

Immunoelectron Microscopic Localization of Neural Cell Adhesion Molecules (L1, N-CAM, and MAG) and Their Shared Carbohydrate Epitope and Myelin Basic Protein in Developing Sciatic Nerve

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Abstract. The cellular and subcellular localization of the neural cell adhesion molecules L1, N-CAM, and myelin-associated glycoprotein (MAG), their shared carbohydrate epitope L2/HNK-1, and the myelin basic protein (MBP) were studied by pre- and post-embedding immunoelectron microscopic labeling procedures in developing mouse sciatic nerve. L1 and N-CAM showed a similar staining pattern. Both were localized on small, non-myelinated, fasciculating axons and axons ensheathed by non-myelinating Schwann cells. Schwann cells were also positive for L1 and N-CAM in their non-myelinating state and at the onset of myelination, when the Schwann cell processes had turned ~ 1.5 loops. Thereafter, neither axon nor Schwann cell could be detected to express the L1 antigen, whereas N-CAM was found in the periaxonal area and, more weakly, in compact myelin of myelinated fibers. Compact myelin, Schmidt-Lanterman incisures, paranodal loops, and finger-like processes of Schwann cells at nodes of Ranvier were L1-negative. At the nodes of Ranvier, the axolemma was also always L1- and N-CAM-negative. The L2/HNK-1 carbohydrate epitope coincided in its cellular and subcellular localization most closely to that observed for L1. MAG appeared on Schwann cells at the time L1 expression ceased. MAG was then expressed at sites of axon-myelinating Schwann cell apposition and non-

compacted loops of developing myelin. When compaction of myelin occurred, MAG remained present only at the axon-Schwann cell interface; Schmidt-Lanterman incisures, inner and outer mesaxons, and paranodal loops, but not at finger-like processes of Schwann cells at nodes of Ranvier or compacted myelin. All three adhesion molecules and the L2/HNK-1 epitope could be detected in a non-uniform staining pattern in basement membrane of Schwann cells and collagen fibrils of the endoneurium. MBP was detectable in compacted myelin, but not in Schmidt-Lanterman incisures, inner and outer mesaxon, paranodal loops, and finger-like processes at nodes of Ranvier, nor in the periaxonal regions of myelinated fibers, thus showing a complementary distribution to MAG. These studies show that axon-Schwann cell interactions are characterized by the sequential appearance of cell adhesion molecules and MBP apparently coordinated in time and space. From this sequence it may be deduced that L1 and N-CAM are involved in fasciculation, initial axon-Schwann cell interaction, and onset of myelination, with MAG to follow and MBP to appear only in compacted myelin. In contrast to L1, N-CAM may be further involved in the maintenance of compact myelin and axon-myelin apposition of larger diameter axons.

DURING development of peripheral nerves, cell-to-cell interactions follow a sequence of events that involve surface contacts of axons with the mesenchymal environment and peripheral glia, the Schwann cells, which are derived from the neural crest. Proliferating Schwann cells have been observed to migrate along outgrowing axons of peripheral nerves and withdraw from the mitotic cycle to form the myelin sheath or assume their roles as non-myelin-forming Schwann cells (Peters et al., 1976). Formation of myelin occurs around axons of larger diameter, whereas small diameter axons remain unmyelinated either as fascicles or ensheathed singly by Schwann cell processes. Myelin forma-

tion involves the ensheathment of the axon by loops of a Schwann cell process whose cytoplasmic faces of membranes are initially separated by Schwann cell cytoplasm but, upon extrusion of the cytoplasm and reduction of extracellular space to 2–2.5 nm between surface membranes, form the tightly apposed membranes of the adult compact myelin (Peters et al., 1976). The formation of compact myelin is the basis for rapid propagation of action potentials in the axon. Propagation of action potentials is saltatory at the nodes of Ranvier, where the internodal myelin is interrupted leaving the axolemma covered loosely by the finger-like processes of adjacent Schwann cells. The cellular and molecular signals

Table I. List of Primary Antibodies

Antibody* against	Reference for production and specificity	Concentration or dilution used for	
		Pre-embedding	Post-embedding
Mouse L1	Rathjen and Schachner, 1984; Faissner et al., 1984	0.3 mg IgG/ml	0.6 mg IgG/ml
Mouse L1, immunoadfinity purified	See Materials and Methods	0.2–0.6 mg IgG/ml	
Mouse N-CAM	Goridis et al., 1983	1:50, 1:100 [‡]	1:25, 1:50 [‡]
Mouse MAG	Poltorak M., G. Keilhauer, A. Meyer, C. Landa, and M. Schachner, manuscript submitted for publication	1:50 [‡]	1:25, 1:50 [‡]
Mouse MBP	Bologa-Sandru et al., 1981	1:25 [‡]	1:25 [‡]
Human hemoglobin	See Materials and Methods	1:100 [‡]	1:25, 1:50 [‡]
L2 epitope	Kruse et al., 1984	1:50 [§]	1:25 [§]
HNK-1 epitope	Kruse et al., 1984	1:50 [§]	—

* All antibodies were polyclonal antibodies prepared in rabbits except for monoclonal antibody L2, which is from rat, and HNK-1, which is from mouse.

[‡] Dilution of antiserum.

[§] Dilution of hybridoma clone supernatant after (NH₄)₂SO₄ precipitation and dialysis against PBS. The dialysate was approximately five times more concentrated than the supernatant.

that underlie the association of Schwann cells with axonal processes, fasciculation of small diameter axons, formation of compact myelin, and maintenance of unmyelinated axolemma at the nodes of Ranvier have remained obscure.

