Expanded CNS Myelin Sheaths Formed *In Situ* in the Presence of an IgM Antigalactocerebroside-Producing Hybridoma

Jack Rosenbluth, Wei-Lan Liang, Zhiguang Liu, Danzhu Guo, and Rolf Schiff

Department of Physiology and Neuroscience and Institute of Rehabilitation Medicine, New York University School of Medicine, New York, New York 10016

When O1 hybridoma cells, which produce an IgM antigalactocerebroside, are implanted into the dorsal columns of 4–8 d rat spinal cord, some of the myelin that subsequently develops in the immediate vicinity displays an abnormal periodicity. The spacings that are seen cluster at ~19 nm and 31 nm, roughly two and three times the normal 11 nm spacing. In the expanded sheaths, major dense lines are separated by broad extracellular spaces containing a dense material in which single or double rows of ~10 nm circular profiles can be identified, consistent with the “central rings” of IgM molecules. Because IgM is

multivalent, it may serve to link adjacent lamellae together in place of intrinsic myelin molecules that normally interact at close range. Extensive direct contact between myelin components of successive myelin lamellae is thus not essential to signal the growth of the oligodendrocyte membrane or the spiral wrapping of that membrane around axons during myelinogenesis, or to stabilize the myelin spiral that forms.

Key words: demyelination; remyelination; myelinogenesis; multiple sclerosis; IgM gammopathy; paraproteinemia; immunoglobulin M; glycolipid; neuropathy

The structure of compact myelin is thought to be stabilized by the interaction of intrinsic myelin components in successive layers with one another. In addition, some of these elements may serve as guideposts during myelin formation (Lemke and Axel, 1985), permitting successive lamellae to recognize and follow those laid down previously.

A recent study of a knockout mouse (Boison et al., 1995) showed, however, that in the absence of at least one major constituent of CNS myelin, proteolipid protein (PLP), which is thought to mediate interlamellar interaction, CNS myelin can form and remain stable, albeit with some abnormality. Continuous intermediate lines were reported to be absent, but focal contacts between successive lamellae were still present, indicating that close interlamellar interaction still occurs in PLP- animals and is presumably mediated by other intrinsic myelin components.

In the current study, we implanted the O1 hybridoma (Sommer and Schachner, 1981) into the CNS of neonatal rats to explore the role of antimyelin IgM antibodies on myelin formation *in vivo*. Our results show that an abnormal form of myelin develops in the presence of O1 that displays a wide separation of lamellae from one another over broad areas, precluding interlamellar interaction. Thus, in such regions, myelin was able to develop without apparent direct contact between lamellae and therefore without direct interaction between the myelin components of successive lamellae. Moreover, the abnormal myelin that formed was stable enough to persist, at least temporarily, indicating that close interlamellar interaction is not required for either the formation or maintenance of the sheath.

In this paper, we consider the manner by which expanded myelin sheaths form in the presence of IgM and their relevance to the mechanism of myelinogenesis. The physiological consequences of expanded myelin are considered elsewhere (Rosenbluth, et al., 1995a).

A preliminary report of this study has been published previously (Rosenbluth et al., 1995b).

MATERIALS AND METHODS

Cultures of the O1 hybridoma, kindly provided by S. Pfeiffer (University of Connecticut), courtesy of I. Sommer (University of Glasgow), were maintained at 36–37°C in Falcon flasks in a CO₂ incubator by standard methods. For implantation, cells were shaken free, spun down, and resuspended in L-15 medium at a concentration of ~10⁶–10⁸ cells/mm³. Only live cells were counted (identified by trypan blue exclusion).

Rat pups at ages P4–8 were anesthetized by hypothermia (–20°C for 5–10 min). Laminectomy was performed in the lower thoracic region, and the dorsal spinal cord was exposed; 0.01 ml of the cell suspension was injected into the dorsal columns through a 28 gauge needle, and the skin was closed over the operative site. Cyclosporine (10 mg/kg/d, i.p.) was administered beginning on the day of operation. After surgery, animals were watched until they awakened and then were returned to the maternal cage. Six to eighteen days later, animals were reanesthetized and fixed by vascular perfusion with a mixture of 3% glutaraldehyde and 2% formaldehyde (3 + 2) in 0.1 M cacodylate buffer or with buffered 4% formaldehyde alone, followed by 3 + 2 after dissection. Spinal cords were removed, sliced, rinsed, postfixed in 1–2% osmium tetroxide, dehydrated, and sectioned by standard methods for examination by light and electron microscopy.

