

# Down-Regulation of Myelin-Associated Glycoprotein on Schwann Cells by Interferon- $\gamma$ and Tumor Necrosis Factor- $\alpha$ Affects Neurite Outgrowth

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## Summary

To investigate the influence of inflammatory cytokines on the potential of peripheral nerves to regenerate, we analyzed the effect of interferon- $\gamma$  (IFN- $\gamma$ ) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) on the ability of immortalized Schwann cells to mediate outgrowth of neurites from primary DRG neurons. We found that IFN- $\gamma$  and TNF- $\alpha$  synergistically inhibited the neurite outgrowth-promoting properties of the Schwann cells by specifically down-regulating myelin-associated glycoprotein (MAG) at the levels of mRNA and cell surface protein by approximately 60%. Antibodies to MAG inhibited the outgrowth of neurites on Schwann cells to the same extent as treatment with the two cytokines. Since MAG appears to be involved in both neurite outgrowth and myelination, our findings may provide evidence for a mechanism, by which inflammatory cytokines interfere with Schwann cell-neuron interactions.

## Introduction

After nerve trauma and during inflammatory responses, macrophages and lymphocytes infiltrate the nervous system at the site of a lesion and produce cytokines that may act on cells of the immune system as well as the nervous system. Activated macrophages are the source of interleukin-1 and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), whereas T lymphocytes secrete interferon- $\gamma$  (IFN- $\gamma$ ) and interleukins. A direct functional link between the immune and nervous systems is provided by interleukin-1, which stimulates the synthesis of nerve growth factor (NGF) by Schwann cells (Lindholm et al., 1987). This increase in NGF synthesis has been suggested to replace partially the interrupted supply of NGF from the target tissue to the NGF-

responsive reinnervating sensory and sympathetic neurons (Brown et al., 1991; Heumann et al., 1987). NGF, in turn, influences the expression of the neural recognition molecule L1 on neurons and Schwann cells (Seilheimer and Schachner, 1987).

The cytokines TNF- $\alpha$  and IFN- $\gamma$  have been shown to induce the expression of major histocompatibility complex (MHC) class I and II molecules on a variety of neural cell types, which normally do not express these molecules (Fontana et al., 1984; Giulian, 1987; Yu et al., 1990). Expression of MHC class II molecules on the surface of nonmyelinating Schwann cells can be stimulated by IFN- $\gamma$  and TNF- $\alpha$  or by cocultivation of T cells and antigen with Schwann cells (Wekerle et al., 1986; Samuel et al., 1987; Kingston et al., 1989). Both cytokines frequently act together in a synergistic manner supporting the immune response by antigen presentation to MHC-restricted T lymphocytes and may contribute to the initiation of autoimmune responses to myelin proteins (for reviews, see Brostoff, 1984; Quarles, 1989). However, direct effects of these cytokines on the expression of neural recognition molecules and functional consequences for myelination and regeneration have not been investigated.

Peripheral nerve regeneration depends on intercellular recognition events between neurons and Schwann cells, which are mediated by several cell surface molecules belonging to the families of cadherins, integrins, and immunoglobulin superfamily molecules (Bixby et al., 1988; Doherty et al., 1989; Kleitman et al., 1988; Martini and Schachner 1988; Matsunga et al., 1988; Seilheimer and Schachner, 1988; Seilheimer et al., 1989; Tomaselli et al., 1988). The immunoglobulin superfamily molecules N-CAM, L1, myelin-associated glycoprotein (MAG) and the major peripheral myelin protein P<sub>0</sub> have all been shown to mediate neurite outgrowth in vitro (Bixby et al., 1988; Johnson et al., 1989; Schneider-Schaulies et al., 1990; Seilheimer and Schachner, 1988). During development, N-CAM and L1 are already expressed by Schwann cells before myelination, whereas MAG and P<sub>0</sub> become detectable with the onset of myelination (Jessen and Mirsky, 1991; Martini and Schachner, 1986; Martini et al., 1988; Owens and Bunge, 1989). In the adult, MAG remains present at the axon-myelin interface and in paranodal loops and may thus be decisive for the maintenance of intact myelin.

Since neural recognition molecules play such important roles in development, maintenance, and regeneration of peripheral nerves, it is important to know whether inflammatory cytokines affect the expression of these molecules by Schwann cells. We used a Schwann cell line, which was immortalized by transfecting primary rat Schwann cells with a plasmid expressing simian virus 40 (SV40) T antigen under the control of the metallothionein promoter (Peden et al., 1989). These cells proliferate well in the presence of

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Table 1. Marker Profile of Immortalized Schwann Cells

Molecule	Antibody <sup>a</sup>	Fluorescence Intensity <sup>b</sup>	Percentage of Fluorescent Cells (%) <sup>c</sup>	Apparent Mol. Mass (kd) <sup>d</sup>
N-CAM	Poly	++	75 ± 8	140 and 120
L1	Poly	++	81 ± 11	230 and 200
MAG	Mono 513	+++	79 ± 12	95
Class I	Mono Ox18	+++	95 ± 4	45
P <sub>0</sub>	Poly	+	50 ± 12	ND
GFAP	Poly	+++	70 ± 8	ND
Ran1	Mono 217c	++	90 ± 10	ND
O1	Mono 59	+++	1 ± 1	ND
O4	Mono 81	+++	1 ± 1	ND
O10	Mono 479	-	0 ± 0	ND

<sup>a</sup> Antibody designations are given in Experimental Procedures; mono, monoclonal antibody; poly, immunoaffinity-purified rabbit anti-serum.

<sup>b</sup> Fluorescence intensities: (-) negative; (+) low; (++) medium; (+++) high, as determined by indirect immunofluorescence using a fluorescence microscope.

<sup>c</sup> The percentage of immunofluorescent cells was determined by flow cytometry for N-CAM, L1, MAG, and MHC class I molecules. For the other molecules, immunofluorescent cells were scored by fluorescence microscopy.

<sup>d</sup> Apparent molecular mass (kd) was determined by immunoprecipitation and subsequent SDS-PAGE. ND, not determined.

Zn<sup>2+</sup> and slow down proliferation and differentiate morphologically after removal of Zn<sup>2+</sup>. Besides displaying the typical spindle-shape morphology of Schwann cells in primary culture, these cells have been described as expressing several properties characteristic of Schwann cells *in vivo* and *in vitro* (Peden et al., 1989). In the present study, we investigated the effects of IFN- $\gamma$  and TNF- $\alpha$  on neurite outgrowth-promoting properties of these Schwann cells and on the expression of the neural recognition molecules N-CAM, L1, and MAG.

