

# Expression of Recombinant Myelin-associated Glycoprotein in Primary Schwann Cells Promotes the Initial Investment of Axons by Myelinating Schwann Cells

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**Abstract.** Myelin-associated glycoprotein (MAG) is an integral membrane protein expressed by myelinating glial cells that occurs in two developmentally regulated forms with different carboxyterminal cytoplasmic domains (L-MAG and S-MAG). To investigate the role of MAG in myelination a recombinant retrovirus was used to introduce a MAG cDNA (L-MAG form) into primary Schwann cells in vitro. Stably infected populations of cells were obtained that constitutively expressed MAG at the cell surface without the normal requirement for neuronal contact to induce expression. Constitutive expression of L-MAG did not affect myelination. In long term co-culture with purified sensory neurons, the higher level of MAG expression on infected Schwann cells was reduced to control levels on cells that formed myelin. On the other hand, unlike normal Schwann cells, infected Schwann cells as-

sociated with nonmyelinated axons or undergoing Wallerian degeneration expressed high levels of MAG. This suggests that a posttranscriptional mechanism modulates MAG expression during myelination. Immunostaining myelinating cultures with an antibody specific to L-MAG showed that L-MAG was normally transiently expressed at the earliest stages of myelination. In short term co-culture with sensory neurons, infected Schwann cells expressing only L-MAG segregated and ensheathed larger axons after 4 d in culture provided that an exogenous basal lamina was supplied. Similar activity was rarely displayed by control Schwann cells correlating with the low level of MAG induction after 4 d. These data strongly suggest that L-MAG promotes the initial investment by Schwann cells of axons destined to be myelinated.

**M**YELINATION presents a striking example of cellular differentiation that depends upon the interaction between two cell types, namely an appropriate glial cell and neuron. In the PNS, the first anatomically resolved step in myelination involves the segregation, by Schwann cells, of the larger axons that are to be myelinated from the smaller axons which will remain unmyelinated. The Schwann cell then commits itself to a particular axon by enveloping 100–200  $\mu\text{m}$  of the axon's length with a single layer of cytoplasm. Hereafter, we will refer to this step as establishment of the 1:1 association between Schwann cell and axon (28, 40). The advance of one lip of this cytoplasmic process over the surface of the axon initiates the myelin spiral (4). For the Schwann cell to envelop selectively an axon destined to be myelinated specific recognition and adhesion mechanisms must exist. The location of myelin associated glycoprotein (MAG)<sup>1</sup> in the periaxonal collar of Schwann cell cytoplasm in mature myelin has led to a suggested role

in glial/axonal interaction (36). Recent elucidation of MAG cDNA sequences has shown that the extracellular region of MAG shares homology with the neural cell adhesion molecule N-CAM and other members of the immunoglobulin gene superfamily (1, 18, 33). This homology with N-CAM, together with the recent demonstration that liposomes containing purified MAG exhibit MAG-dependent binding to neurites (15, 30), has strengthened the idea that MAG functions as a cell adhesion molecule in myelination. Sequencing of MAG cDNAs has also shown that the two forms of the molecule (11) arise from a single gene by alternative splicing to yield two polypeptides that only appear to differ in the size of their carboxyterminal cytoplasmic domains (18, 33). We refer to the two forms as large-MAG (L-MAG) and small-MAG (S-MAG), respectively (33). It has been reported that mRNA encoding S-MAG is much more abundant than L-MAG mRNA throughout development in the PNS (12, 38). In the central nervous system (CNS) higher levels of L-MAG mRNA have been found and peak expression coincides with the period of myelination (12, 18, 38).

We previously reported that MAG expression by Schwann

1. *Abbreviations used in this paper:* EHS, Engelbreth-Holm-Swarm; MAG, myelin-associated glycoprotein; NGF, nerve growth factor.

cells is regulated by axonal interaction (26). Schwann cells maintained in the absence of neurons do not express MAG; however, MAG is detectable after 7 d of co-culture with sensory neurons. Neuronal induction of MAG expression can occur before the onset of myelination and does not require assembly of a basal lamina by the Schwann cell. The finding that ensheathment and myelination by Schwann cells do depend upon basal lamina formation (3) suggests that MAG may not function in these processes unless the Schwann cell assembles a basal lamina. We previously hypothesized, therefore, that the basal lamina is required for the Schwann cell to develop polarized cell surfaces, and thereby allow a critical number of MAG molecules to interact with receptors on an axon destined to be myelinated (26). Recently, we have found that Schwann cells arrested at the 1:1 stage of Schwann cell/axon association by depletion of galactocerebroside from the cell surface express MAG in the single layer of cytoplasm that surrounds the axon (27). This indicates that MAG is available to participate in the interaction with the axon at this early stage of myelination.

In mature peripheral myelin sheaths, MAG is only found in uncompacted regions of the sheath, including the paranodes, Schmidt-Lanterman incisures and, as previously mentioned, the periaxonal collar of Schwann cell cytoplasm (36). In the hypomyelinating mutant mouse quaking, axons have been observed to be surrounded by uncompacted lamellae that contain both P<sub>0</sub> and MAG immunoreactivity (37). This observation has led to the suggestion that MAG maintains uncompacted membranes, possibly by a homophilic interaction, and must be removed from a membrane for compaction mediated by P<sub>0</sub> to occur (37).

With these considerations in mind, we decided to overexpress MAG in Schwann cells and examine the consequences on myelination. By infection with a retrovirus containing the L-MAG cDNA, we established populations of primary rat Schwann cells that constitutively expressed MAG at the cell surface without neuronal contact. We combined these infected Schwann cells with purified sensory neurons in long and short term co-culture experiments. In the long term co-cultures, we did not detect any difference in the quantity and morphology of the myelin formed by infected cells as compared to normal Schwann cells. Using an L-MAG specific antibody, we found that L-MAG was normally expressed in nascent internodes formed by both control and infected Schwann cells. In mature myelin segments, however, L-MAG was only detectable in segments formed by infected Schwann cells. Further, unlike normal Schwann cells, infected Schwann cells associated with unmyelinated axons or undergoing Wallerian degeneration expressed high levels of MAG.

In short term co-culture experiments in which Schwann cells were grown for 4 d with purified sensory neurons in the presence of an exogenous basal lamina, we found that infected cells had segregated and ensheathed many more larger axons compared with uninfected Schwann cells. In the control cul-

ture, only 1–2% of the cells had upregulated MAG after this limited time of contact with neurons. This result, together with observed expression of L-MAG in nascent internodes formed by uninfected Schwann cells, leads us to propose that L-MAG normally promotes the initial investment of axons by myelinating Schwann cells.

## Materials and Methods

### Production of a Recombinant Retrovirus Encoding MAG

Hind III linkers were added to a 2042 bp Apa I cDNA fragment containing the complete L-MAG coding region (33). The resulting fragment was inserted into the retroviral vector pMV7 (16) at the Hind III site, and was transfected into the amphotropic packaging cell line PA317 (22) using the calcium phosphate coprecipitation method (13). G418 resistant clones were screened for the surface expression of MAG with an anti-MAG monoclonal antibody (30). Supernatant from a MAG positive clone was used to infect the ecotropic packaging cell line Psi2 (20), and several MAG positive, G418 resistant clones were selected. A cell line, MAG/Psi2 with an estimated titer of  $1.6 \times 10^6$  cfu/ml on NIH 3T3 cells was used to infect primary Schwann cells.