To study cell-surface interactions between neurons and glia during development of peripheral nerves, we have previously analyzed the expression of the neural cell adhesion molecules L1 and N-CAM and their common carbohydrate epitope L2/HNK-1 in the mouse sciatic nerve (Nieke and Schachner, 1985). These cell surface constituents have been of interest, since, in the central nervous system, they have been found to mediate cell-to-cell interactions involving the fasciculation of neurites and the migration of granule cells in the developing mouse cerebellar cortex (Fischer et al., 1986; Lindner et al., 1983, 1986). Both cell surface molecules are involved in Ca⁺⁺-independent adhesion and aggregation of neural cell bodies (Edelman, 1985; Schachner et al., 1985, for reviews). L1, which is immunohistochemically identical to the nerve growth factor-inducible large external protein NILE (Bock et al., 1985), which in turn has been shown to be the mammalian equivalent of chicken Ng-CAM (Friedlander et al., 1986), has been found in the central nervous system to be expressed only by post-mitotic neurons and to mediate neuron-to-neuron but not neuron-to-glia interactions (Keilhauer et al., 1985; Lindner et al., 1983; Rathjen and Schachner, 1984). On the other hand, N-CAM has been observed not only on pre- and post-mitotic neurons, but also on predominantly immature astrocytes and oligodendrocytes (Keilhauer et al., 1985). It is involved in neuron-to-neuron, neuron-to-astrocyte, and astrocyte-to-astrocyte adhesion (Keilhauer et al., 1985). Despite their different molecular properties, L1 and N-CAM share a common carbohydrate epitope consisting of a sulfated glucuronic acid that is detected by the monoclonal antibodies L2 and HNK-1 (Chou et al., 1985; Noronha et al., 1986). These antibodies also react with an unknown number of glycoproteins including the novel cell adhesion molecule J1 (Kruse et al., 1985) and the myelin-associated glycoprotein MAG¹ (Kruse et al., 1984). In agreement with our previous hypothesis that all glycoproteins expressing the L2/HNK-1 epitope are cell adhesion molecules, MAG has recently been shown to mediate neu-

ron-to-oligodendrocyte and oligodendrocyte-to-oligodendrocyte interaction (Poltorak, M., G. Keilhauer, A. Meyer, C. Landa, and M. Schachner, manuscript submitted for publication). MAG had previously been suggested to mediate axon-myelinating cell interactions in the central and peripheral nervous system (Quarles, 1983/84, for review). Not all L1, N-CAM, and MAG molecules express the L2/HNK-1 epitope, which is thought to be itself a ligand in adhesion (Keilhauer et al., 1985).

The aim of the present study was to gain insight into the molecular signals that underlie cell-to-cell interactions in peripheral nerves. We chose to study the three neural cell adhesion molecules L1, N-CAM, and MAG and their shared carbohydrate epitope L2/HNK-1 during development of the mouse sciatic nerve. Myelin basic protein (MBP) was also investigated as an antigen that appears late developmentally and does not belong to the L2/HNK-1 family. A detailed analysis of the developmental expression of cell adhesion molecules in the three-dimensional context of the intact tissue appears to be essential for our understanding of morphogenesis in the peripheral nervous system.

Materials and Methods

Animals and Tissue Processing

NMRI mice (newborn, 8- and 21-d-old) were anesthetized by intraperitoneal injection of an aqueous solution of chloral hydrate (3%, 0.01 ml/g body weight) and perfused through the heart with ~50–100 ml 4% paraformaldehyde and 0.25% glutaraldehyde in 0.12 M Palay buffer (Palay and Chan-Palay, 1974). Sciatic nerves were then removed and immersed in 4% paraformaldehyde in 0.12 M Palay buffer for at least 2 h at 4°C. Postfixation could be extended up to 24 h with no influence on tissue integrity and preservation of antigen reactivity. Sciatic nerves were embedded in 3% agar agar (E. Merck, Darmstadt, FRG) in phosphate-buffered saline, pH 7.3 (PBS). Sections 40–100- μ m thick were cut on an Oxford vibratome (Ted Pella, Inc., Irvine, CA) and rinsed for at least 2 h in PBS containing 0.1% bovine serum albumin (PBS/BSA). For some experiments a blocking solution of 1% BSA in PBS was used with similar results.

Antibodies

Except for the monoclonal antibodies to the L2/HNK-1 epitope, polyclonal primary antibodies were used, since they stained more strongly than the corresponding monoclonal ones. Production and specificity of the primary antibodies have been described and the pertinent information is summarized in Table I. Immunoaffinity purification of polyclonal rabbit antibodies to mouse L1 was performed by passing hyperimmune serum over a Sepharose

1. *Abbreviations used in this paper:* MAG, myelin-associated glycoprotein; MBP, myelin basic protein; N-CAM, neural cell adhesion molecule.

4B column conjugated to immunoaffinity-purified L1 antigen by cyanogen bromide activation and elution with glycine buffer at pH 2.5. Antibodies to human hemoglobin (Sigma Chemical GmbH, Munich, FRG) were prepared in rabbits by three injections of 1 mg hemoglobin each, in complete Freund's adjuvant for the first immunization, incomplete Freund's adjuvant for the second immunization, and no adjuvant for the third immunization. The first two injections were subcutaneous and the third intraperitoneal. Animals were bled 7 d after the last immunization.

For pre-embedding staining, polyclonal antibodies were visualized by peroxidase-coupled protein A (Sigma Chemical GmbH) at a dilution of 0.75 $\mu\text{g}/\text{ml}$. The monoclonal antibodies to the L2 and HNK-1 epitopes were visualized by peroxidase-coupled goat antibodies to rat and mouse IgG, respectively (72 $\mu\text{g}/\text{ml}$; Cappel Laboratories, Cochranville, PA). For post-embedding staining antibodies adsorbed to colloidal gold (5 and 15-nm diam; Janssen Pharmaceutica, Beerse, Belgium) were used at dilutions of 1:25. Polyclonal antibodies were visualized by goat antibodies to rabbit IgG and monoclonal antibodies by goat antibodies to rat IgG.