## Results

### Marker Profile and Neural Recognition Molecule Expression of Immortalized Schwann Cells

All experiments described were performed after culturing the Schwann cell line MT<sub>4</sub>SV-H1 (Peden et al., 1989) in the absence of Zn<sup>2+</sup> for at least 7 days to allow down-regulation of SV40 T antigen and formation of the characteristic Schwann cell morphology. To verify that the immortalized Schwann cells express the neural recognition molecules previously recognized on Schwann cells in primary culture, they were characterized using cell type-specific antibodies. Using indirect immunofluorescence, a high percentage of Schwann cells expressed considerable levels of the neural recognition molecules N-CAM (75% ± 8%), L1 (81% ± 11%), MAG (79% ± 12%), MHC class I molecules (95% ± 4%), P<sub>0</sub> (50% ± 12%), glial fibrillary acidic protein (GFAP) (70% ± 8%), and Ran-1 (90% ± 10%) (Table 1). Only a very small percentage of cells (1% ± 1%) expressed the cell surface antigens O1 and O4. The O10 antigen could not be detected under the conditions of this study.

The fact that approximately 70% of these cells express GFAP, as a marker for nonmyelinating Schwann cells (Jessen and Mirsky, 1991), and approximately

80% and 50% of the cells express MAG and P<sub>0</sub>, respectively, as markers for myelinating Schwann cells, is interesting. This cell line might provide the possibility of further differentiation into either nonmyelinating or myelinating Schwann cells.

Expression of N-CAM, L1, MAG, and MHC class I molecules was confirmed by immunoprecipitation after [<sup>35</sup>S]methionine biosynthetic labeling of cells and preparation of detergent extracts (Table 1). N-CAM was detectable as two components of 140 and 120 kd. As in primary cultures of Schwann cells, the 180 kd N-CAM component could not be detected. L1 was immunoprecipitated as a double band of 230 and 200 kd. MAG was detectable as a main band of 95 kd, which was converted into a single band of 67 kd after deglycosylation with glycopeptidase F. These results showed that N-CAM, L1, and MAG are expressed by the immortalized Schwann cells as molecular species described for Schwann cells in primary cultures.

### Induction of MHC Class II Molecule Expression by IFN- $\gamma$ and TNF- $\alpha$ on Immortalized Schwann Cells

The inflammatory cytokines IFN- $\gamma$  and TNF- $\alpha$  have been reported to stimulate MHC class II molecule expression synergistically in primary cultures of nonmyelinating Schwann cells (Kingston et al., 1989; Wekerle et al., 1986). These cytokines also induced MHC class II expression by immortalized Schwann cells, as analyzed by indirect immunofluorescence using flow cytometry (Figure 1). When Schwann cells were maintained for 3 days in the presence of increasing concentrations of IFN- $\gamma$  (up to 300 U/ml), at most 21% ± 9% of the cells were induced to express MHC class II molecules. TNF- $\alpha$  (10 ng/ml) did not induce expression of MHC class II molecules (data not shown). However, when increasing concentrations of IFN- $\gamma$  (up to 300 U/ml) and a constant concentration of TNF- $\alpha$  (10 ng/ml) were added together to the cultures, up to 70% ± 8%

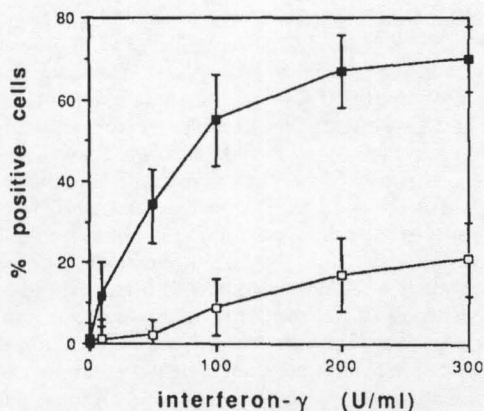


Figure 1. Flow Cytometric Determination of MHC Class II Molecule Expression by Cytokine-Treated Immortalized Schwann Cells

Schwann cells were maintained for 3 days in culture medium in the presence of increasing concentrations of IFN- $\gamma$  and in the absence (open squares) or the presence (closed squares) of 10 ng/ml TNF- $\alpha$ . Cells were stained with anti-MHC class II monoclonal antibodies Ox6 and Ox17 and FITC-conjugated second antibodies and analyzed with a flow cytometer. The percentage of MHC class II immunofluorescent cells (% positive cells) was plotted as a function of IFN- $\gamma$  concentration. Mean values (%)  $\pm$  standard deviation from three independent experiments are shown. TNF- $\alpha$  alone did not stimulate MHC class II expression on Schwann cells (data not shown).

of the cells expressed MHC class II molecules. The synergistic effects of IFN- $\gamma$  and TNF- $\alpha$  were the same in the absence or presence of 100 ng/ml NGF. These observations support the notion that the immortalized Schwann cells respond to IFN- $\gamma$  and TNF- $\alpha$  as Schwann cells in primary cultures do.

#### Quantitative Determination of Neurite Outgrowth on Immortalized Schwann Cells as a Function of Cytokine Treatment

The immortalized Schwann cells provide an excellent substrate for neurite outgrowth from dorsal root ganglion (DRG) neurons, leading to a maximal total length of neurites per neuron of up to 5000  $\mu$ m and an average total length of neurites per neuron of approximately 1500  $\mu$ m within 20 hr of coculture (Figure 2A). To determine the effects of IFN- $\gamma$  and TNF- $\alpha$  on the neurite outgrowth-promoting capacity of the Schwann cells, cultures were pretreated with the cytokines 3 days prior to coculture with neurons and then maintained either in the absence or presence of the cytokines. Alternatively, the cytokines were applied only during the 20 hr of coculture of Schwann cells with neurons. In all cases, cultures were maintained in the presence of high concentrations of NGF (100 ng/ml) to exclude an indirect effect of the cytokines on endogenously produced NGF, which itself could modify neurite outgrowth (Seilheimer and Schachner, 1988). Under these conditions, application of IFN- $\gamma$  and TNF- $\alpha$  individually inhibited neurite outgrowth by

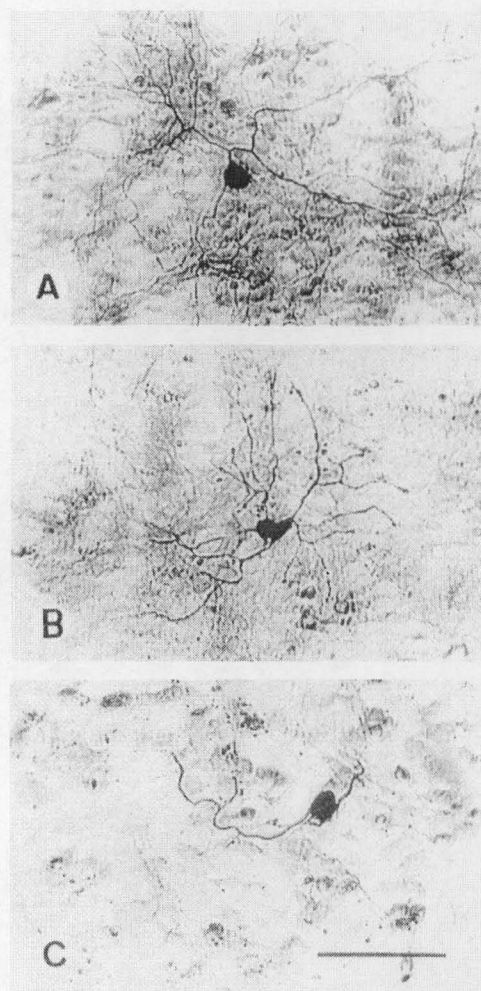


Figure 2. Neurite Outgrowth from DRG Neurons on Schwann Cell Monolayers as Visualized by Immunoperoxidase Staining of Neurofilaments

DRG neurons were plated on monolayers of immortalized Schwann cells and maintained for 20 hr in the presence of 100 ng/ml NGF. Cocultures were maintained in standard medium (A), in 100 U/ml IFN- $\gamma$  and 10 ng/ml TNF- $\alpha$  after pretreatment of Schwann cells with both cytokines (B), or in the presence of antibodies against N-CAM, L1, and MAG (C). Cultures were stained with anti-neurofilament antibodies by the indirect immunoperoxidase method. Bar, 100  $\mu$ m.