### Primary Cell Culture

Schwann cells were prepared according to Brookes et al. (2) but with 2 arabinoside C treatments as previously described (26). To infect Schwann cells, cells were plated on poly-L-lysine coated glass coverslips at a density of  $\sim 1.5 \times 10^5$  cells/coverslip and were pretreated for 2 h with polybrene (5  $\mu$ g/ml). The Schwann cells were rinsed with media and then incubated with filtered culture media from subconfluent packaging cells for two consecutive periods of 24 h in the presence of glial growth factor and forskolin (31). After a further 48 h, G418 (200  $\mu$ g/ml final effective concentration) was added; and 14–21 d later, small groups of resistant cells were observed; in general 1–2 colonies were obtained per coverslip. Forskolin and glia growth factor were removed 21–28 d post infection to avoid the onset of unregulated growth due to continuous mitogen stimulation (32). To expand the number of infected cells, depending upon their size, colonies were either pooled or plated individually onto purified dorsal root ganglion neuron cultures prepared as described (8, 26). In previous work, Schwann cells were expanded on neurites in defined medium (8, 26); however, due to the low plating density, it was observed that neurites that were not occupied by Schwann cells degenerated after several weeks in defined medium. This did not occur if serum containing medium was used. Therefore, all Schwann cells in co-culture with neurons were propagated in MEM containing nerve growth factor (NGF) (7S nerve growth factor; Boehringer Mannheim Diagnostics, Inc., Indianapolis, IN); glucose (315 mg %); FBS (10%); Hyclone Laboratories, Logan, UT). This medium did not promote basal lamina assembly and myelination. After 6–8 wk, cells had repopulated the outgrowth of neurites and were transferred to either new neuronal cultures or to poly-L-lysine-coated coverslips. To recover the Schwann cells from a culture, the neuronal cell bodies were first cut out, the culture was treated with collagenase (0.1%, 30 min, 37°C) followed by trypsin (0.1%, 30 min, 37°C), and then transferred to serum containing medium, triturated, and replated. To promote myelination, cultures were switched to MEM, NGF, glucose, FBS (15%) and ascorbic acid (50  $\mu$ g/ml). Cultures were allowed to form myelin for up to 4 wk. In the experiment using an exogenous basal lamina matrix (Matrigel, an extract of Engelbreth-Holm-Swarm [EHS] mouse tumor extracellular matrix; Collaborative Research, Bedford, MA)  $\sim 3 \times 10^5$  Schwann cells were plated in serum containing medium onto a single neuronal culture and were allowed to adhere for 3 h at 37°C. The medium was removed and 0.35 ml of ice-cold basal lamina matrix was pipetted onto the cells and allowed to gel for 30 min at 37°C. Myelin promoting medium was then added, and the culture was maintained for 4 d.

### Immunocytochemistry and Electron Microscopy and Confocal Microscopy

Immunocytochemistry and electron microscopy were carried out as previously described (26). To reduce background staining, it was necessary to preadsorb the L-MAG specific antipeptide antibody P6 (25) against a homogenate of Schwann cells prepared by disrupting the cells in a Dounce



**Figure 1.** Structure of the recombinant retrovirus encoding L-MAG. LTR, Maloney murine leukemia virus long terminal repeat; TK, thymidine kinase; NEO, neomycin phosphotransferase.

homogenizer in PBS containing protease-inhibitors (PMSF, benzamide, pepstatin, and aprotinin). To quantitate the surface expression of MAG on infected and control Schwann cells, cells in randomly selected microscope fields were imaged using a scanning laser confocal microscope (MRC-500 confocal imaging system; Bio-Rad Laboratories, Cambridge, MA). 20 consecutive scans were averaged to obtain the final image of each field. Relative fluorescence intensity was measured from the digitized images by integrating the pixel intensities within a defined cell boundary.

### Protein and RNA Blotting

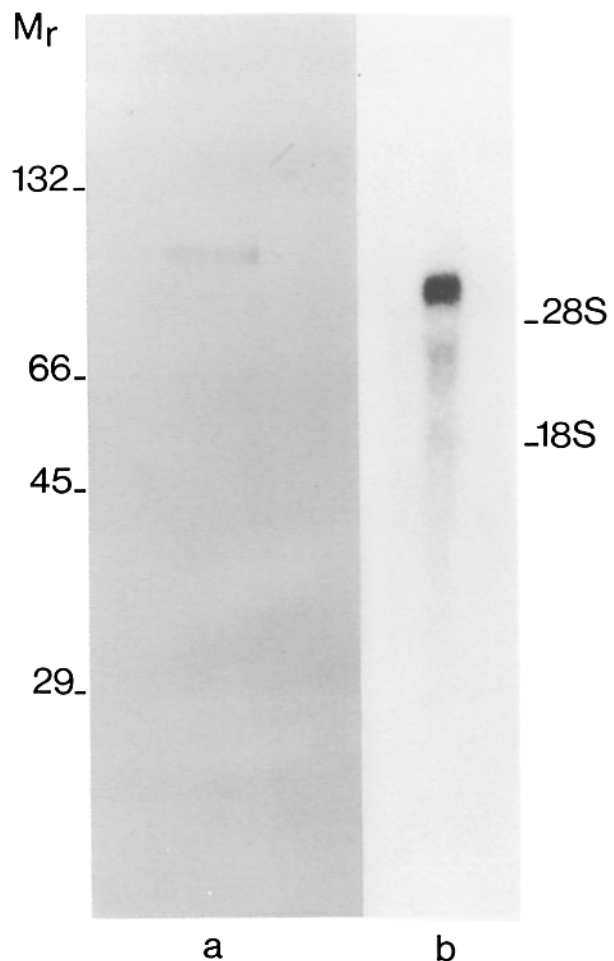
A crude plasma-membrane fraction was obtained by differential centrifugation. Cells were scraped from tissue culture dishes and pelleted in PBS containing protease inhibitors. The cells were disrupted with a polytron (setting 5, 1 min), and Dounce homogenizer (50 times) in 0.15 M sucrose/PBS plus protease inhibitors. After centrifugation (10,000 *g*, 20 min) membranes were pelleted from the supernatant at 130,000 *g* for 90 min. The pellet was resuspended in Tris HCl (25 mM, pH 7.5) with MgCl<sub>2</sub> (5 mM) and protease inhibitors, and an aliquot was boiled in 2× sample buffer, fractionated on SDS PAGE, and electroblotted (17, 35). The nitrocellulose was blocked with PBS containing 2% BSA, 1% nonfat dry milk, 0.5% Tween-20, and incubated with anti-MAG mAbs GenS1 and GenS3 (1:1,000 dilution) (24). Binding was visualized with an affinity purified biotinylated secondary antibody and a streptavidin/beta-galactosidase conjugate followed by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. Total RNA was isolated according to the method of Cathala et al. (6) and was fractionated on an 0.8% agarose gel containing 2.2 M formaldehyde. A MAG cDNA probe was oligo labeled (10) and hybridization was performed at 42°C in 50% formamide and 5× SSPE (19).

## Results

### Expression of L-MAG cDNA in Primary Schwann Cells

We constructed a retrovirus encoding the L-MAG form of the molecule under the transcriptional control of the 5' long terminal repeat (Fig. 1) and established an ecotropic packaging cell line harboring the recombinant provirus that expressed MAG at the cell surface. Western and RNA blot analyses confirmed that authentic MAG RNA and corresponding protein were expressed by this cell line (Fig. 2). The incidence of two immunoreactive bands resolved by SDS-PAGE with estimated molecular masses of 101 and 104 kD are likely to result from differences in glycosylation (15).