Pre-embedding Staining

Vibratome sections were treated with 0.1 M NaIO_4 in PBS followed by NaBH_4 reduction (10 mg/ml PBS) and 5% dimethyl sulfoxide in PBS at room temperature as described (Schachner et al., 1977). After washing in PBS/BSA the vibratome sections were incubated for 2 h with primary antibodies at room temperature. Control sections were immersed in PBS/BSA or with rabbit anti-hemoglobin antiserum diluted in PBS/BSA. Sections were washed for 60 min at room temperature in PBS/BSA and incubated for 2 h with peroxidase-coupled protein A or peroxidase-coupled antibodies to rat or mouse IgG. After washing in PBS for 60 min at room temperature sections were fixed with 1% glutaraldehyde in 0.01 M phosphate buffer, pH 7.2. Sections were washed again in PBS overnight at 4°C and immersed in a solution of 2 mg diaminobenzidine hydrochloride (DAB; Sigma Chemical GmbH) in 10 ml 0.04 M Tris-buffer, pH 7.6, for 30 min. Treatment with the same DAB solution containing 0.005% H_2O_2 followed for 15 min. Incubations with DAB were performed in the dark at room temperature. Sections were postfixed with 2% OsO_4 in 0.01 M phosphate buffer, pH 7.2, dehydrated in an ascending series of acetone, and embedded in Spurr's medium. Thin sections, 50–70-nm thick, were cut on an LKB III ultramicrotome and mounted on formvar-coated copper grids. Some sections were counterstained with lead citrate for 3 min and uranyl acetate for 15 min at room temperature. Sections were examined in a Zeiss EM 10C.

Post-embedding Staining

Vibratome sections were dehydrated in an ascending series of ethanol at low temperatures (30% at 0°C for 30 min; 50% at -20°C for 60 min; 70% at -35°C for 60 min; 100% at -35°C twice for 60 min). Infiltration with Lowicryl and polymerization by ultraviolet light (UV) was carried out for 24 h at -30°C. UV irradiation was continued for 2–3 d at room temperature to improve the sectioning properties (Roth et al., 1981). Ultrathin sections, 50–60-nm thick, were cut, mounted on formvar-coated copper grids, and rinsed in PBS containing 1% BSA for 2 h. After incubation with the first antibodies for 2 h the sections were washed for 2 h and incubated for 30 min with the second antibodies complexed with colloidal gold. All incubations were carried out at room temperature. Control sections were treated as described under "Pre-embedding Staining." Sections were washed and counterstained with an aqueous solution of uranyl acetate for 10 min at room temperature.

Results

Sciatic nerves of newborn and 8- and 21-d-old mice were taken for pre- and post-embedding immunoelectron microscopic procedures. At each age, fasciculating, unmyelinated axons, axons with compact and uncompacted myelin, and non-myelinating and myelinating Schwann cells could be observed. However, compact myelin was more abundant in the 21-d-old nerve, whereas unmyelinated axons, non-myelin forming Schwann cells, and uncompacted myelin were less frequently seen. Mitotic Schwann cells, which were always found associated with axons, were only seen in newborn mice.

L1 and N-CAM

Since both antigens showed a very similar localization at non-myelinated stages, they will be described together.

Detectable levels of both antigens were seen on small, non-myelinated, fasciculating axons (Figs. 1 *a* and 2 *c*). Axons separated from each other by intervening Schwann cell processes also expressed L1 and N-CAM (Figs. 1 *b* and 2 *c*). In their non-myelinating state, Schwann cells expressed the antigens at the sites of axonal apposition (Figs. 1 and 2 *c*). Mitotic Schwann cells (in metaphase) recognized by the absence of nuclear membrane around chromatin expressed L1 and N-CAM at the site of neuronal contact (Fig. 2 *c*, *inset*). Large axons were only positive at early developmental stages, when they were surrounded by the mesaxon by not more than ~ 1.5 loops (Fig. 1 *c*, *inset*). Schwann cells also remained L1- and N-CAM-positive until they had turned around the axon for ~ 1.5 loops. Thereafter, L1 could not be detected on either axon or Schwann cell (Fig. 1 *a*). Compact myelin, Schmidt-Lanterman incisures, paranodal loops, and finger-like processes of Schwann cells at the nodes of Ranvier were always L1-negative (Figs. 1, *b* and *c*). In myelinated fibers N-CAM was, in contrast to L1, detectable at the periaxonal region, and, more weakly, in compact myelin (Fig. 2 *d*), particularly when smaller gold particles were used as second antibody label. It is our impression that the larger diameter myelinated axons express N-CAM in their periaxonal region and in compact myelin more than the smaller myelinated ones. At the nodes of Ranvier, the axolemma was always L1- and N-CAM-negative (Fig. 1 *b*). These results were the same in pre- and post-embedding staining procedures. In general, the density of immunoreactive material was higher and more continuous in pre-embedding than in post-embedding procedures, except for compact myelin, which was never stained in pre-embedding techniques.

In 8- and 21-d-old mice, staining with L1 and N-CAM was also found in the basement membranes and collagen fibrils surrounding the myelinating and non-myelinating Schwann cells, when compared with sections that had been treated either with antibodies to human hemoglobin, MBP, (see below) or no primary antibodies at all (Figs. 1, *a* and *b* and 2, *a* and *b*). However, immunostaining was not uniformly distributed around Schwann cells, so that regions with high and low immunoreactivity could be seen adjacent to each other in one and the same section (Figs 1, *a* and *b*). This uneven immunostaining was more obvious in sections obtained by pre- than by post-embedding procedures, but could definitively be observed also by post-embedding methods, thus precluding artifactual staining by diffusion of peroxidase reaction product in pre-embedding methods (Fig. 1 *c*). In newborn mice staining of the extracellular matrix was either very weak or absent.