14%  $\pm$  5% and 5%  $\pm$  2%, respectively (see Figure 3A for one representative experiment and Table 2 for mean values of three independent experiments). When IFN- $\gamma$  and TNF- $\alpha$  were added together to the Schwann cell cultures 3 days prior to plating of neurons and the cocultures were then maintained in the absence or presence of the cytokines, neurite outgrowth was reduced by 36%  $\pm$  9% and 39%  $\pm$  5%, respectively (Figure 2B; Figure 3B; Table 2). When neurons were cocultured for 20 hr together with the two cytokines without cytokine pretreatment of Schwann

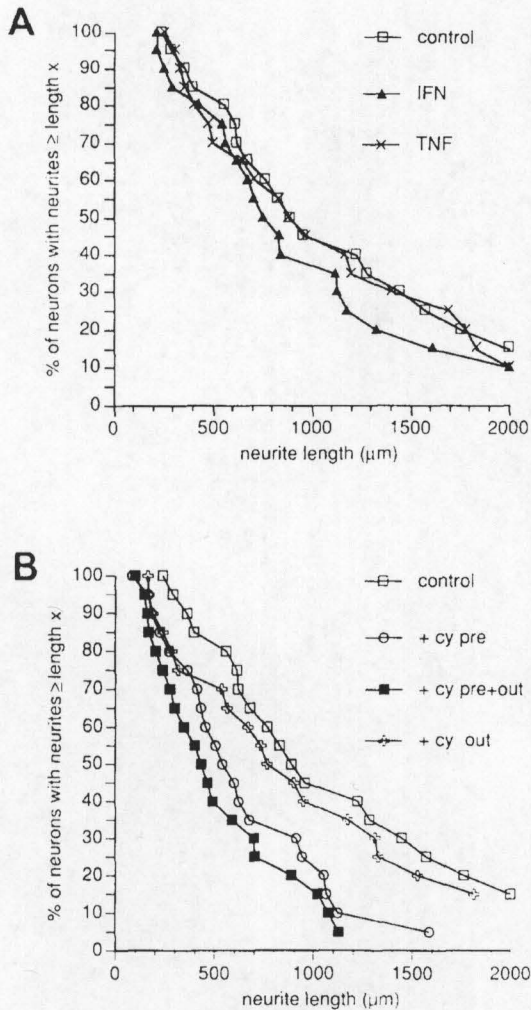


Figure 3. Cumulative Frequency Distribution Plot of the Neurite Lengths of DRG Neurons Maintained on Schwann Cell Monolayers in the Presence of Cytokines

The percentage of neurons with neurites greater than or equal to a certain length  $x$  (vertical axis) was plotted as a function of neurite length  $x$  (horizontal axis). The values of one representative experiment are shown (for mean values of three independent experiments, see Table 2).

(A) Control, culture medium containing 100 ng/ml NGF; IFN, pretreatment of Schwann cells with IFN- $\gamma$  and presence of IFN- $\gamma$  during cocultivation with neurons; TNF, pretreatment of Schwann cells with TNF- $\alpha$  and presence of TNF- $\alpha$  during cocultivation. Inhibition of neurite outgrowth in IFN- $\gamma$ -treated (IFN) but not in TNF- $\alpha$ -treated (TNF) cultures was significantly different from outgrowth of neurites in control medium ( $p < 0.01$ ).

(B) Control, culture medium containing 100 ng/ml NGF (same values as in [A]); +cy pre, pretreatment of Schwann cells with 100 U/ml IFN- $\gamma$  and 10 ng/ml TNF- $\alpha$ ; +cy pre + out, pretreatment of Schwann cells with both cytokines and presence of both cytokines during coculture; +cy out, presence of both cytokines during coculture. Inhibition of neurite outgrowth on Schwann cells treated with both cytokines (+cy pre and +cy pre + out) was significantly different from outgrowth of neurites in control medium ( $p < 0.001$ ).

cells, neurite outgrowth was inhibited by  $12\% \pm 8\%$  (Figure 3B; Table 2). Survival of DRG neurons on Schwann cell monolayers or collagen as substrate was not affected by IFN- $\gamma$  and TNF- $\alpha$  (data not shown).

Thus, the effect of the combined cytokines on neurite outgrowth was found to be more than additive, i.e., synergistic, when compared with the effects of IFN- $\gamma$  or TNF- $\alpha$  alone. Furthermore, the reduction in neurite outgrowth appeared to be due to a direct effect of the cytokines on the Schwann cells, since pretreatment of Schwann cells with the cytokines and absence during coculture resulted in a reduction of neurite outgrowth similar to that seen with pretreatment of Schwann cells and further maintenance of cocultures with the cytokines. To determine whether the cytokine-induced inhibitory effect on neurite outgrowth was due to a modification in recognition molecule expression by Schwann cells, expression of N-CAM, L1, and MAG on the Schwann cell surfaces was quantified.

#### Expression of Neural Recognition Molecules on the Surface of Immortalized Schwann Cells as a Function of Cytokine Treatment

When Schwann cells were maintained for 3 days in the presence of IFN- $\gamma$  or TNF- $\alpha$  alone and expression of N-CAM, L1, and MAG was quantified by flow cytometry after indirect immunofluorescence staining, no significant changes in neural recognition molecule expression were detectable when compared with cultures maintained in the absence of cytokines (data not shown). However, when the two cytokines were added together to the Schwann cell cultures, the mean intensity of MAG immunofluorescence decreased by  $54\% \pm 6\%$  (from 227 to 124 units) after 3 days and by  $62\% \pm 7\%$  (from 227 to 97 units) after 6 days (Figure 4). The reduction in mean fluorescence intensity could be attributed to a shift of the population of cells with high expression of MAG to a population of cells with low expression of MAG. Simultaneously, the percentage of MAG-positive cells decreased from 79% to 73% after 3 days and to 57% after 6 days in the presence of the two cytokines. The down-regulation of MAG was fully reversible within 7 days after removal of the cytokines from the culture medium (data not shown).