Culture medium containing the recombinant retrovirus was used to infect primary Schwann cells in the presence of soluble mitogens, and G418 was added 48 h later. After 14 d, small colonies of resistant cells were evident; however, we found that it was not possible to expose the cells to stimulation by soluble mitogens for longer than 28 d without risking the onset of unregulated growth. Therefore, to expand the limited numbers of stably infected cells, we took advantage of the capacity of sensory neurons to induce Schwann cell proliferation (42). Under these conditions, Schwann cells retain their normal function including the ability to myelinate appropriate axons. In most infections, several resistant colonies were pooled and the cells were plated onto duplicate neuronal cultures. Thus, populations of infected cells were obtained rather than clonal lines. The neuronal cell bodies were removed from one such repopulated culture and the expression of MAG was examined after 3 d. Normal Schwann cells deprived of neuronal contact for this length of time have down regulated MAG (26). As shown in Fig. 3, infected Schwann cells continued to express MAG. Some cells expressed higher amounts of MAG than others that may be a consequence of the different insertion sites of the recom-

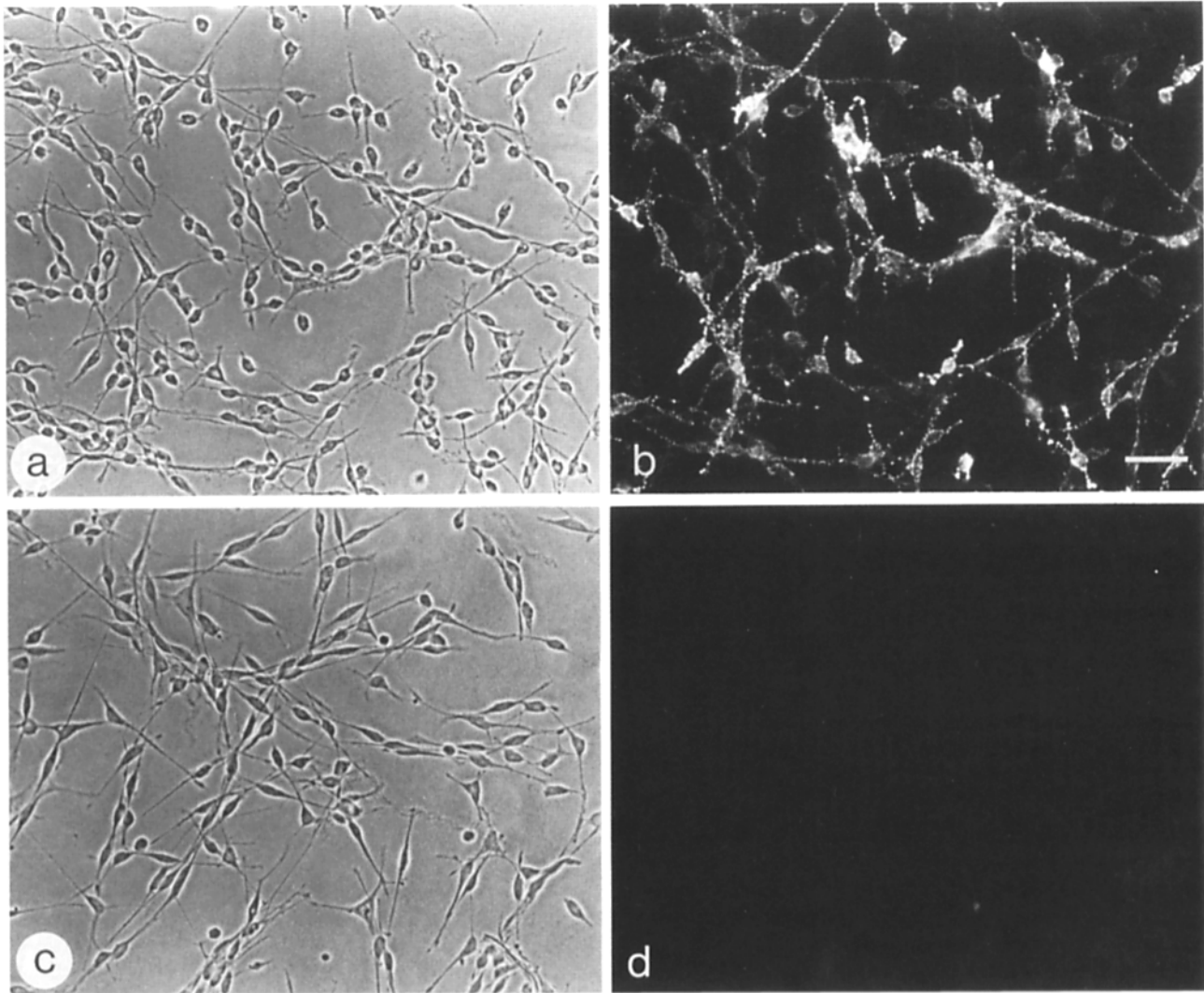


**Figure 2.** Characterization of the packaging cell line expressing L-MAG. (a) Protein blot of crude plasma membranes probed with anti-MAG mAbs GenS1 and GenS3 (24). The antibody binding was visualized with a biotinylated secondary antibody and streptavidin conjugated beta-galactosidase. Molecular mass standards are in kilodaltons. (b) RNA blot of total RNA probed with <sup>32</sup>P-labeled MAG cDNA. The major band corresponds in size to a full length retroviral transcript.

binant retrovirus in the original population of infected cells (14). We transferred infected Schwann cells back onto glass coverslips and verified that they were still resistant to G418 after mitogenic stimulation. G418 added directly to the co-culture proved to be toxic to the neurons at the concentration used initially to select for the infected Schwann cells. We have maintained populations of Schwann cells that constitutively express MAG for up to 9 mo by serially passaging them on purified neuronal cultures.

### Myelination by Infected Schwann Cells

As previously described, MAG expression is induced on Schwann cells by co-culture with sensory neurons under conditions that support Schwann cell proliferation but do not allow basal lamina formation and myelination (26). Schwann cells that are plated onto a neuronal culture and allowed fully to repopulate the outgrowth of neurites begin to form myelin within 3 d after transfer to media containing ascorbic acid, and continue to myelinate for several weeks. When we exam-

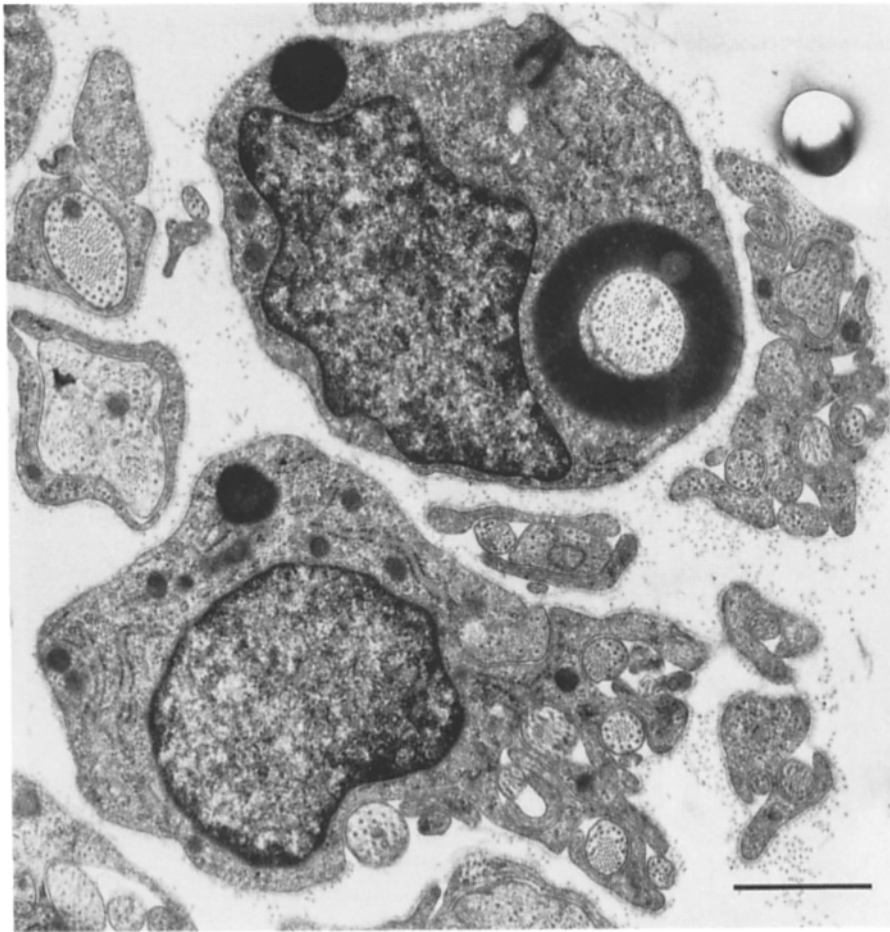


**Figure 3.** Infected G418 resistant primary Schwann cells constitutively express MAG. Neuronal cell bodies were removed from a culture of dorsal root ganglion neurons and infected Schwann cells resulting in rapid degeneration of the neurites. After 3 d, surface staining with an anti-MAG mAb (30), showed the continued expression of MAG(*b*). Under the same conditions, MAG was downregulated on control cells (*d*). *a* and *c* are the corresponding phase images. Bar, 40  $\mu$ m.