The L2/HNK-1 Carbohydrate Epitope

This carbohydrate epitope showed a cellular and subcellular distribution similar to the one observed for L1 and N-CAM (Fig. 3, *a* and *b*). It was consistently detectable on some, but not all small, fasciculating axons, axons surrounded by Schwann cells, and axons at the onset of myelin formation. It was present on axon and adjacent Schwann cell before completion of ~ 1.5 –2 loops of Schwann cell membrane, but disappeared thereafter (Fig. 3 *b*). No coincident localization

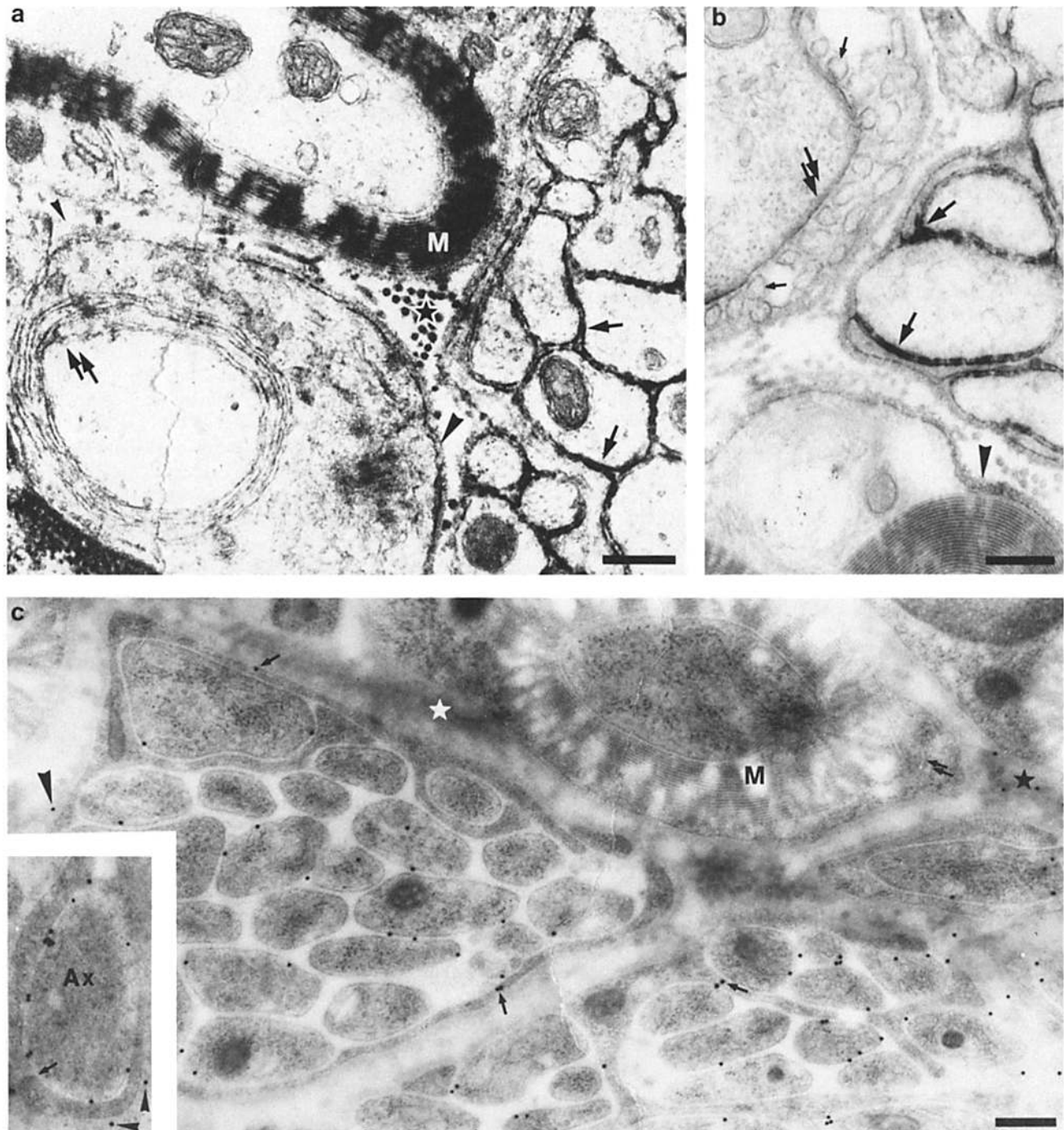


Figure 1. Immunoelectron microscopic localization of L1 antigen in sciatic nerve of 21- (*a-c*) and 8- (*inset in c*) d-old mice. (*a*) Immunolabeling was performed by pre-embedding staining procedures. Section was counterstained with lead citrate and uranyl acetate. Note the peroxidase reaction product on fasciculated axons (*arrows*). Developing myelin sheets with two turns of non-compacted Schwann cell membrane and corresponding axon are L1-negative (*double arrows* at inner mesaxon). Basement membrane and collagen fibrils are antigen-positive in some areas (*large arrowhead* and *asterisk*, respectively), while negative in others (*small arrowhead* for basement membrane). *M*, compacted myelin. (*b*) Immunolabeling was performed by pre-embedding staining procedures. Section was not counterstained. Note the peroxidase reaction product on axons and non-myelinating Schwann cells at the sites of apposition (*arrows*) and on basement membrane of myelin-forming Schwann cell (*arrowhead*). At the node of Ranvier finger-like processes of Schwann cell (*small arrows*) and axolemma (*double arrows*) are antigen-negative. The axolemma shows an electron-dense undercoating, which is also seen in control sections (see Fig. 2 *a*). (*c*) Immunolabeling was performed by post-embedding staining procedures (15-nm gold particles). Note that L1 is detectable both on the non-myelinated axons and non-myelin-forming Schwann cells (*arrows*). The localization of L1 on these Schwann cell processes could be unequivocally determined, since a distance of only 15 nm accounts for the size of two antibodies in the indirect immunolabeling procedure. Staining of basement membrane of non-myelinating Schwann cell (*arrowhead*) and collagen fibrils is seen in some areas (*black asterisk*), but not in others (*white asterisk*). Neither the axolemma nor the compact (*M*) or non-compacted myelin (*double arrows*) are L1-positive. (*Inset*) Large axon surrounded by a single Schwann cell loop. Both axon and Schwann cell are stained. Mesaxon (*arrow*), staining of basement membrane (*arrowheads*). Bars, 0.25 μm .