In contrast to MAG, expression of N-CAM and L1 was not significantly reduced after 3 days of treatment with the two cytokines. Neither mean fluorescence intensity nor percentage of immunofluorescent cells was significantly affected (Figure 4). After 6 days in the presence of IFN- $\gamma$  and TNF- $\alpha$ , the mean fluorescence intensity of N-CAM was reduced by  $14\% \pm 5\%$  (from 118 to 101 units) and the mean fluorescence intensity of L1 by  $31\% \pm 7\%$  (from 141 to 96 units). The percentage of N-CAM and L1 immunofluorescent cells was not significantly affected after 6 days of cytokine treatment. MHC class I molecule expression was strongly stimulated, and MHC class II molecule expression was

Table 2. Quantitative Determination of Neurite Lengths of Neurons from DRGs Grown out on Schwann Cell Monolayers

Pretreatment of Schwann Cells with Cytokines <sup>a</sup>	Maintenance of Cocultures with <sup>b</sup>		Total Length of Neurites per Neuron (μm) <sup>c</sup>	Inhibition of Neurite Outgrowth (%) <sup>d</sup>
	Cytokines	Antibodies		
-	-	-	1472 ± 39	0 ± 7
IFN	IFN	-	1266 ± 24	14 ± 5
TNF	TNF	-	1392 ± 10	5 ± 2
IFN + TNF	IFN + TNF	-	897 ± 24	39 ± 5
IFN + TNF	-	-	939 ± 44	36 ± 9
-	IFN + TNF	-	1295 ± 39	12 ± 8
-	-	Anti-M	870 ± 14	41 ± 3
IFN + TNF	IFN + TNF	Anti-M	822 ± 39	44 ± 8
-	-	Anti-N + L	1041 ± 68	29 ± 14 <sup>e</sup>
IFN + TNF	IFN + TNF	Anti-N + L	544 ± 29	63 ± 6
-	-	Anti-N + L + M	483 ± 44	67 ± 9
IFN + TNF	IFN + TNF	Anti-N + L + M	513 ± 58	65 ± 12

Abbreviations: IFN, IFN-γ; TNF, TNF-α; anti-M, MAG monoclonal antibodies to MAG; anti-N + L, polyclonal antibodies to N-CAM and Anti-L1; anti-N + L + M, polyclonal antibodies to N-CAM and L1 and monoclonal antibodies to MAG added together.

<sup>a</sup> Schwann cells were maintained in culture medium in the absence or presence of cytokines (100 U/ml IFN-γ and 10 ng/ml TNF-α) for 3 days before the addition of DRG neurons.

<sup>b</sup> Cocultures of Schwann cells and DRG neurons were maintained (20 hr) in the absence or presence of cytokines (100 U/ml IFN-γ and 10 ng/ml TNF-α) or antibodies (300 μg/ml each). During cocultivation, 100 ng/ml NGF was present.

<sup>c</sup> Values were determined by image analysis and are given as mean values ± standard deviation obtained from three independent experiments.

<sup>d</sup> The percent inhibition of neurite outgrowth was determined by reference to the control culture (0% inhibition). Mean values are from three independent experiments ± standard deviation.

<sup>e</sup> This value is lower than that reported by Seilheimer and Schacher (1988), probably because of the use of Schwann cells from rat rather than mouse and antibodies generated against mouse and not rat N-CAM and L1.

induced after treatment with IFN-γ and TNF-α for 3 and 6 days (Figure 4).

A dose-response analysis of surface molecule expression by Schwann cells as a function of increasing amounts of IFN-γ in the presence of constant concentrations of TNF-α is shown in Figure 5. Down-regu-

lation of MAG in the presence of TNF-α was half-maximal at approximately 5 U/ml IFN-γ, while induction of MHC class II molecule expression was half-maximal at approximately 50 U/ml IFN-γ (Figure 5). Expression of N-CAM and L1 was not significantly modified after treatment with the cytokines. Similar results were ob-

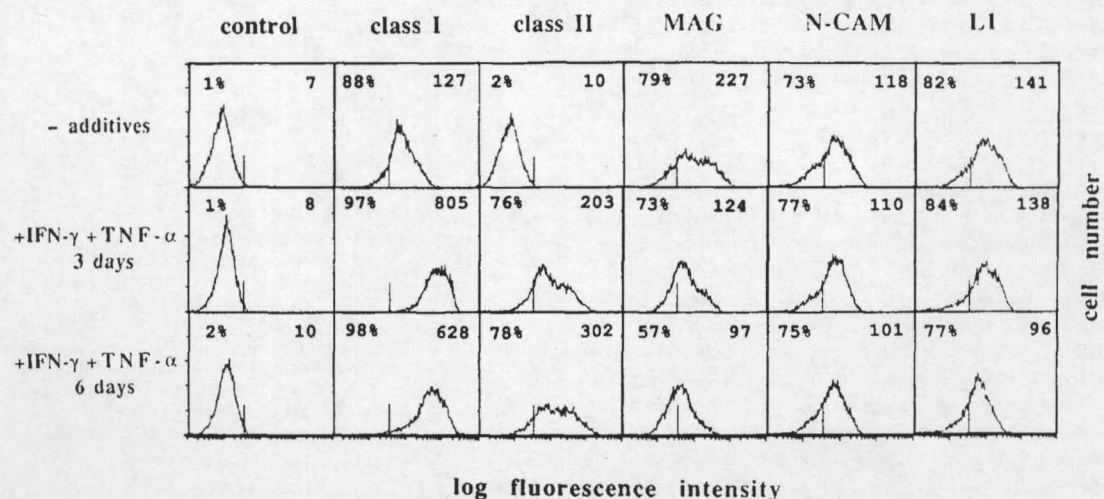
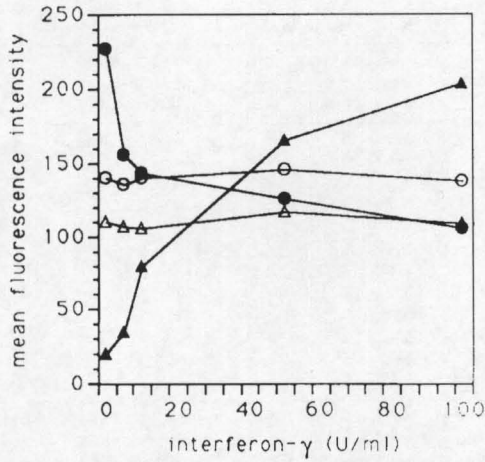


Figure 4. Quantitative Determination of Recognition Molecule Expression by Cytokine-Treated Immortalized Schwann Cells  
Flow cytometric analysis of the expression of MHC class I and II molecules, MAG, N-CAM, and L1 on the surface of Schwann cells maintained in culture medium (- additives) and in the presence of 100 U/ml IFN-γ and 10 ng/ml TNF-α for 3 or 6 days. Cells were stained by indirect immunofluorescence. Cell number (vertical axis, linear scale) was plotted versus fluorescence intensity (horizontal axis, logarithmic scale). The percentage of cells with fluorescence intensities higher than the negative control values is given in the upper left corner, and values of mean fluorescence intensity (arbitrary units) are given in the upper right corner of each panel.