ined the ability of infected Schwann cells to form myelin in the above paradigm, we found no difference in the rate of myelination, nor in the structure of the myelin compared to control cells (Fig. 4). Ensheathment by nonmyelinating infected Schwann cells appeared to be normal (Fig. 4). As expected live staining such a culture with anti-MAG antibodies showed that, unlike normal Schwann cells, surface expression of MAG was not confined to myelin segments (Fig. 5); however, it seemed that the amount of MAG at the surface of the myelin segments formed by infected cells was equivalent to that of myelinating Schwann cells in control cultures (Fig. 5). This was surprising since we had envisaged that expression of the recombinant MAG would have had an additive effect. We therefore measured the amount of MAG on the surface of cells in parallel control and infected Schwann cell: neuronal cultures, maintained in nonmyelinating and myelinating media, by quantitating immunofluorescence images obtained with a confocal microscope. Table I presents the data from two separate experiments using two independently de-

rived populations of infected Schwann cells. We found that under nonmyelinating conditions about twice as much MAG was found at the surface of the infected cells, reflecting the expression of the L-MAG cDNA. However, there was no difference between the amount of MAG on infected myelinating and normal myelinating Schwann cells.

We had previously observed that the level of MAG expression at the abaxonal surface of mature myelin segments was less than that of early myelinating Schwann cells (26). This is also reflected in the quantitative data in Table I in which cultures were maintained in myelin promoting media in experiment 1 for 16 d and in experiment 2 for 5 d. Although each set of measurements were made using slightly different photomultiplier gains and aperture settings, the similar levels of expression measured in nonmyelinating cultures in both experiments suggests that a valid comparison can be made between each data set. We conclude, therefore, that the mechanism that normally reduces MAG expression at the abaxonal surface probably accounts for the modulation of



**Figure 4.** Infected Schwann cells myelinate and communally ensheath axons. A culture of infected Schwann cells and sensory neurons maintained in myelinating media for 24 d was prepared for electron microscopy. Transverse section shows a myelinated axon and an example of non-myelinating ensheathment. Bar, 1  $\mu\text{m}$ .

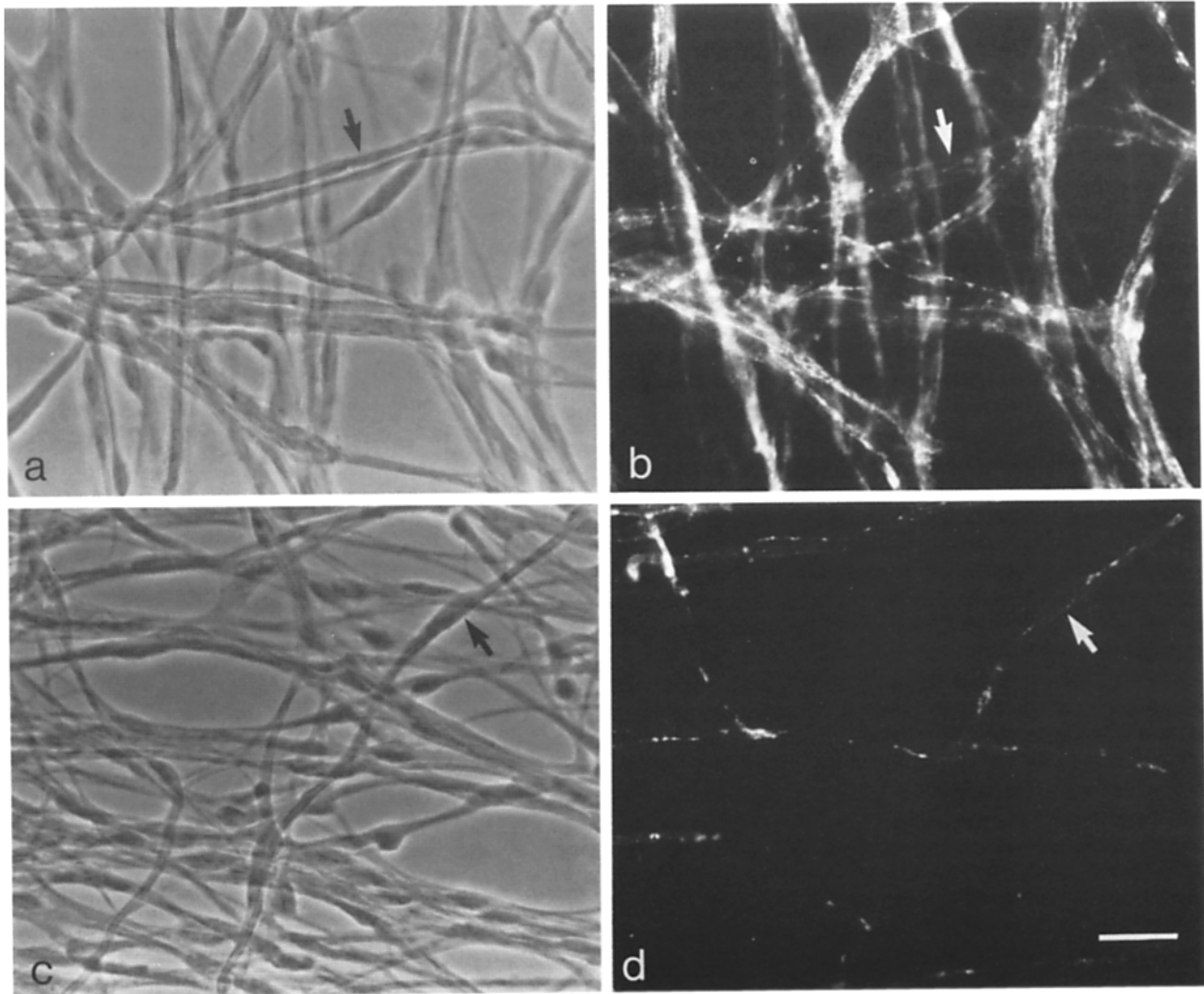
recombinant MAG expression at the abaxonal surface of infected cells that form myelin.