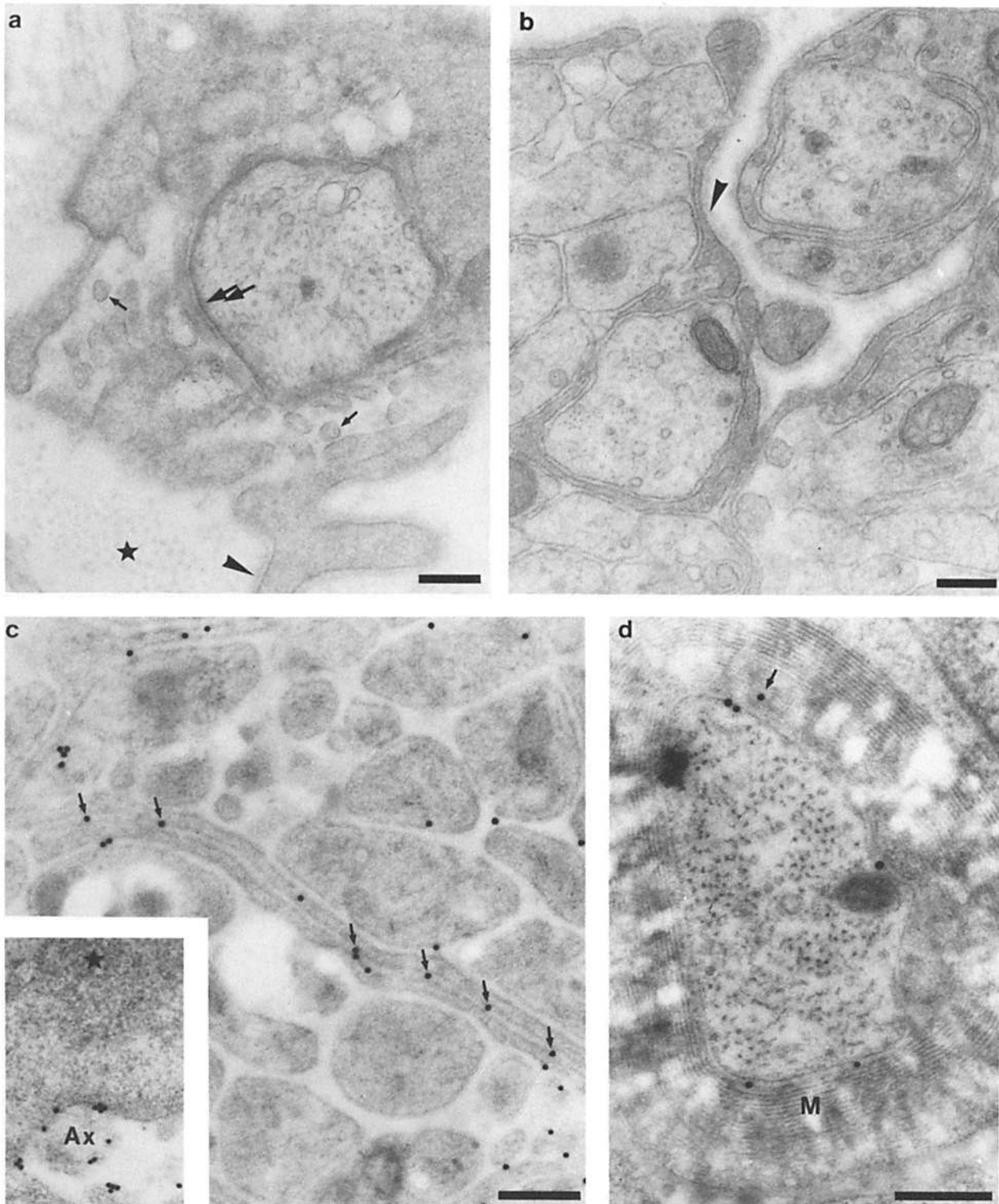


Figure 2. Immunoelectron microscopy of control sections treated with antibodies to human hemoglobin (*a* and *b*) and localization of N-CAM (*c* and *d*) in sciatic nerve of 8-d-old (*a* and *b*), 21-d-old (*c* and *d*), and newborn (*inset* in *c*) mice. Immunolabeling was performed by pre-embedding staining procedures and sections were not counterstained (*a* and *b*) and by post-embedding staining procedures with 15-nm gold particles (*c* and *d*). (*a* and *b*) Note the absence of peroxidase reaction product on finger-like processes (*arrows*), basement membrane (*arrowhead*), and collagen fibrils (*asterisk*). Electron-dense undercoating of the nodal axolemma (*double arrows*). (*b*) Neither axons nor non-myelinating Schwann cells and their associated basement membrane (*arrowhead*) show peroxidase reaction product. (*c*) Unmyelinated axons are N-CAM-positive. Plasma membranes of insheathing Schwann cells are also N-CAM-positive at sites of apposition of two Schwann cell processes (*arrows*). (*Inset*) Contact between mitotic (metaphase) Schwann cell and axon is N-CAM-positive. Chromatin of Schwann cell (*asterisk*). (*d*) N-CAM is often observed periaxonally between axolemma and compact myelin (*M*) and in compact myelin (*arrow*). Bars, 0.25 μ m.