Equivalent experiments were carried out using a control hybridoma, CRL 8018 (ATCC), which produces an IgM monoclonal antibody to an irrelevant antigen, in place of the O1 hybridoma. Culture media were characterized using a monoclonal antibody isotyping kit (Boehringer Mannheim, Indianapolis, IN). Both O1 and CRL8018 were found to produce IgMs with κ light chains.

Measurements were made on prints enlarged to 300,000 $\times$, in which myelin lamellae were cut normally and did not show signs of separation, splitting, or other artifact. Wherever possible, the myelin period was measured across multiple lamellae. Spacings were grouped, and means were calculated within each group. Measurements were weighted according to the number of lamellae measured.

For immunocytochemical analysis, thin sections of specimens prepared

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Correspondence should be addressed to Dr. J. Rosenbluth, RR 714, New York University Medical Center, 400 East 34th Street, New York, NY 10016.

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Figure 1. Distribution of myelin spacings in expanded myelin sheaths. Myelin periods are confined within narrow ranges at three preferred spacings: 11 nm, 19 nm, and 31 nm (means). Measured spacings were assorted into groups, each spanning 1 nm. The ordinate shows the number of examples (*n*) in each group.

as described and mounted on electron microscopy grids were blocked with 1% bovine serum albumin/glycine for 1 hr, stained from 2 hr to overnight with 0.1–0.01 anti-IgM coupled to colloidal gold (~10 nm), and rinsed five times in PBS. Examination of these grids showed sparse labeling with little background. In electron micrographs of prints, myelin sheaths were identified as either normal (N) or expanded (E) and were measured with a planimeter to determine their area. Particles were counted over the respective sheath regions and over nonmyelin areas (B) to obtain background levels. Individual counts were pooled to arrive at average labeling densities. Statistical analysis was carried out by Poisson regression.

RESULTS

Within the immediate vicinity of the hybridoma cells, fields can be found in which a large proportion of the myelin sheaths that are present display an expanded myelin period (Figs. 1, 2). This phenomenon was observed in animals that had received the hybridoma implants at ages before or during myelin formation. Thus, the large number of contiguous fibers showing abnormal myelin in Figure 2 must have become myelinated *after* the hybridoma was already in place. Older animals that received O1 implants at a time when myelin had already formed did not develop this abnormal form of myelin over the same time period (Rosenbluth et al., 1995a).

Clearly, the spacing of lamellae within the expanded myelin sheaths is not graded continuously. On the contrary, measurement of the myelin period reveals that the spacings fall within narrow size ranges, with no examples in the intervening positions (Fig. 1).

The smallest spacing averages ~11 nm $\pm$ 0.7 (SD), which corresponds to the period of normal CNS myelin. Morphologically, regions displaying this period consist of typical major dense lines alternating with single intermediate lines (see Fig. 6A).

The second spacing seen (Fig. 3) is approximately double the normal spacing, averaging ~19 nm $\pm$ 1.2. This type of myelin consists of major dense lines, each flanked by the outer dense laminae of the constituent unit membranes. These paired membrane elements are separated by an extracellular space, ~8 nm wide, containing a material of moderate density, within which roughly circular profiles compressed into the gap (Figs. 3, 6C) are often discernible.

The third spacing (Fig. 4) is approximately triple the normal period, approximating 31 nm $\pm$ 3.3. In this case, the paired membrane elements are identical to those in the second case but are separated by a band of extracellular dense material with a mean width of ~20 nm, within which double layers of ~10 nm circular profiles are often discernible. Generally, each such profile

is closely associated with one or the other myelin membrane in an asymmetric manner. Occasional profiles are situated midway between the lamellae, however (Figs. 4, 6D).

In some regions (not used for measurement), the gap is wider still (Figs. 5, 6F), and the intercellular material seems to be divided into three layers: a central one of low density between two moderately dense layers that are directly apposed to the membrane elements and contain the ~10 nm circular structures described above. The central paler band also contains spotty dense material, including circular profiles, but they occur only sparsely here in comparison with the denser bands on either side (Figs. 5, 6F).

Examination of control spinal cords, into which an irrelevant IgM-producing hybridoma (CRL 8018) had been implanted, did not show evidence of expanded myelin sheaths.

Pooled data from anti-IgM-colloidal gold-stained thin sections (Table 1) showed an eightfold increase in labeling of the expanded myelin sheaths (E) compared with background (B). Statistical analysis showed this difference to be highly significant ($p < 0.001$). In contrast, labeling of normal myelin (N) was not significantly different from background. Presumably IgM in interstitial spaces was washed out during specimen preparation, whereas IgM in the expanded myelin sheaths remained trapped within the myelin coils.