We did not distinguish between the two forms of MAG at the abaxonal surface since they differ only in their intracellular portions. We previously found that permeabilizing myelinating Schwann cells with methanol before immunostaining abolishes abaxonal sites of MAG expression and exposes sites within the myelin sheath including the adaxonal surface (26, 27). We therefore examined the contribution of recombinant L-MAG to the pattern of MAG expression within the myelin sheath, using an antipeptide polyclonal antibody specific for L-MAG (25), and an anti-MAG monoclonal antibody (30). We compared the pattern of immunostaining in control and infected myelinating Schwann cells. As illustrated by Fig. 6, early myelinating control or infected Schwann cells, which are characterized by continuous MAG staining along the forming internode (26, 27), stained with both antibodies. These forming segments are most abundant in cultures that have been recently transferred to myelin promoting media (Fig. 6, *e* and *f*), but we occasionally encountered them in older cultures (Fig. 6, *a-d*). Nascent segments formed by infected Schwann cells appeared to contain higher amounts of L-MAG immunoreactivity than corresponding controls. In mature myelin segments, L-MAG was not found in paranodes and Schmidt-Lanterman incisures of segments formed by uninfected Schwann cells. Therefore, L-MAG is normally expressed in myelinating Schwann cells at an early stage but

is later downregulated compared to S-MAG. By contrast, in infected Schwann cells, it would appear that L-MAG becomes localized in regions of mature myelin where S-MAG normally predominates (Fig. 6). As also illustrated in Fig. 6, however, L-MAG was not invariably detectable in the mature myelin segments formed by infected Schwann cells, which may reflect heterogeneity in the level of recombinant L-MAG expression in the original population of infected cells. To confirm the stability of the inserted MAG sequences, we removed the neuronal cell bodies from a culture of infected Schwann cells that had been in myelin promoting media for 3 wk and induced Wallerian degeneration. Immunostaining after 1 wk revealed that all the cells examined expressed MAG (Fig. 7). As expected, control cells did not express MAG (Fig. 7).

#### **Segregation of Large Axons by Schwann Cells Expressing Recombinant MAG**

The observation that L-MAG expression by normal Schwann cells is confined to forming internodes suggests that this form of MAG plays a role early in myelination. We reasoned, therefore, that differences between the behavior of normal and infected cells might be observed if we co-cultured the cells with neurons for a short period of time. This would limit the normal induction of MAG expression on control cells. If we supplied a basal lamina, any restriction on Schwann cell differentiation imposed by the rate of assembly of the en-



**Figure 5.** Surface expression of MAG by infected and normal Schwann cells during myelination. Myelinating cultures containing infected (*a* and *b*) or normal Schwann cells (*c* and *d*) were stained in the living state with anti-MAG mAbs. In contrast to the control Schwann cells (*c* and *d*) where MAG expression is restricted to myelin segments, infected Schwann cells (*a* and *b*) expressed MAG irrespective of whether they had formed myelin. Arrows indicate myelin segments. Bar, 20  $\mu\text{m}$ .

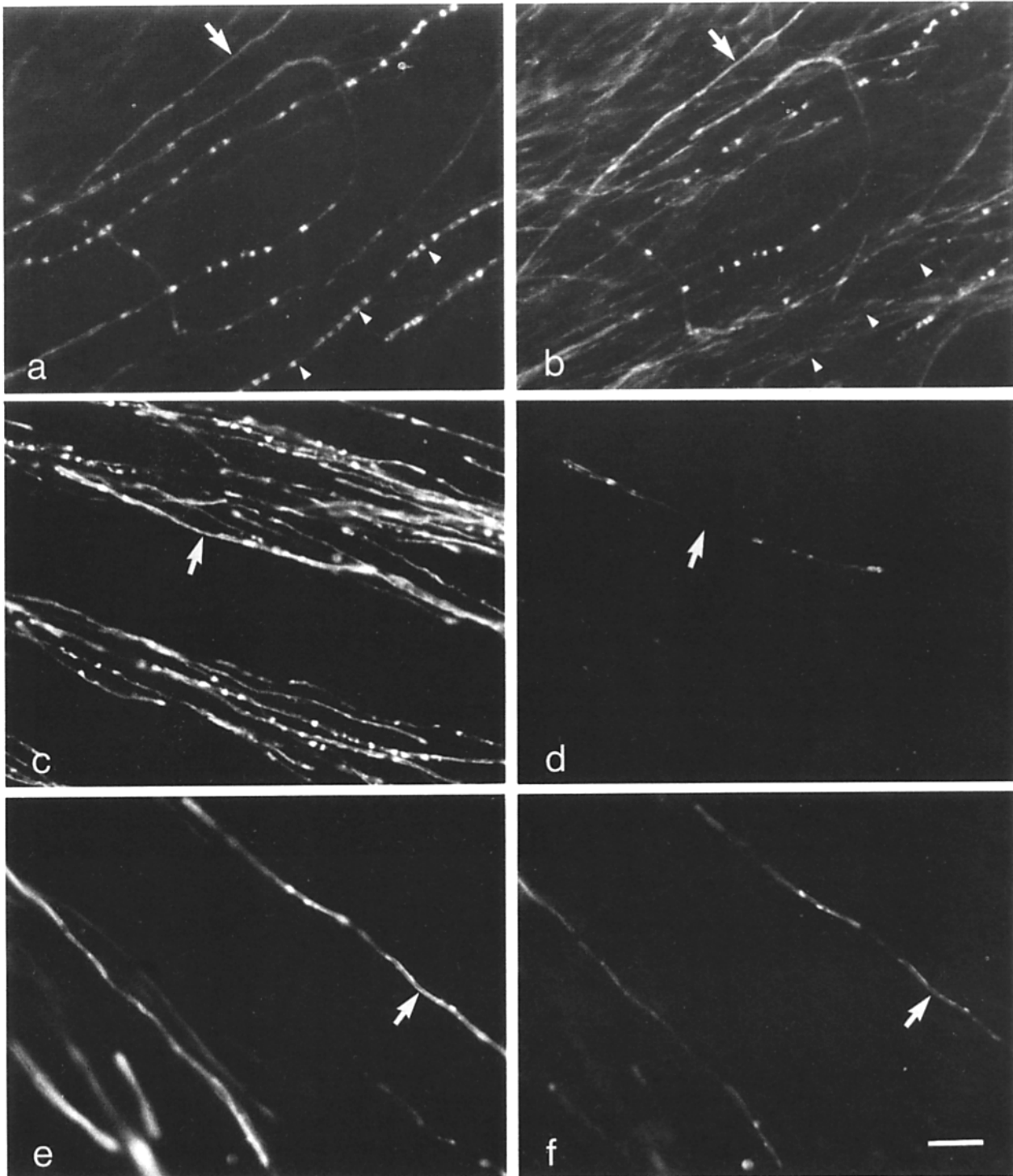
**Table I. Quantitation of MAG Expressed by Infected and Control Schwann Cells**

	Nonmyelinating	Myelinating
<b>Experiment 1</b>		
Experimental	33.9 $\pm$ 1.6	8.2 $\pm$ 0.4
Control	15.6 $\pm$ 0.5	9.1 $\pm$ 0.3
Ratio of means	2.17	1.1
<b>Experiment 2</b>		
Experimental	38.9 $\pm$ 9.3	20.7 $\pm$ 1.3
Control	15.5 $\pm$ 1.28	20.9 $\pm$ 0.7
Ratio of means	2.51	0.99

Data are expressed as arbitrary units of relative fluorescence intensity per  $\mu\text{m}^2$  and each value is the mean (with SEM) of measurements made on five cells. The four sets of data were recorded on separate occasions using slightly different photomultiplier gains and aperture settings. In experiment 1, the cells were maintained in nonmyelinating and myelinating media for 21 d and 16 d, respectively. In experiment 2, the corresponding times were 38 d and 5 d, respectively. In both experiments, cultures were switched to the myelinating media 14 d after the Schwann cells were added to the neuronal cultures.