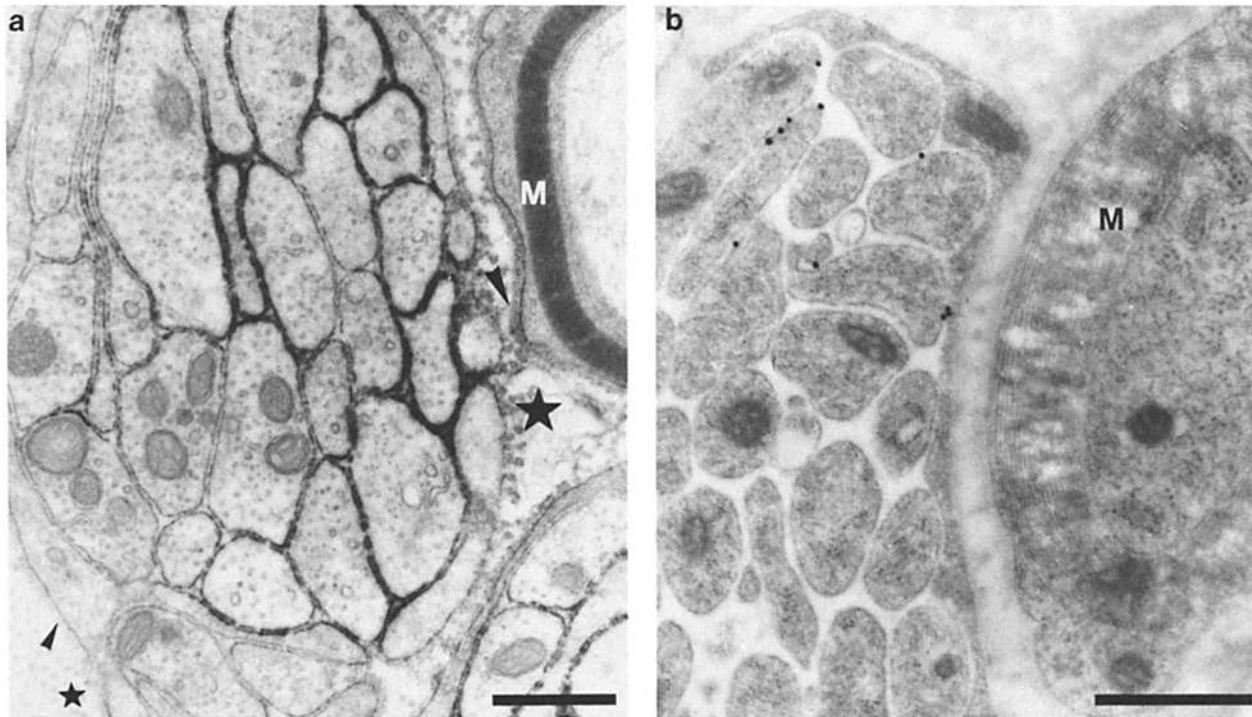


Figure 3. Immunoelectron microscopic localization of the L2 epitope in sciatic nerve of 8-d-old mice. (a) Immunolabeling was performed by pre-embedding staining procedures. Section was not counterstained. Non-fasciculating axons and insheathing Schwann cell show immunoperoxidase reaction product as observed for L1 or N-CAM. Note, however, that the labeling intensities vary between axons. Labeling of basement membranes is also not uniform, with labeling seen in some areas (*large arrowhead*), but not in others (*small arrowhead*). Collagen fibrils can be epitope-positive (*large asterisk*) and epitope-negative (*small asterisk*). M, compacted myelin. (b) Immunolabeling was performed by post-embedding staining procedures with 15-nm gold particles. Some axons are immunogold labeled, while others are not. Compact myelin (M) and periaxonal regions of myelinated fibers are never stained. On this micrograph no labeling of basement membranes is seen. Bars, 0.5 μ m.

of the carbohydrate epitope was observed with MAG or MBP using the post-embedding staining method (see below). Basement membranes of Schwann cells and collagen fibrils were intensely stained by L2 and HNK-1 antibodies at both ages examined (Fig. 3 a). Both antibodies gave similar staining patterns.

MAG

MAG was not detectable on non-myelinating Schwann cells. It first appeared on the myelinating membranes of Schwann cells when the mesaxon had formed \sim 1.5–2 loops (Fig. 4 a). When compaction of myelin occurred, MAG disappeared from the compacting membranes and was confined to the periaxonal region and to non-compact structures, i.e., the Schmidt–Lanterman incisures, inner and outer mesaxon, and paranodal loops (Fig. 4, b and c). The finger-like processes at the nodes of Ranvier were also antigen-negative in the fully myelinated nerve. In rarer cases, however, these finger-like processes were transiently stained at early stages of myelination. MAG immunoreactivity was observed on basement membranes of the myelin-forming and non-myelinating Schwann cells and on collagen fibrils (Fig. 4, b and c). This staining pattern was particularly prominent in the pre-embedding technique.

MAG could be detected reliably only by the post-embedding method. However, MAG immunoreactivity in the extracellular matrix and transient appearance on finger-like processes at the node of Ranvier could be seen also by pre-

embedding methods. Furthermore, when the tissue had been damaged or the myelin was abnormally vacuolated, MAG was detected at the axon–Schwann cell interface by pre-embedding staining procedures.

MBP

MBP was reliably detectable also only by post-embedding staining procedures. In contrast to MAG it was amply present in compact myelin (Fig. 4 d), but absent from Schmidt–Lanterman incisures, inner and outer mesaxon, paranodal loops, and finger-like processes at nodes of Ranvier. It was never seen in periaxonal regions of myelinated nerves. During myelin formation it was first seen in the compacting membranes. It was never seen on basement membranes or collagen fibrils.

Discussion

In this study we have shown that the developmental appearance of three cell adhesion molecules (L1, N-CAM, and MAG) and MBP, an intracellularly localized protein, follows a particular pattern that seems to be coordinated in time and space. L1 and N-CAM are the first molecules, detectable during development, with an almost identical cellular and subcellular localization. The fasciculating axons and the non-myelinating Schwann cells are L1- and N-CAM-positive at the time neurons and Schwann cells have made contact with each other. Both antigens are expressed on Schwann