DISCUSSION

In the present study, we show that an abnormal “expanded” form of CNS myelin can develop even when successive lamellae are separated widely and therefore presumably too far apart to interact via intrinsic myelin components. This result was seen in the presence of an antigalactocerebroside IgM capable of bridging between successive myelin lamellae by virtue of its multivalency. Expanded myelin was not found after implantation of an IgG antigalactocerebroside (Rosenbluth et al., 1994), however, or after implantation of a control hybridoma that secretes an IgM directed against an irrelevant, nonmyelin antigen.

Thus, direct extensive interaction between intrinsic myelin molecules in successive layers is not required for myelin formation. Linkage between successive lamellae, even at a distance much greater than normal, is adequate to support myelinogenesis and to provide some stability to the resulting sheaths. The presence of an irrelevant IgM has no effect on myelinogenesis, but the presence of an antimyelin IgG, capable of binding myelin but not capable of bridging between successive lamellae, blocks myelin formation.

Figure 2. Electron micrograph showing portions of nine myelin sheaths, all of which display expanded myelin, except for the seven-layer sheath at the lower right, which is normally compacted. Magnification: 33,000 $\times$.

Figure 3. Electron micrograph of a 13-layer expanded myelin sheath. The innermost layer contains cytoplasm (*asterisk*), and the next three lamellae are normally compacted. The remaining lamellae display an expanded spacing, except for lamellae 11 and 12, which are nearly apposed at the right. In the region of expanded myelin, the interlamellar spaces are filled with a moderately dense material within which circular profiles are visible (*arrows*). Magnification: 290,000 $\times$.

Incorporation of IgM into myelin

The presence of a dense material between the lamellae of the expanded myelin sheaths distinguishes these sheaths from those generated by exposure of myelin to hypotonic solutions (Robertson, 1958) and indicates the presence of an intercalated material. A similar interlamellar density is present in the wide-spaced peripheral myelin seen in IgM paraproteinemias (King and Thomas, 1984; Prineas, 1985; Kusunoki et al., 1987; Jacobs and Scadding, 1990; Lach et al., 1993; Latov, 1994) and certain experimental immune diseases of the CNS (Bornstein and Raine, 1976;

Raine et al., 1978) or peripheral nervous system (Raine and Bornstein, 1979; Tatum, 1993). There as well, immunogold studies have demonstrated IgM within the expanded regions of peripheral myelin sheaths (Lach et al., 1993).

How such a large molecule might gain access to the interior recesses of a myelin sheath is unclear. Its size would presumably prevent diffusion along the minuscule extracellular space normally present within an already formed myelin sheath, unless the molecule actively intrudes itself or unless myelin is a much more dynamic structure than is thought.

Figure 4. Electron micrograph showing portions of three myelin sheaths. The portion occupying most of the field consists of 13 lamellae, which are alternately separated by wide gaps or intermediate lines. The innermost layer (*IL*) contains cytoplasm. The second layer is compact and forms an intermediate line with the first layer. The wide spaces between layers 2–3, 4–5, 6–7, 8–9, 10–11, and 12–13 contain a dense material, most of which seems to consist of circular profiles in double rows. Intermediate lines form between layers 1–2, 3–4, 5–6, 7–8, 9–10, and 11–12. Dense material also appears between the outermost layer of this sheath (*OL*) and the sheaths at the *upper left* and *extreme right* (*arrow*). Magnification: 210,000 $\times$.

Figure 5. Electron micrograph showing myelin with expanded interlamellar spaces in which rows of circular profiles can be seen attached to each flanking membrane, but are separated from one another by a clear gap. The myelin period here is ~ 44 nm. In some places, a circular profile is visible within the clear gap midway between the adjacent membranes (*arrows*). Magnification: 230,000 $\times$.