dogenous basal lamina would be circumvented. It had previously been shown that extracellular matrix derived from EHS mouse tumor cells could substitute for an endogenous basal lamina (5, 9). Infected and normal Schwann cells that had been maintained in the absence of neurons such that the latter did not express MAG were plated at high density onto neuronal cultures and allowed to adhere. The cells were then overlaid with an exogenous basal lamina and maintained in myelinating media for 4 d. This time period was chosen since it had been shown that in long term co-cultures in defined media morphological changes were induced by added basal lamina by this time (5). Further, in initial studies very few control cells were found to be expressing MAG after this short period of time in co-culture (see below). A control and experimental culture were processed for electron microscopy. The amount of exogenous matrix that was added resulted in a high background that prohibited visualizing MAG expression by immunostaining. However, immunostaining a parallel culture of normal Schwann cells and neurons after 4 d of



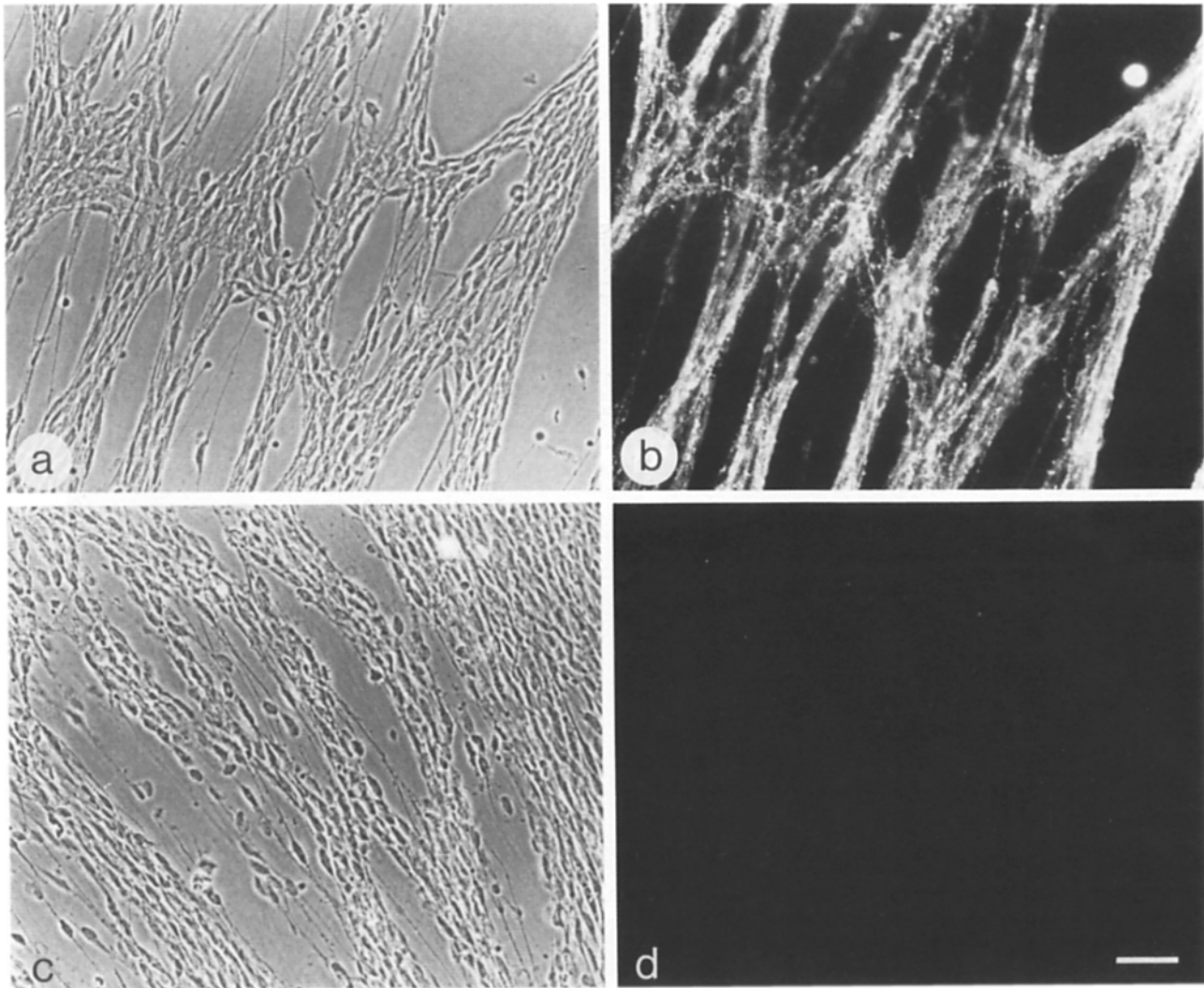


**Figure 6.** L-MAG expression by control and infected Schwann cells. Cultures of infected myelinating Schwann cells (*a* and *b*) and control myelinating Schwann cells (*c-f*) were double stained with an anti-MAG mAb (*a*, *c*, and *e*) and L-MAG specific anti-peptide polyclonal antibody (25) (*b*, *d*, and *f*) after fixation and permeabilization. The two cultures (*a*, *b*; *c*, *d*) were maintained in myelin promoting media for 26 d, and the culture (*e* and *f*) was maintained in myelin promoting media for 5 d. Nascent internodes (*arrows*) co-stain with both antibodies, but only mature internodes formed by infected Schwann cells contain detectable L-MAG immunoreactivity. Arrowheads in *a* and *b* denote a segment that only stains with the anti-MAG mAb. Bar, 30  $\mu$ m.

co-culture without exogenous basal lamina matrix revealed detectable MAG expression on only 1.4% of the cells examined (16 out of 1,133 cells counted).

In preparation for electron microscopy transverse sections

were taken from two areas of the control and experimental cultures at as closely corresponding positions with respect to the neuronal cell bodies as possible. Figs. 8 and 9 present representative images of the activity of Schwann cells in the



**Figure 7.** Surface expression of MAG by infected Schwann cells during Wallerian degeneration. The neuronal cell bodies were removed from cultures of neurons and infected (*a* and *b*) or control (*c* and *d*) Schwann cells after 3 wk in myelinating media. 1 wk later, staining with anti-MAG mAbs revealed that loss of neuronal interaction had resulted in the downregulation of MAG in normal Schwann cells (*d*) but not infected Schwann cells (*b*). *a* and *c* are the corresponding phase images. Bar, 40  $\mu$ m.

experimental and control culture respectively. A striking difference is seen in the extent of segregation of axons by the Schwann cells. As seen in Fig. 8, Schwann cells expressing recombinant MAG have completely surrounded individual axons with a single layer of cytoplasm that is reminiscent of the first step in myelination. By contrast, control cells, although closely apposed to axons, are apparently restricted in the extent of their interaction with them. The cells appeared to be relating to groups of axons but, in general, had not segregated individual axons. Table II details the number of singly ensheathed axons that we counted in randomly selected fields taken from the two areas of sampling in the experimental and control cultures. As seen in Figs. 8 and 9, there is evidence that the exogenous basal lamina matrix was organized around the Schwann cells. We repeated this experiment with a second independently derived population of infected Schwann cells, and, in the limited number of fields examined in the electron microscope, we again found exam-