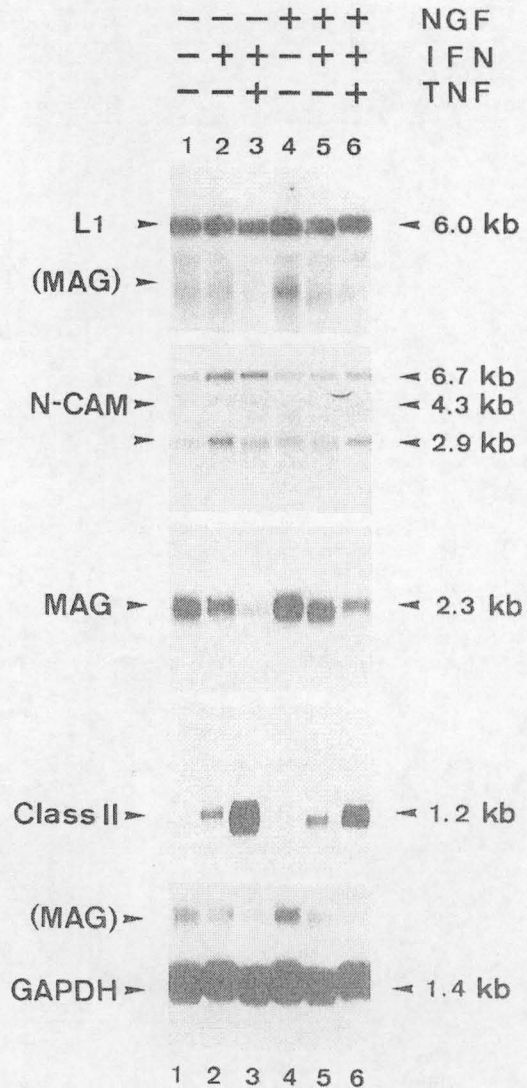
**Figure 5. Quantitative Determination of Recognition Molecule Expression by Schwann Cells as a Function of IFN-γ Concentration**

Flow cytometric analysis of the expression of MAG (closed circles), MHC class II molecules (closed triangles), N-CAM (open triangles), and L1 (open circles) on immortalized Schwann cells after treatment with increasing concentrations of IFN-γ in the constant presence of 10 ng/ml TNF-α for 3 days. Cells were stained by indirect immunofluorescence. The mean fluorescence intensity (vertical axis) was plotted as a function of the IFN-γ concentration (horizontal axis).

tained when cytokine treatment was performed in the absence (Figure 5) or presence (data not shown) of 100 ng/ml NGF.

**Determination of mRNA Levels of Neural Recognition Molecules as a Function of Cytokine Treatment**

To determine whether the reduction in the expression of MAG at the surface of Schwann cells was due to regulation at the mRNA level, Northern blot analyses were performed. RNA was isolated from Schwann cells maintained for 48 hr in control medium, in the presence of IFN-γ alone, or in the presence of IFN-γ and TNF-α. Total RNA was hybridized to probes specific for N-CAM, L1, MAG, MHC class II molecules, and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) (Figure 6). Densitometric quantifications of X-ray films from Northern blots showed that MAG-specific mRNA levels of cells maintained in the presence of both cytokines were reduced by approximately 80% when compared with mRNA levels of Schwann cells cultured without cytokines. This reduction was seen independently of whether Schwann cells were maintained in the absence or presence of NGF, although a slightly stronger signal for MAG-specific mRNA was detected in cells cultured in the presence of NGF (Figure 6, compare lanes 3 and 6). The hybridization signals for L1 did not show significant changes after maintenance of cells in IFN-γ and TNF-α. The mRNA level of N-CAM was slightly enhanced in the presence of IFN-γ and IFN-γ plus TNF-α in the absence of NGF. MHC class II-specific mRNA was



**Figure 6. Northern Blot Analyses of Recognition Molecule Expression by Immortalized Schwann Cells**

Schwann cells were maintained in culture medium for 48 hr in the absence (lanes 1-3) or the presence (lanes 4-6) of 100 ng/ml NGF, in 100 U/ml IFN-γ (lanes 2 and 5), or in 100 U/ml IFN-γ in combination with 10 ng/ml TNF-α (lanes 3 and 6). Total RNA (20 μg per lane) was separated on agarose gels, blotted on Hybond N filters, and hybridized to the recognition molecule-specific probes as indicated. Two filters hybridized to the MAG-specific probe were rehybridized with L1- and GAPDH-specific probes, showing the remaining bands of MAG in the autoradiogram, as indicated by the brackets (MAG). No N-CAM-specific mRNA of 7.2 kb encoding the 180 kd form of N-CAM could be detected.

strongly induced after treatment of Schwann cells with IFN-γ alone or with the combination of both cytokines.

**Demonstration of the Specific Contribution of MAG to Neurite Outgrowth**

To investigate whether the reduction in neurite out-

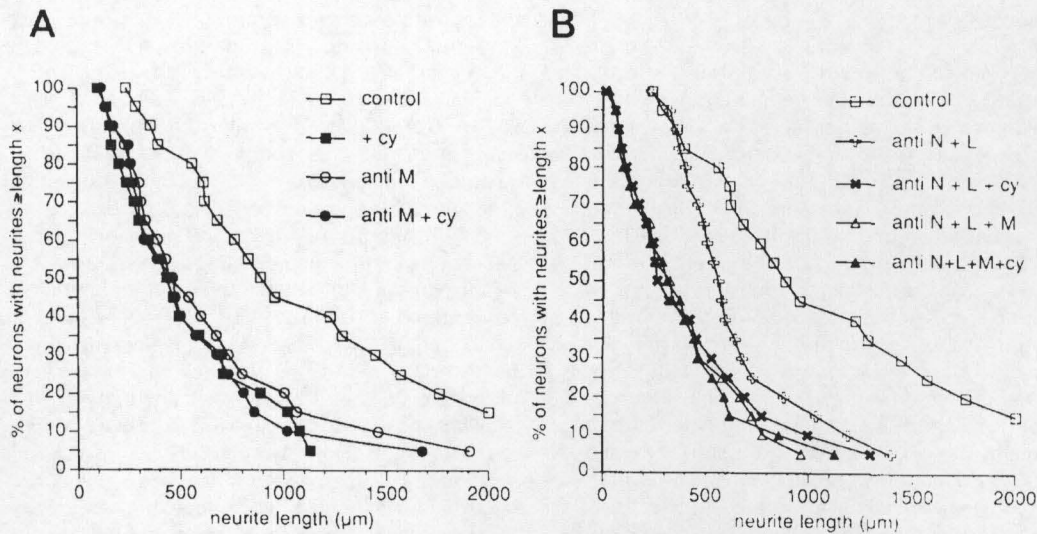


Figure 7. Cumulative Frequency Distribution Plot of the Neurite Lengths of DRG Neurons Maintained on Schwann Cell Monolayers in the Presence of Antibodies and Cytokines

The percentage of neurons with neurites greater than or equal to a certain length  $x$  (vertical axis) was plotted as a function of neurite length  $x$  (horizontal axis). The values of one representative experiment are shown (for mean values of three independent experiments, see Table 2).