Figure 6. Diagram summarizing proposed models of expanded myelin spacings in relation to IgM molecules. *A*, Two major dense lines from a region of normal myelin (11 nm period). The intermediate line between them represents the fusion of the outer dense components of the adjacent plasma membranes. *B*, IgM molecule in planar-star conformation composed of a central ring (parallel to the page) and five arms. *C*, Expanded myelin (19 nm period) in which the outer dense components of the adjacent plasma membranes are widely separated by an intervening material resembling a single row of IgM central rings, some of which are seen *en face* and some rotated around a vertical axis (and therefore foreshortened). The arms are presumably flattened against the membranes or penetrate into them and are thus not visible. (cf. Fig. 3.) *D*, Expanded myelin (31 nm period) in which the adjacent membranes are separated by an intervening material resembling a double row of IgM central rings. Each row is apposed to one or the other membrane. In the center, one IgM is shown midway between the two membranes in a staple conformation, with arms extending toward both membranes. (cf. Fig. 4.) *E*, IgM in the staple conformation. The central ring is perpendicular to the page and appears as a linear bar. Arms extend both up and down. *F*, Expanded myelin (>31 nm period) in which the adjacent membranes are separated by two bands of dense material and a central clear band. This is consistent with two rows of IgM molecules adherent to one or the other membrane, but separated from each other. In the center, one IgM is shown midway between the two membranes in the planar star conformation, with arms extending toward both membranes. (cf. Fig. 5.)

Alternatively, the data presented here suggest that IgM is incorporated as the myelin is formed. O1-coated oligodendroglial processes may thus enwrap axons, carrying the IgM coating along as the myelin spiral is generated. Such expanded myelin sheaths once formed might then permit further movement of IgM within the widened interlamellar clefts.

Relation of IgM structure to expanded myelin period

Earlier investigations of isolated IgM have shown it to display a planar, star-shaped structure, composed of a central Fc ring or disc, ~85–100 Å in diameter, from which five Fab arms, ~100 Å long, extend (Feinstein and Munn, 1969; Green, 1969; Metzger, 1970; Feinstein et al., 1971, 1986). These arms are clearly flexible, in view of the existence of “staple” conformations of IgM (Feinstein and Munn, 1969) in which the whole molecule, instead of being planar, has arms bent at nearly a right angle to the central ring, in the way that legs project from a table. Bending is presumed to occur at the hinge region located at the junction of the arms with the central ring (Feinstein et al., 1971).

Table 1. Anti-IgM immunogold label over expanded myelin, normal myelin, or background in electron micrographs

	Expanded myelin (E)	Normal myelin (N)	Background (B)	Ratio of densities	<i>p</i>
Total gold particles (P)	17	3	15		
Total area (cm ²) (A)	1254	869	8907		
Particle density (P/A) × 1000	13.6	3.5	1.7		
E vs B (densities)				8.1	<0.001
N vs B (densities)				2.1	n.s.

Labeling density over expanded myelin profiles is significantly greater than background labeling. n.s., Not significant.

Of the spacings found after O1 implantation, the 11 nm period represents myelin in which adjacent lamellae seem to contact one another with apparent fusion of the outermost lamellae (Fig. 6*A*), as is typical of fixed, dehydrated, epoxy-embedded myelin from mammalian CNS.

The next two larger spacings are reminiscent of the two separations seen previously between flagella linked by IgM antibodies (Feinstein et al., 1971). In that study, a ~20 nm separation was found when the linking antibody was in the staple conformation, and a ~30 nm separation was found when the IgM was in the planar star conformation.

In the expanded myelin sheaths, however, the ~19 nm spacing results from the presence of an extracellular cleft of only ~8 nm between successive myelin lamellae, far smaller than the separations seen between IgM-linked flagella. Moreover, if IgM molecules in the staple form were present within this cleft, and if the inner rings were parallel to the myelin lamellae, the rings should be seen aligned, edge-on, forming an intermediate density midway between the lamellae. What we see instead, however, are circular structures with no evidence of an intermediate density. The circular structures within the extracellular space in the expanded myelin sheaths are thus more consistent with *en face* views of the central rings alone. The arms may extend into the depths of the myelin membranes or lie compressed and flattened along their length.

Because IgM is multivalent, such intercalated IgM molecules could serve to anchor adjacent myelin lamellae together, in the same way that molecules normally present within myelin lamellae are assumed to do, but across a much greater distance. Indeed, it seems unlikely that the molecules intrinsic to myelin that normally subserve this role could interact over such a great distance. Specific association between the intrinsic myelin components themselves therefore seems not to be necessary for elaboration of the myelin membrane or for spiral wrapping of that membrane around the axon or for stabilization of the resulting spiral.

If in the regions of expanded myelin sheaths displaying a 31 nm period the lamellae were separated by a single row of planar stars with arms extended, circular structures would be expected in roughly the middle of the cleft. What is seen, however, are double rows of circles, each row closely applied to one or the other myelin membrane. These could again represent *en face* images of the central rings of IgM molecules, with their arms flattened, interposed in double rows between successive myelin lamellae.