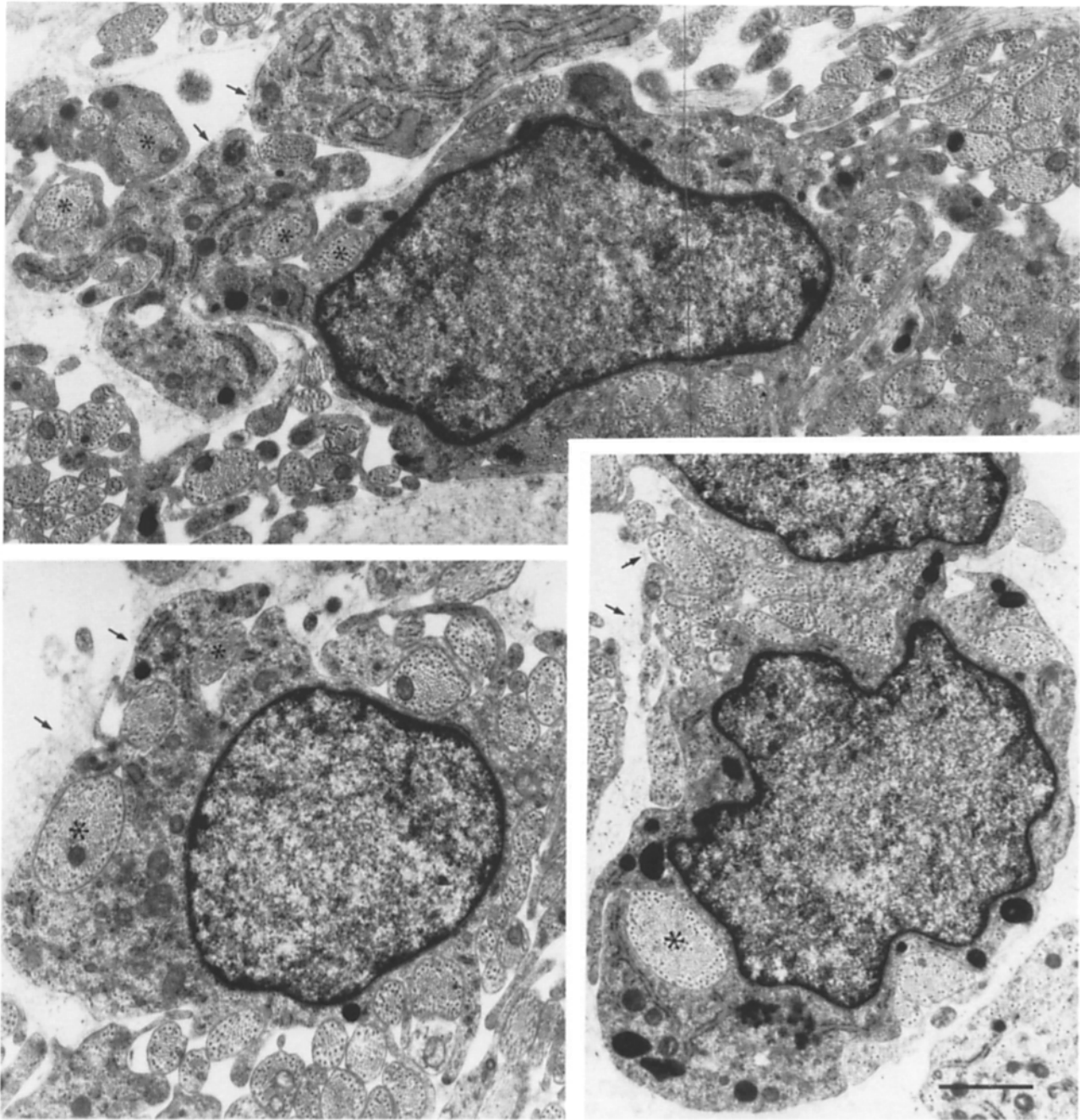
ples of segregated axons in the culture of infected Schwann cells and no case of a singly ensheathed axon in the control culture. Without the addition of a basal lamina matrix, we did not observe any segregation of large axons (data not shown). We conclude that L-MAG promotes the establishment of 1:1 associations between Schwann cells and axons to be myelinated, provided that a basal lamina is assembled.

### Discussion

We have used a novel experimental strategy that combines an *in vitro* myelinating co-culture system with retrovirus mediated cDNA expression to investigate the role of MAG in peripheral myelination.

In culture conditions that do not permit myelination, we measured about twice as much MAG on the surface of infected cells compared to normal cells indicating that an additional  $\sim 100\%$  MAG was inserted into the plasma mem-



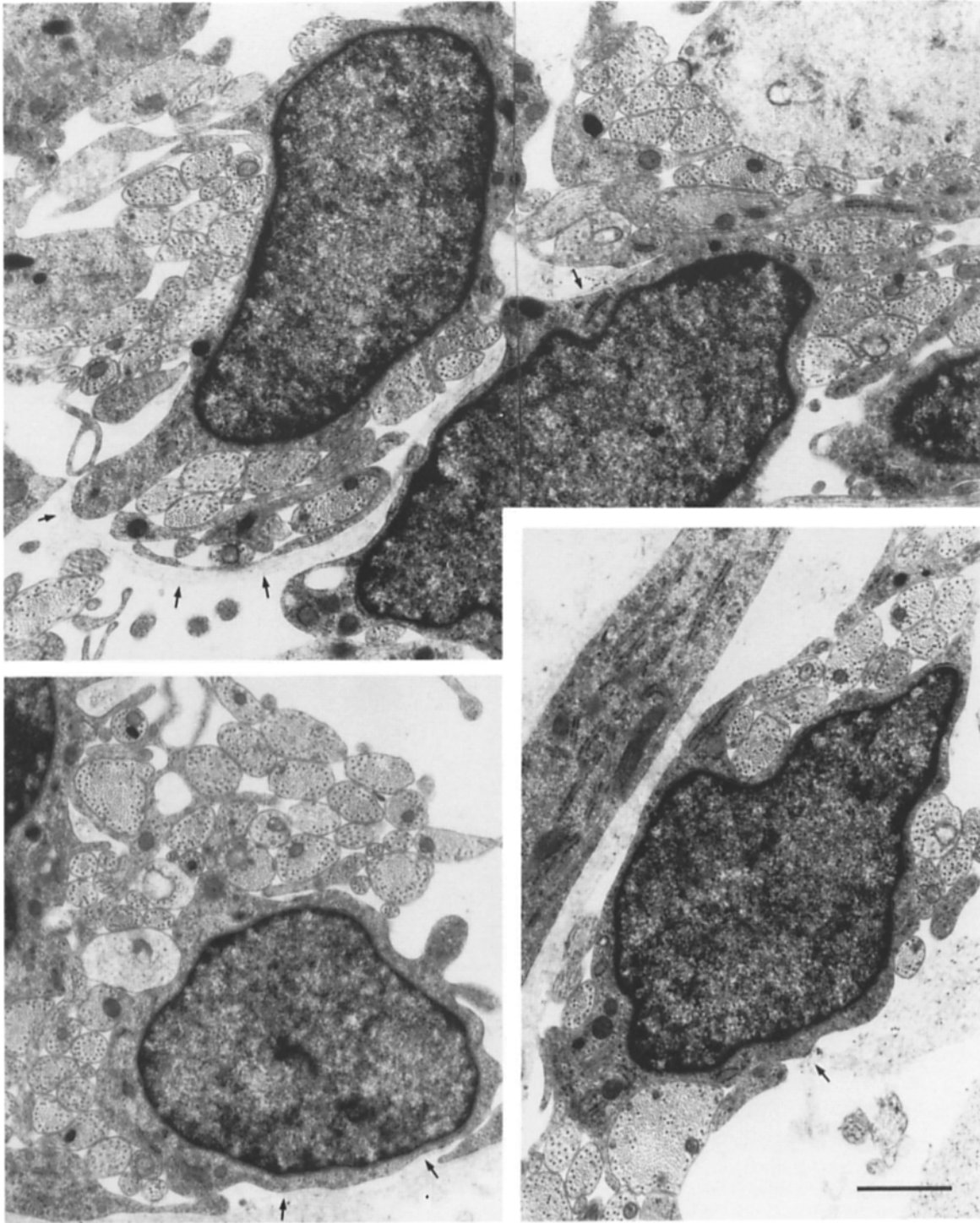


**Figure 8.** MAG promotes single ensheathment of axons. After 4 d of co-culturing MAG expressing Schwann cells with sensory neurons in the presence of an exogenous basal lamina matrix, the culture was prepared for electron microscopy. Transverse sections showing extensive interaction between the infected Schwann cells and axons including clear examples of segregated axons (\*). Arrows indicate basal lamina matrix juxtaposed to the Schwann cell surface. Bar, 1  $\mu\text{m}$ .

brane. Nevertheless, when switched to media that promotes Schwann cell differentiation, these infected cells formed morphologically normal, fully compacted myelin segments (Fig. 4). Nonmyelinating ensheathment was unaffected by the expression of MAG on the Schwann cell surface. Normally, nonmyelinating Schwann cells do not express MAG (26). Expression of MAG is therefore not sufficient to promote myelination of axons that would not normally be myelinated. This suggests that a MAG receptor is only found

on the surface of myelinated axons or that MAG by itself cannot promote myelination.