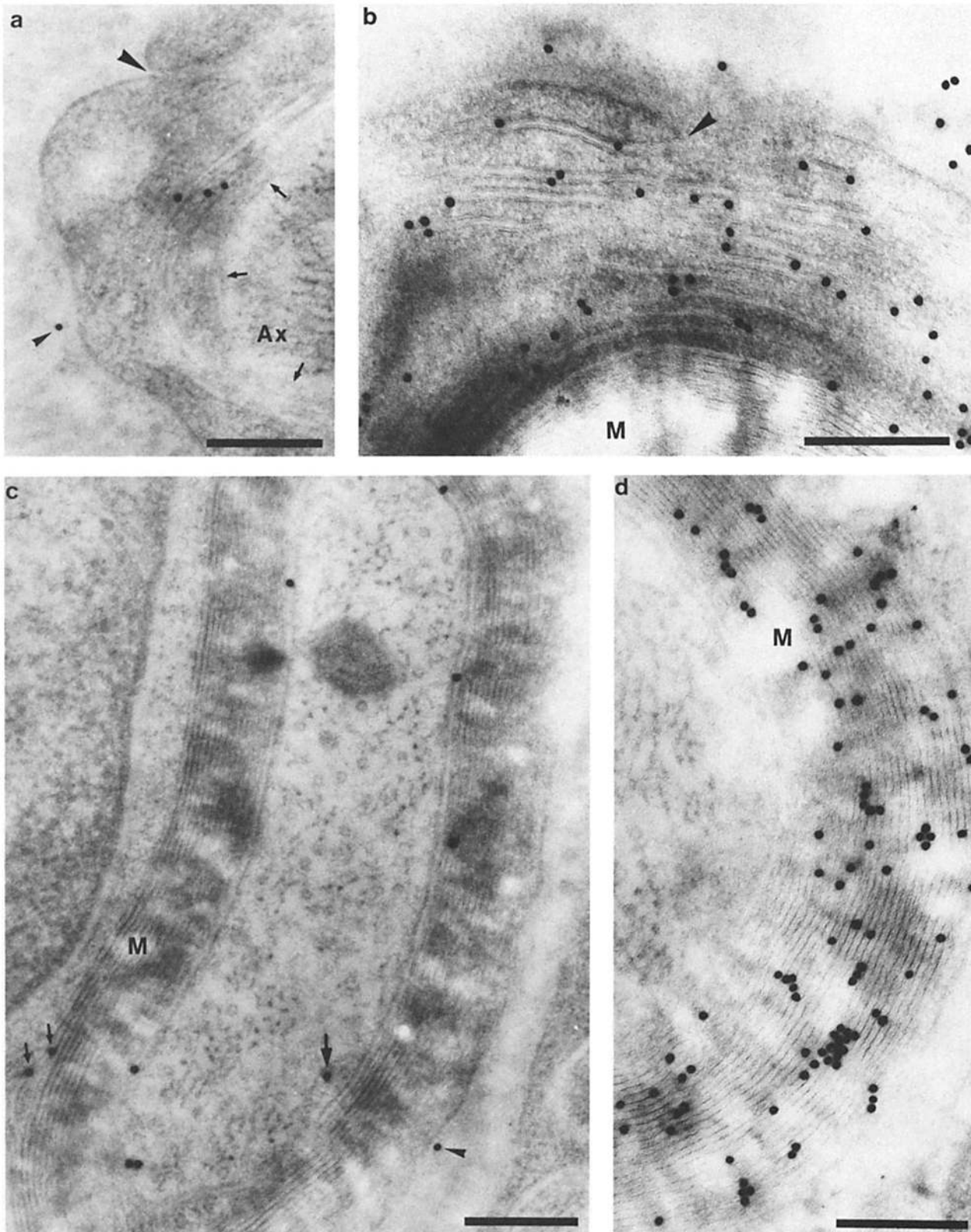


Figure 4. Immunoelectron microscopic localization of MAG (*a–c*) and MBP (*d*) in sciatic nerve of 8-d-old (*a* and *b*) and 21-d-old (*c* and *d*) mice. Immunolabeling was performed by the post-embedding staining procedures with 15-nm gold particles. (*a*) Axon (*Ax*) is not MAG-positive at axolemma (*arrows*). Loops of Schwann cells are antigen-positive. The basement membrane is also stained (*small arrowhead*). Outer mesaxon (*large arrowhead*). (*b*) Uncompact myelin is heavily stained by MAG antibodies, whereas compact myelin (*M*) is not stained. Note the antigen-positivity in the extracellular matrix. Outer mesaxon (*large arrowhead*). (*c*) MAG is detectable in the periaxonal region of a myelinated axon (compact myelin, *M*). Inner mesaxon (*large arrow*) and uncompact myelin near the outer mesaxon (*small arrows*) are also MAG-positive. Basement membrane is also labeled (*arrowhead*). Compact myelin (*M*) is not MAG-positive. (*d*) MBP is only present in compact myelin (*M*). Bars, 0.25 μ m.

cells during mitosis, when the cells remain in surface contact with axons (Martin and Webster, 1973; Peters et al., 1976). L1 ceases to be expressed on both axon and myelinating Schwann cell, when the Schwann cell processes have turned for ~ 1.5 loops around the axon. N-CAM expression is reduced when myelination proceeds (see also Covault and Sanes, 1986; Mirsky et al., 1986; Nieke and Schachner, 1985; Noble et al., 1985), but is still detected periaxonally in mature fibers. It is at the stage, when L1 disappears, that MAG is first detectable not only at the axon-Schwann cell interface, but also on the opposing cell surface membranes of the turning glial loops. At more advanced developmental stages, MAG remains expressed in non-compacted myelin and at sites of axon apposition, but is lost from the compacting Schwann cell membranes. This loss coincides with the appearance of MBP, which is only detectable in the compact myelin. This finding, however, is in contrast to the results of Schwob et al. (1985) and Omlin et al. (1982), who observed MBP not only in the major dense line, but also diffusely in the cytoplasm of developing processes of oligodendroglia or Schwann cells. It is possible that our failure to detect MBP in the cytoplasm is due to a different fixation protocol (Sternberger, 1984, for review).