(A) Control, culture medium containing 100 ng/ml NGF; + cy, pretreatment of Schwann cells with IFN- $\gamma$  and TNF- $\alpha$  and presence of the cytokines during coculture; anti M, coculture in the presence of MAG antibodies; anti M + cy, pretreatment of Schwann cells with IFN- $\gamma$  and TNF- $\alpha$  and presence of the cytokines and MAG antibodies during coculture. Inhibition of neurite outgrowth by cytokines (+ cy), MAG antibodies (anti M), and MAG antibodies together with cytokines (anti M + cy) was significantly different from outgrowth of neurites in control cultures ( $p < 0.001$ ). Differences between cultures treated with cytokines, MAG antibodies, and MAG antibodies in the presence of cytokines were not significant.

(B) Control, culture medium containing 100 ng/ml NGF (same values as in [A]); anti N + L, presence of N-CAM and L1 antibodies during coculture; anti N + L + cy, pretreatment of Schwann cells with IFN- $\gamma$  and TNF- $\alpha$  and presence of the cytokines and N-CAM and L1 antibodies during coculture; anti N + L + M, presence of N-CAM, L1, and MAG antibodies during coculture; anti N + L + M + cy, pretreatment of Schwann cells with IFN- $\gamma$  and TNF- $\alpha$  and presence of cytokines and N-CAM, L1, and MAG antibodies during coculture. Differences between control culture and cultures treated with additives were highly significant ( $p < 0.001$ ).

growth on Schwann cells maintained in the presence of IFN- $\gamma$  and TNF- $\alpha$  was due to the reduction of MAG expression, the molecular specificity was investigated by addition of antibodies to N-CAM, L1, and MAG. Antibodies were used in concentrations known to block the neurite outgrowth-promoting functions of these molecules. Pretreatment of Schwann cells with the combination of IFN- $\gamma$  and TNF- $\alpha$  for 3 days and maintenance of the cocultures in the presence of both cytokines inhibited neurite outgrowth by approximately the same extent as addition of MAG antibodies to the untreated cocultures ( $39\% \pm 5\%$ , and  $41\% \pm 3\%$ , respectively) (see Figure 7A for one representative experiment and Table 2 for mean values of three independent experiments). When neurite outgrowth was determined on Schwann cells pretreated with the combination of the two cytokines and maintained in the presence of the cytokines and MAG antibodies during the coculture with neurons, no further reduction of neurite outgrowth was seen ( $44\% \pm 8\%$ ), when compared with outgrowth of neurites in the presence of MAG antibodies alone ( $41\% \pm 3\%$ ) or treatment with the combination of both cytokines ( $39\% \pm 5\%$ ).

Antibodies to N-CAM and L1 inhibited neurite outgrowth by  $29\% \pm 14\%$ . When neurite outgrowth in cytokine-pretreated cultures was measured after maintenance in the presence of antibodies to N-CAM and L1 and the continuing presence of cytokines, a further inhibition was observed ( $63\% \pm 6\%$ ), the extent of which appeared to be equivalent to the sum of the individual inhibitory effects of cytokines and antibodies (Figure 7B; Table 2). Addition of all three antibodies to Schwann cells pretreated with the two cytokines did not lead to a more pronounced reduction of neurite outgrowth ( $65\% \pm 12\%$ ), when compared with neurite outgrowth on cytokine-treated Schwann cells in the presence of antibodies to N-CAM and L1 ( $63\% \pm 6\%$ ). After addition of the three antibodies together to Schwann cells maintained in the absence of cytokines, neurite outgrowth was found to be inhibited to a similar extent ( $67\% \pm 9\%$ ) (Figure 7C; Figure 7B; Table 2). Thus, the reduction of neurite outgrowth on Schwann cells maintained in the presence of IFN- $\gamma$  and TNF- $\alpha$  can be specifically attributed to the reduction of MAG expression on the surface of Schwann cells.

## Discussion

The exact role of the neural recognition molecule MAG during myelination and regeneration *in vivo* is not well known. *In vitro* experiments have shown that MAG is necessary for interactions of oligodendrocytes with neurons and oligodendrocytes (Poltorak et al., 1987) and confers adhesion and neurite outgrowth-promoting functions (Johnson et al., 1989; Sadoul et al., 1990). Also, interactions between Schwann cells and neurons, especially during the process of myelination, require the presence of MAG (Owens and Bunge, 1989). The function of MAG is difficult to assess in cultures of primary Schwann cells, since MAG is not expressed at the age when Schwann cells are taken for cultures, or is down-regulated after a short time *in vitro* in the absence of neurons (Jessen and Mirsky, 1991; our own observations). The immortalized rat Schwann cells used in this study express MAG and respond to the inflammatory cytokines IFN- $\gamma$  and TNF- $\alpha$  by up-regulation of MHC molecules, as reported for Schwann cells in primary cultures (Kingston et al., 1989; Wekerle et al., 1986). Thus, this cell line provided the possibility to investigate the function of MAG in its natural molecular context with other adhesion molecules on the surface of Schwann cells.

We have shown that the cytokines IFN- $\gamma$  and TNF- $\alpha$  down-regulate the expression of MAG on these Schwann cells by approximately 60%. This down-regulation was detected at the levels of both surface glycoprotein and mRNA and led to a considerable reduction in the neurite outgrowth-promoting properties of these cells. Antibody blocking experiments confirmed the specificity of this MAG-mediated effect on neurite outgrowth. An indirect effect of the cytokines on neurite outgrowth via endogenous NGF production could be excluded, since neurite outgrowth was measured in the presence of more than saturating concentrations of NGF. However, indirect effects of IFN- $\gamma$  and TNF- $\alpha$  on the synthesis of other Schwann cell-derived cytokines, which may influence the expression of MAG, cannot be excluded. Nevertheless, our results indicate that inflammatory cytokines affect the expression of a neural recognition molecule and thus provide evidence for a further link between the immune and nervous systems.

It is noteworthy that IFN- $\gamma$  and TNF- $\alpha$  not only lead to the down-regulation of MAG, but also stimulate the expression of MHC class I and II molecules on the surface of the immortalized as well as primary Schwann cells. The differentiation state of cytokine-treated Schwann cells switches from a more neurally to a more immunologically oriented one, which enables Schwann cells to present antigen to MHC-restricted T lymphocytes (Kingston et al., 1989; Wekerle et al., 1986). It is likely that such a switch leads to considerable functional consequences in the physiology of peripheral nerves.