When we quantitated the amount of MAG at the abaxonal surface of infected Schwann cells that had formed myelin there was no difference compared to control myelinating cells. It would appear that a mechanism that normally modulates the level of MAG at the abaxonal surface during myelination (26) affected the expression of the recombinant L-MAG as well. A retroviral long terminal repeat is considered to be



**Figure 9.** Control Schwann cells not expressing MAG are restricted in the extent of their interaction with axons in short term co-culture. After 4 d of co-culturing normal Schwann cells with sensory neurons in the presence of an exogenous basal lamina matrix, the culture was prepared for electron microscopy. Transverse sections show extension of Schwann cell processes around groups of axons but virtually no segregation of individual axons. Arrows indicate basal lamina matrix in close proximity to the Schwann cell surface. Bar, 1  $\mu\text{m}$ .

a strong promoter that is active in a variety of differentiated cell types (39). Consequently, it seems likely that MAG was downregulated in the infected cells by a posttranscriptional mechanism. Persistent expression of recombinant MAG at the surface of nonmyelinating cells indicates that the mechanism only operates in myelinating Schwann cells. Con-

versely, the absence of MAG expression in normal nonmyelinating Schwann cells but the continuous expression of MAG under the transcriptional control of the long terminal repeat in infected nonmyelinating Schwann cells and in infected cells undergoing Wallerian degeneration points to transcriptional control of MAG by neuronal interaction.

**Table II. Relative Number of Axons Segregated by Infected and Control Schwann Cells**

Conditions	No. of axons counted (>0.25 $\mu\text{m}$ in diameter)	No. of segregated axons	Percentage of segregated axons
Experimental	567	76	13.4
Control	810	9	1.1

Only axons with estimated diameters >0.25  $\mu\text{m}$  were counted. The mean diameter of nonmyelinated axons was reported to be 0.24  $\mu\text{m}$  for dorsal root ganglion neurons in culture (41); therefore, it was assumed that in a given field any axon <0.25  $\mu\text{m}$  in diameter would never become singly ensheathed. None of the segregated axons that were counted were <0.25  $\mu\text{m}$  in diameter. The minimum criterion used to record a segregated axon was complete investment of the axon by one layer of Schwann cell membrane.

Run-off transcription experiments using nuclei prepared from isolated Schwann cells and Schwann cells in co-culture with neurons would address this question.

The primary transcript of the MAG gene is differentially spliced to yield L-MAG and S-MAG (18, 33). In the CNS, the level of L-MAG mRNA is highest and that of S-MAG mRNA lowest during the period of myelination (18, 38). In later postnatal life S-MAG mRNA predominates although both forms of the polypeptide are found in adult CNS myelin (25, 33). In the PNS, L-MAG mRNA is much less abundant than S-MAG mRNA at all stages of development, but, again, its expression coincides with the period of myelination (38). In examining the pattern of recombinant L-MAG expression, we found that L-MAG is normally expressed by early myelinating Schwann cells. To our knowledge, this is the first demonstration that L-MAG is a normal, albeit transient, component of peripheral myelin. Consistent with its reported absence from extracts of adult peripheral myelin (25), L-MAG was not detectable in mature myelin segments. Constitutive transcription of the recombinant L-MAG mRNA results in the persistent expression of L-MAG in myelin formed by most infected Schwann cells. Therefore, a change in the splicing of the MAG pre-mRNA to favor S-MAG mRNA production, coupled with turnover of the L-MAG polypeptide, probably accounts for the decrease in L-MAG compared to S-MAG that normally occurs as myelination proceeds.

In view of its expression during the early phase of myelination in the CNS, it seems reasonable to suggest, therefore, that L-MAG plays a similar role early in the formation of myelin by both Schwann cells and oligodendrocytes. In support of this contention, it is worth noting that Schwann cells and oligodendrocytes will myelinate CNS and PNS axons (7, 43) and that in both Schwann cells and oligodendrocytes the myelin spiral forms in the same way, by advance of the inner axon related glial process (4). The higher steady level of L-MAG mRNA found at the time of myelination in CNS tissue compared with PNS tissue (38) might be explained by the fact that in contrast to Schwann cells, a single oligodendrocyte can myelinate 30–50 axons (29). If this occurred synchronously, then the amount of L-MAG expressed by a myelinating oligodendrocyte would be higher.

The short term culture experiment provides direct evidence that L-MAG functions in Schwann cells to promote the initial investment of axons destined to be myelinated. We found that Schwann cells expressing only recombinant L-MAG because of no prior interaction with neurons to induce the endogenous gene segregated and singly ensheathed larger

axons provided that an extracellular matrix was supplied to the cells. In contrast, there was considerably less evidence of segregated axons in control cultures. Control cells were apparently more restricted in their interaction with axons. The incidence of a few segregated axons in the control may be explained by the limited upregulation of MAG (1–2%) on Schwann cells that occurred during the time course of the experiment.

To promote axonal investment, L-MAG may function both by interacting with a specific receptor present on large diameter axons (15) as well as by interacting with cytoskeletal elements via its unique cytoplasmic domain, perhaps to enhance process formation by the Schwann cell. Although we are making a case for L-MAG function, we do not know the normal stoichiometry of L-MAG and S-MAG at the surface of early myelinating Schwann cells; thus, we do not exclude a role for S-MAG in axonal investment. Furthermore, it is likely that other cell surface adhesion molecules are involved in the interaction between Schwann cells and axons destined to be myelinated. LI/NgCAM and N-CAM are expressed by Schwann cells before axonal contact and are subsequently downregulated on myelinating Schwann cells (21, 23). In recent studies, antibodies against LI have been shown to block ensheathment and myelination in vitro (reference 34; Wood, P. M., M. Schachner, and R. P. Bunge, manuscript submitted for publication). The inhibited Schwann cells did not collectively or singly ensheath axons, suggesting an important role for LI/NgCAM in steps that lead to myelination. Nevertheless, the results of this study and those of a parallel study in which expression of antisense MAG RNA in Schwann cells inhibited MAG expression and arrested myelination at the stage of axon segregation (Owens, G. C., and R. P. Bunge, manuscript in preparation) strongly supports a critical role for MAG in establishing the 1:1 stage of Schwann cell/axon association.

We wish to thank Drs. Melitta Schachner, Rob Milner, Greg Sutcliffe, and Norman Latov for anti-MAG antibodies, and Dr. Bernard Weinstein for pMV7. We also thank Dr. Dorothy Schafer for advice on the confocal microscopy and Rose Hays for preparing the manuscript.

This work was supported by National Multiple Sclerosis Society grant RG 1995-A-1 to G. C. Owens, National Institutes of Health (NIH) grant NS 19923 to R. P. Bunge, and NIH grant NS 26001 and March of Dimes grant 1-1127 to J. L. Salzer. The confocal microscope was purchased with funds provided to Washington University Medical School by the Lucille P. Markey Charitable Trust.

Received for publication 8 March 1990 and in revised form 21 May 1990.

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