Interestingly, the L2/HNK-1 carbohydrate epitope shows coincident localization with L1 and N-CAM at the three stages examined, with the exception of the periaxonal region, where N-CAM but no L2/HNK-1 epitope is detectable. Since we do not know which other members of the L2/HNK-1 family are also expressed in sciatic nerve, the molecules carrying the epitope remain unidentified. Coincident labeling with MAG was, however, never observed, suggesting that the epitope is not involved in the activity of MAG in myelination of the sciatic nerve. The L2/HNK-1 epitope is also never detectable in the finger-like processes of Schwann cells at the nodal region. It is therefore unlikely that an L2/HNK-1 epitope carrying molecule on these processes assumes a similar function as the cell adhesion molecule J1 on astrocytic processes, which are thought to play a role in the maintenance of nodal architecture in the adult rat optic nerve (French-Constant et al., 1986). It should be noted also that not all non-myelinated axons express the L2/HNK-1 epitope, thus pointing to a cellular heterogeneity that has been noted previously in the peripheral nervous system for other carbohydrate structures (Dodd and Jessell, 1985).

The presence of L1, N-CAM, MAG, and L2/HNK-1 epitope on basement membranes and collagen fibrils is detectable in 8- and 21-d-old, but not in newborn mice. This localization is considered specific, since it is not seen in control sections and sections treated with antiserum to MBP. Furthermore, these structures are also immunostained in the post-embedding staining procedure, where leakage of the peroxidase reaction product is avoided. In light of these observations, the localization of L1 at the nodes of Ranvier in the sciatic nerve of the rat at the light microscopic level (Mirsky et al., 1986) can now be attributed to the particularly strong expression of L1 in basement membranes of the rat peripheral nervous system (unpublished observations) and renders a localization of L1 on the surface membrane of the nodal finger-like processes unlikely.

Our present observations are in agreement with previous results on the expression of L1 and N-CAM during development of the mouse sciatic nerve at the light microscopic level

(Nieke and Schachner, 1985). However, at this level of resolution, it was difficult to see the localization of L1 and N-CAM on fasciculating nerves and the overlap of the localization of the shared carbohydrate epitope L2/HNK-1 with L1 and N-CAM. Since the L2/HNK-1 epitope is most strongly localized in basement membranes and in collagen fibrils, a less prominent staining in other structures may have been overlooked. On the other hand, in previous studies the HNK-1 epitope has been described to be associated with neurons of peripheral nerves (McGarry et al., 1985; Schuller-Petrovic et al., 1983).

Of particular importance is the observation that MAG is not present in compact myelin, but is unequivocally observed at the myelin-axon interface, Schmidt-Lanterman incisures, outer and inner mesaxon, and paranodal loops. These findings are in agreement with those by Trapp and Quarles (1982, 1984) and Trapp et al. (1984), but contradict results obtained by Webster et al. (1983, 1985) and Favilla et al. (1984), who observed MAG in compact myelin of oligodendroglia. We feel that the present study gives the first unequivocal evidence of the absence of MAG in compact myelin and thus settles a controversy. Also, in contrast to a previous immunocytochemical study in the peripheral nervous system of the chicken, we have not found detectable levels of MAG on neurons (Omlin et al., 1985; Philippe et al., 1986).

Although functional relationships in cell interactions cannot be deduced from morphological data, our observations suggest that L1 and N-CAM are involved in axon fasciculation as it has been observed previously in the central nervous system (Fischer et al., 1986) and mediate initial contacts between axons and Schwann cells. In agreement with this notion, both L1 and N-CAM antibodies have recently been shown to interfere with adhesion of neurons to Schwann cells (Seilheimer, B., A. Faissner, G. Keilhauer, and M. Schachner, manuscript submitted for publication). It is, furthermore, conceivable that N-CAM and L1 mediate surface interactions during the initial turnings of apposing Schwann cell loops. MAG appears to take over Schwann cell-axon and Schwann cell-Schwann cell interactions initiated by L1 and N-CAM. It is interesting in this context that, in the central nervous system, MAG has been shown to mediate not only neuron-oligodendrocyte, but also oligodendrocyte-oligodendrocyte adhesion (Poltorak, M., G. Keilhauer, A. Meyer, C. Landa, and M. Schachner, manuscript submitted for publication). Since in the adult sciatic nerve the localization of MBP in compact myelin is a virtual mirror image of MAG, we would like to propose that MBP is involved in the apposition of the cytoplasmic sites of the Schwann cell membranes (Schwob et al., 1985), whereas MAG is characteristic of surface contacts at sites where the cytoplasm is retained (Quarles, 1983/84).

It may be worthwhile to point out that other than "homophilic" binding partners for the cell adhesion molecules have to be postulated from our observation that N-CAM, L1, and MAG are all localized in basement membranes and collagen fibrils. It is intriguing that the intensity of the immunostaining reaction is not uniform within one section, particularly for the collagen fibrils. Since staining artifacts can be excluded in our study we would like to suggest that the adhesion molecules are secreted by unevenly localized cellular sources and retained by the extracellular matrix, where

they subserve yet unknown functions. It has been observed that the basement membrane or extracellular matrix material guides regrowing axons under regenerative conditions (Longo et al., 1984; Schwab and Thoenen, 1985). It is therefore interesting that the adhesion molecule J1 has been observed on collagen fibrils in the proximity of denervated, but not innervated neuromuscular junctions (Sanes et al., 1986). It is worth noting here that a rigorous classification of adhesion molecules into those that mediate interactions with other cells and those mediating interactions with the extracellular matrix becomes futile, as adhesion molecules are now found both as integral membrane constituents and as part of the extracellular matrix.

It is still not known which molecular signals lead an axon-Schwann cell interaction to myelination in one case, but to no myelination in the other. MAG and MBP expression seem to be the consequence of the commitment to myelination, while L1 and N-CAM expression appear to precede this decision. The signals that determine the realization of these distinct differentiation pathways may involve surface bound or diffusible signals between axon and Schwann cells. Expression of L1 and N-CAM could therefore provide a basis for axon-Schwann cell relationships upon which other modulating events may be superimposed that trigger a Schwann cell to myelinate or remain non-myelinating.

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