin-associated glycoprotein in the peripheral nervous system. *J. Neurochem.* 37:749-758.
9. Griffin, J. W., P. N. Hoffman, A. W. Clark, P. T. Carroll, and D. L. Price. 1978. Slow axonal transport of neurofilament proteins: impairment by B,B'-iminodipropionitrile administration. *Science (Wash. DC).* 202:633-635.
10. Griffin, J. W., and D. L. Price. 1981. Segmental demyelination in experimental IDPN and hexacarbon neuropathies: evidence for an axonal influence. *Lab. Invest.* 45:130-141.
11. Griffin, J. W., and D. L. Price. 1981. Schwann cell and glial responses in B,B'-iminodipropionitrile intoxication. I. Schwann cell and oligodendrocyte ingrowths. *J. Neurocytol.* 10:995-1007.
12. Griffin, J. W., K. E. Fahnestock, D. L. Price, and P. N. Hoffman. 1983. Microtubule-neurofilament segregation produced by B,B'-iminodipropionitrile: evidence for the association of fast axonal transport with microtubules. *J. Neurosci.* 3:557-566.
13. Hirano, A., and H. M. Dembitzer. 1976. Eosinophilic rod-like structures in myelinated fibers of hamster spinal roots. *Neuropathol. Appl. Neurobiol.* 2:225-232.
14. Hirano, A., and H. M. Dembitzer. 1981. The periaxonal space in an experimental model of neuropathy: the mutant Syrian hamster with hindleg paralysis. *J. Neurocytol.* 10:261-269.
15. Hirano, A. 1983. Reaction of the periaxonal space to some pathological processes. In *Progress in Neuropathology*. H. M. Zimmerman, editor. Raven Press, New York. 5:99-112.
16. Johnson, D., R. H. Quarles, and R. O. Brady. 1982. A radioimmunoassay for the myelin-associated glycoprotein. *J. Neurochem.* 39:1356-1362.
17. Quarles, R. H., D. Johnson, R. O. Brady, and N. H. Sternberger. 1981. Preparation and characterization of antisera to the myelin-associated glycoprotein. *Neurochem. Res.* 6:1109-1121.
18. Quarles, R. H., G. R. Barbarash, D. A. Figlewicz, and L. J. McIntyre. 1983. Purification and partial characterization of the myelin-associated glycoprotein from adult rat brain. *Biochem. Biophys. Acta* 757:140-143.
19. Shimono, M., K. Izumi, and Y. Kuroiwa. 1978. 3,3'-iminodipropionitrile induced centrifugal segmental demyelination and onion bulb formation. *J. Neuropathol. Exp. Neurol.* 37:375-386.
20. Spencer, P. S., and P. K. Thomas. 1974. Ultrastructural studies of the dying back process. II. The sequestration and removal by Schwann cells and oligodendrocytes of organelles from normal and diseased axons. *J. Neurocytol.* 3:763-783.
21. Spencer, P. S., D. Couri, and H. H. Schaumburg. 1980. n-hexane and methyl n-butyl

- ketone. In *Experimental and Clinical Neurotoxicology*. P. S. Spencer and H. H. Schaumberg, editors. Williams and Wilkins, Baltimore. 456-475.
22. Sternberger, N. H., R. H. Quarles, Y. Itoyama, and H. deF. Webster. 1979. Myelin-associated glycoprotein demonstrated immunocytochemically in myelin and myelin-forming cells of developing rats. *Proc. Natl. Acad. Sci. USA.* 76:1510-1514.
 23. Trapp, B. D., N. H. Sternberger, R. H. Quarles, Y. Itoyama, and H. deF. Webster. 1979. Immunocytochemical localization of rat peripheral nervous system myelin proteins: P₂ protein is not a component of all peripheral nervous system myelin sheaths. *Proc. Natl. Acad. Sci. USA.* 76:3552-3556.
 24. Trapp, B. D., Y. Itoyama, N. H. Sternberger, R. H. Quarles, and H. deF. Webster. 1981. Immunocytochemical localization of Po protein in Golgi membranes and myelin of developing rat Schwann cells. *J. Cell Biol.* 90:1-6.
 25. Trapp, B. D., and R. H. Quarles. 1982. Presence of the myelin-associated glycoprotein correlates with alterations in the periodicity of peripheral myelin. *J. Cell Biol.* 92:877-882.
 26. Trapp, B. D., and J. W. Griffin. 1982. Myelin-associated glycoprotein and Schwann cell-axon interactions in chronic IDPN neuropathy. IX International Congress of Neuropathology, Vienna, Austria. 49.
 27. Trapp, B. D., R. H. Quarles, J. W. Griffin, and K. Suzuki. 1983. The myelin associated glycoprotein and axon-myelinating Schwann cell contact. *J. Neuropathol. and Exp. Neurol.* 42:357.
 28. Trapp, B. D., R. H. Quarles, and K. Suzuki. 1983. The myelin-associated glycoprotein and myelin forming Schwann cell-axon interaction in Quaking mice. *Society for Neuroscience.* 9:451a. (Abstr.)
 29. Webster, H. deF., C. G. Palkovits, G. L. Stoner, J. T. Favilla, D. E. Frail, and P. E. Braun. 1983. Myelin-associated glycoprotein: electron microscopic immunocytochemical localization in compact developing and adult central nervous system myelin. *J. Neurochem.* 41:1469-1479.