Although Schwann cells express several molecules

with neurite outgrowth-promoting properties, such as N-CAM, L1, cadherins, integrins, and J1/tenascin (Bixby et al., 1988; Doherty et al., 1989; Kleitman et al., 1988; Martini and Schachner, 1988; Martini et al., 1990; Matsunga et al., 1988; Seilheimer and Schachner, 1988; Seilheimer et al., 1989; Tomaselli et al., 1988), our observations indicate that down-regulation of a single molecule on the Schwann cell surface can exert strong effects on neurite outgrowth. It has been reported that neurite outgrowth-promoting activities of N-CAM, N-cadherin, and L1 can be separately inhibited by their antibodies (Doherty et al., 1991; Seilheimer and Schachner, 1988). For N-CAM it has been described that a certain minimal level on the cell surface is required to evoke neurite outgrowth and that relatively small changes above or below this level can have pronounced effects (Doherty et al., 1990). Our data suggest that in the presence of inflammatory cytokines, the level of MAG at the cell surface drops below the threshold necessary for MAG-mediated neurite outgrowth, whereas the expression and functions of other neurite outgrowth-promoting molecules are not affected. Thus, our data support the view that individual recognition molecules contribute in an additive manner to the neurite outgrowth-promoting properties of the Schwann cell surface.

These phenomena may be important *in vivo* for regenerative processes in peripheral nerves, where cytokines are synthesized by cells present in the nerve and infiltrating at the site of a lesion. Interleukin-1, synthesized by infiltrating macrophages, appears to play a positive role for regenerative processes, inducing NGF expression (Brown et al., 1991; Heumann et al., 1987; Lindholm et al., 1987) and thus increasing the expression of L1 by both NGF-receptive neurons and Schwann cells (Seilheimer and Schachner, 1987). On the other hand, if during regeneration an inflammatory response is also generated, infiltrating macrophages and lymphocytes produce IFN- $\gamma$  and TNF- $\alpha$  and, according to our findings, could inhibit MAG-mediated Schwann cell-neuron interactions necessary for neurite outgrowth and remyelination. Thus, these immune system-derived cytokines might result in rather negative effects on regeneration.

The functional consequences of a cytokine-induced down-regulation of MAG in the adult peripheral nerve during inflammatory diseases are perhaps even more intriguing. MAG remains expressed in the myelin of adult peripheral nerves at the axon-Schwann cell interface and in the uncompacted regions constituting the outer and inner mesaxon, Schmidt-Lanterman incisures, paranodal loops, and extracellular matrix (Martini and Schachner, 1988). Its down-regulation under inflammatory conditions, including responses to viral or bacterial infections, would influence cellular interactions at exactly these sites, possibly resulting in the dissociation of myelin from the axon and the disintegration of myelin. One of the most conspicuous examples of human disorders that appear to affect the peripheral myelinated state is demy-



elinating polyneuritis, or Guillain-Barré syndrome (Brostoff, 1984). This disease is characterized by infiltration of lymphocytes and macrophages, indicative of a pronounced immune response, and by the disruption of myelin (for review see Quarles, 1989). It is tempting to speculate that, as an early step in the pathogenesis of this syndrome, cytokines secreted by infiltrating lymphocytes and macrophages may affect the integrity of myelin and axon-myelin interactions by impairing the expression of MAG.

These arguments point to the possibility that such cytokine-mediated effects may play a role in inflammatory responses not only in the peripheral, but also in the central nervous system. IFN- $\gamma$  and TNF- $\alpha$  have been demonstrated immunohistologically on astrocytes adjacent to brain lesions of multiple sclerosis patients (Hofman et al., 1989; Traugott and Lebon, 1988). If our observations with Schwann cells can be extrapolated to myelin-forming oligodendrocytes, these cytokines could cause a reduction of MAG expression below the levels needed for maintenance of myelin, even before cytotoxic effects are detectable. Indeed, a significant loss of approximately 70% in steady-state levels of MAG has been detected in the vicinity of multiple sclerosis plaques (Itoyama et al., 1980; Johnson et al., 1986; Möller et al., 1987). Furthermore, it has been observed that TNF- $\alpha$  provokes dilations in the myelin lamellae of organotypic cultures of rodent brain tissue (Brosnan et al., 1988). A reduction of MAG expression induced by IFN- $\gamma$  and TNF- $\alpha$  could explain these observations. Thus, our findings may provide evidence for a mechanism by which inflammatory cytokines impair functional cell interactions, leading to the destruction of tissue integrity, even before cellular or humoral immune responses triggered by the disintegrating tissue have come into play.

#### Experimental Procedures

##### Antibodies

The following antibodies were used for indirect immunofluorescence and flow cytometric analyses: anti-MHC class I molecules, mouse monoclonal antibody Ox18 (Serotec); anti-MHC class II molecules, mouse monoclonal antibodies Ox6 and Ox17 (Serotec); anti-MAG, mouse monoclonal antibody 513 (Poltorak et al., 1987); anti-N-CAM, polyclonal immunoaffinity-purified rabbit antisera; anti-L1, polyclonal immunoaffinity-purified rabbit antisera; anti-P<sub>0</sub>, polyclonal antibodies raised against the extracellular domain of P<sub>0</sub> (Schneider-Schaulies et al., 1990); anti-GFAP, rabbit polyclonal antibodies (Serotec); anti-Ran1, mouse monoclonal antibody 217c (Fields and Dammerman, 1985); anti-O1, mouse monoclonal antibody 59; anti-O4, mouse monoclonal antibody 81; anti-O10, mouse monoclonal antibody 479 (Nieke et al., 1988); anti-neurofilament (160 kd), mouse monoclonal antibody (Boehringer Mannheim).

Immunoaffinity purification of polyclonal rabbit antibodies to mouse L1 and N-CAM was performed by passing hyperimmune serum over a Sepharose-4B column conjugated by cyanogen bromide activation to immunoaffinity-purified L1 or N-CAM from adult mouse brain and eluting the bound antibody with glycine buffer at pH 2.5 (Martini and Schachner, 1986).

The following were used as secondary antibodies: fluorescein-isothiocyanate (FITC)-conjugated rabbit anti-mouse immunoglobulin antibodies (1:100) (Cappel); FITC-coupled goat

anti-rabbit immunoglobulin antibodies (1:100) (Cappel); and horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin antibodies (1:100) (Dianova).

##### Cytokines

Recombinant rat IFN- $\gamma$  (Boehringer Mannheim) and recombinant rat TNF- $\alpha$  (Boehringer Mannheim) were used at concentrations of 100 U/ml and 10 ng/ml, respectively, or as indicated in the text. NGF (7S NGF from submaxillary glands of male mice; Boehringer Mannheim) was used at a concentration of 100 ng/ml.

##### Cell Culture

The immortalized Schwann cell line MT<sub>4</sub>SV-H1 was obtained by transfecting primary rat Schwann cells with a plasmid expressing the SV40 T antigen under the control of the metallothionein enhancer (Peden et al., 1989). These cells were grown in RPMI medium supplemented with 10% fetal calf serum and 10 mM HEPES buffer (pH 7.2), or basal medium Eagle's containing 10% horse serum. High expression of SV40 T antigen was achieved in the presence of 100  $\mu$ M Zn<sup>2+</sup> in the culture medium, leading to cell proliferation. In the absence of Zn<sup>2+</sup>, cells had low proliferative activity and displayed the typical spindle-shape morphology of Schwann cells in primary culture. Schwann cells were kept in culture medium without Zn<sup>2+</sup> for 7–10 days before the addition of cytokines or antibodies.