If the respective rows were offset from one another, i.e., in hexagonal array, the separation between the myelin membranes,

caused by the dimensions of the central rings alone acting as spacers, would approximate 14–17 nm (assuming a ring diameter of 8–10 nm). If the rows were aligned, i.e., in square array, the separation would approximate 16–20 nm, roughly comparable to the actual separation of ~20 nm. When two rows of IgMs are intercalated asymmetrically between successive myelin membranes, each row presumably binds preferentially to the closer membrane. Because the adjacent rows of IgMs would not be expected to bind each other, what then holds the lamellae together? Some IgMs may exist in the staple form midway between adjacent myelin lamellae and extend arms to bind *both* facing membranes, thus providing a basis for stabilization at the 31 nm spacing. The central rings of such molecules could be oriented either parallel to the lamellae, as depicted in Fig. 6D, or perpendicular, with arms oriented parallel to the lamellae.

In the expanded myelin sheaths that show a period even larger than 31 nm, the pale central band between successive myelin lamellae may represent a plane along which the double layers of IgM molecules have separated. The occasional densities within this pale band resemble planar stars centered in the clefts (Figs. 4, 6F). Again, these may extend arms to *both* facing membranes and impart some stability to these sheaths as well. Nevertheless, separation or shear at the interface between rows of IgMs could lead to eventual disintegration of the myelin structure. Because the myelin sheath consists of a continuous membrane spiral, alternation of the normal period with the 31 nm period, as seen in Fig. 4, could also impart partial stability to such sheaths.

Effects of other immunoglobulins

In view of the possibility that the formation of expanded myelin in the presence of O1 could be related to its interaction with galactocerebroside specifically, we also implanted the O4 hybridoma, which secretes an IgM directed against other constituents of myelin and oligodendrocytes, including sulfatides and seminolipids (Bansal et al., 1989). Wide-spaced sheaths developed in this case as well (Rosenbluth et al., 1995b); however, after implanting a control hybridoma (CRL8018) that secretes an IgM directed against an irrelevant antigen, not located in oligodendrocyte membranes or myelin sheaths, no expanded myelin was found.

Although multivalent IgMs apparently are able to substitute for intrinsic myelin constituents during myelin formation, we have no evidence that IgG molecules can. Our previous study of the antiGalC hybridoma (Ranscht et al., 1982), which secretes an IgG3, showed failure of myelin formation, resulting in the development of large amyelinated fascicles of axons, but no wide-spaced myelin (Rosenbluth et al., 1994). One possible explanation for this finding is that IgG present during myelin formation may indeed bind to the oligodendrocyte membrane and sterically prevent the intrinsic myelin molecules of adjacent lamellae from interacting, but without cross-linking adjacent lamellae in the way that IgMs do.

Antibodies bound to oligodendrocyte processes could activate signaling pathways, in view of evidence for morphological changes in the processes of cultured oligodendrocytes after addition of antigalactocerebroside antibodies to the culture medium (Dyer and Benjamins, 1989). However, as suggested above, the mere binding of the antiGalC IgG, which is unable to bridge between lamellae in the way that the O1 IgM can, evidently is insufficient to signal myelin formation.

CONCLUSIONS

- (1) CNS myelin having an expanded period is able to develop in the presence of the O1 hybridoma, which produces an IgM antigalactocerebroside.
- (2) The expanded sheaths display periodicities that cluster preferentially at 19 nm and 31 nm.
- (3) Close apposition between successive myelin lamellae, with direct interaction between intrinsic myelin components, is not essential for elaboration of the oligodendrocyte membrane, for the spiral wrapping of that membrane around the axon, or for the stabilization of the sheath thus formed. Interaction at a distance, mediated by multivalent antimyelin IgM molecules, can be substituted.
- (4) The separation between myelin membranes in the 19 nm and 31 nm period sheaths is compatible with the dimensions of a single or double row of IgM central rings between the respective membranes. This interpretation is supported by the presence of visible single and double rows of ring-like structures in electron micrographs.
- (5) Myelin exhibiting a 19 nm period, caused by the intercalation of a single row of IgMs, is likely to be relatively stable because each molecule is capable of binding both myelin membranes across the gap. In contrast, intercalation of two or more rows of IgM molecules, resulting in a period ≥ 31 nm, is likely to be unstable because of the lack of binding of the IgMs to one another and the tendency of the rows to separate or shear apart.
- (6) These observations provide bases for both stability and instability of CNS myelin formed (or reformed) in the presence of IgM antimyelin antibodies.

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