Cultures of DRG neurons were established from 1-day-old NMRI mice as described (Seilheimer and Schachner, 1988). After incubation of DRGs with 0.03% collagenase (Sigma) and 0.5% trypsin (Sigma) for 45 min at room temperature, cells were mechanically dissociated by pipetting up and down with a fire-polished Pasteur pipette and centrifuged through a cushion of 35% Percoll (Sigma) in Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free Hank's balanced salt solution for 20 min at 200  $\times$  g and 4°C to separate neurons from nonneuronal cells. Neurons were maintained in basal medium Eagle's containing 10% horse serum and 100 ng/ml NGF.

##### Cytofluorometric Analysis

Flow cytometry was performed with a fluorescence-activated cell scanner (FACSscan, Becton Dickinson). Immortalized Schwann cells were seeded in 6-well plates (Nunc) coated with 0.01% poly-L-lysine (molecular weight 30,000; Sigma) and grown in RPMI medium containing 10% fetal calf serum and 100  $\mu$ M Zn<sup>2+</sup> until cells were confluent. Cultures were then kept for 7–10 days in culture medium without Zn<sup>2+</sup>. Cytokines were added during the last 3 days before the cells were harvested by a 30 min incubation with Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free Hank's balanced salt solution containing 1 mM EDTA at room temperature to detach cells from the substrate. Cells (1  $\times$  10<sup>5</sup> per tube) were then washed with PBS (pH 7.4), incubated with first antibodies in 300  $\mu$ l of culture medium for 30 min at 4°C, washed twice with PBS, incubated with FITC-coupled second antibodies in 300  $\mu$ l of culture medium, washed twice with PBS, and maintained in the dark on ice until the cytofluorometric analysis was performed. The mean fluorescence intensity and percentage of immunofluorescent cells were evaluated by reference to the values obtained with Schwann cells stained with control antibody (mouse IgG1) and FITC-coupled second antibodies.

##### Immunoprecipitation

Biosynthetic labeling of cell cultures and immunoprecipitation were performed as described (Faissner et al., 1984). In brief, 1  $\times$  10<sup>6</sup> Schwann cells were cultured for 4 hr in methionine-free culture medium containing 10 mM HEPES (pH 7.2) and [<sup>35</sup>S]methionine. Cells were washed once with PBS and incubated for 45 min at 4°C in 1 ml of solubilization buffer (20 mM Tris-HCl, 150 mM NaCl, 20 mM NaF, 25 mM Na<sub>2</sub>P<sub>2</sub>O<sub>7</sub>, 20 mM Na<sub>2</sub>HPO<sub>4</sub>, 20 mM NaH<sub>2</sub>PO<sub>4</sub>, 1 mM EDTA, 1 mM EGTA, 0.5% NP-40, 1 mM PMSF [pH 7.4]). The lysate was then cleared by centrifugation at 100,000  $\times$  g, and the supernatant was incubated with antibody solution (50  $\mu$ g/ml) for 2 hr at 4°C. After addition of 1% protein A-Sepharose and incubation for 1 hr, the Sepharose beads were centrifuged through a cushion of 1 M sucrose in solubilization buffer. Analy-

sis of biosynthetically labeled components was performed by SDS-PAGE.

#### RNA Preparation and Northern Blot Analysis

Total cellular RNA was prepared by the guanidinium-isothiocyanate method as described (Chirgwin et al., 1979) and purified by centrifugation through a CsCl cushion (1.7 g/ml). The RNA (20 µg per lane) was then electrophoresed on 1.5% agarose gels containing 6.3% formaldehyde and blotted onto Hybond N filters (Amersham). The hybridization probes were as follows: MAG, the 2.5 kb BamHI fragment of plasmid pB236-18 (Lai et al., 1987); N-CAM, the 600 bp EcoRI fragment of pM1.3 (Goridis et al., 1985); L1, the 1.0 kb EcoRI fragment of pK21 (Moos et al., 1988); MHC class II molecules, the 830 bp EcoRI-HindIII fragment of pRTIEβ (Robertson and McMaster, 1985); and GAPDH, the 1.3 kb PstI fragment of pGAPDH (Fort et al., 1985). All hybridization probes were [<sup>32</sup>P]dCTP-labeled with a random priming kit (Boehringer Mannheim) to specific activities of 3 × 10<sup>6</sup> cpm/µg. The intensity of bands on X-ray films was quantified by densitometric scanning with a UV/8 spectrophotometer (Beckman). The values for each band were set in relation to GAPDH as a constitutively expressed standard.

#### Quantitative Determination of Neurite Outgrowth

Schwann cells were seeded into 8-well plastic chamber slides (Nunc) and grown in culture medium containing 100 µM Zn<sup>2+</sup> until a complete monolayer was achieved. Then the cells were maintained in culture medium without Zn<sup>2+</sup> for 7–10 days. IFN-γ (100 U/ml) and TNF-α (10 ng/ml) were added for 3 days before addition of DRG neurons. Neurite outgrowth was quantified as described (Seilheimer and Schachner, 1988). In brief, DRG neurons (1 × 10<sup>3</sup> per well in 200 µl of culture medium) were plated on Schwann cell monolayers in basal medium Eagle's containing 10% horse serum and 100 ng/ml NGF and maintained for 20 hr at 37°C. Cultures were then treated with 3.7% formaldehyde in PBS containing 0.1% Triton X-100 for 1 hr at room temperature and stained with anti-neurofilament antibodies by the indirect immunoperoxidase method (Seilheimer and Schachner, 1988). The total length of neurites per neuron was measured with a computer equipped with a graphic tablet and cursor, TV camera, color monitor, and image analysis program (VIDS III, AI Tektron, Meerbusch). Forty cells each from three independent experiments were measured for statistical evaluation, and the significance of the values was determined by the U-test of Mann and Whitney.

#### Acknowledgments

The authors are grateful to I. Bahnmüller for technical assistance, Dr. R. Probstmeier and A. Spitzauer (Institut für Neurobiologie, Heidelberg, Germany) for immunoaffinity-purified antibodies to N-CAM and L1, Dr. G. I. Tennekoon (Johns Hopkins University School of Medicine, Baltimore, Maryland) for providing the Schwann cell line pMT<sub>1</sub>SV-H1, Dr. J. C. Roder (Department of Medical Genetics, University of Toronto, Canada) for the cDNA probe for MAG, and Deutsche Forschungsgemeinschaft (fellowships to J. S.-S. and J. A.), Bundesministerium für Forschung und Technologie, Ciba-Geigy (Basel), and Kommission zur Förderung der wissenschaftlichen Forschung for support.

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Received May 30, 1991; revised August 20, 1991.

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