Astrocytes Inhibit Schwann Cell Proliferation and Myelination of Dorsal Root Ganglion Neurons *in vitro*

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Schwann cells promote the regrowth of nerve fibers in both the PNS and CNS and might thus be of value in strategies to promote repair following injury or demyelination in the CNS. The effectiveness of Schwann cells in promoting repair could, however, be limited by interactions with reactive astrocytes that are prominent at lesioned and demyelinated sites. To investigate this possibility, experiments were performed to determine the influence of cortical astrocytes on Schwann cell proliferation and myelination of dorsal root ganglion (DRG) neurons *in vitro*. DRG neurons from embryonic rats and Schwann cells, astrocytes, and fibroblasts isolated from the sciatic nerve, cerebral cortex, and cranial periosteum, respectively, of neonatal rats were purified and then recombined to provide neuron–Schwann cell, neuron–Schwann cell–astrocyte, and neuron–Schwann cell–fibroblast cultures. Astrocytes inhibited both neuron-dependent Schwann cell proliferation and the myelination of axons by Schwann cells. The expression of galactocerebroside, but not of the O4 antigen, was inhibited by astrocytes, suggesting that astrocytes blocked Schwann cell differentiation prior to the onset of myelination. Ultrastructural analysis of the cultures also indicated that both axonal ensheathment and the segregation of large axons into 1:1 relationships were decreased in the presence of astrocytes. Astrocytes did not affect the expression of the basal lamina components type IV collagen and laminin, and basal lamina formation assessed by electron microscopy was only slightly decreased. Some of these inhibitory effects appear to be mediated by diffusible factors since astrocyte-conditioned medium also reduced Schwann cell myelination. Fibroblasts or fibroblast-conditioned medium did not induce such inhibitory effects, indicating that the effects were astrocyte specific. We conclude that cortical astrocytes release a soluble

factor(s) that inhibits specific aspects of neuron–Schwann cell interactions leading to myelination.

[Key words: astrocyte, Schwann cell, neuron, proliferation, myelination, differentiation]

Physical injuries to the CNS can cause severe disability and numerous complications in victims of such injuries. The loss of nerve fibers across lesioned sites and of myelin sheaths on surviving fibers contributes to the functional deficits. Recent demonstrations that neurons could regenerate new fibers and that demyelinated fibers could be remyelinated have challenged the older belief that repair of damaged CNS could not occur and inspired a renewed research effort to define conditions and approaches to maximize repair in animal models (David and Aguayo, 1981; Wrathall et al., 1982; Duncan et al., 1988; Paino and Bunge, 1991; Xu et al., 1993). It is well established that Schwann cells, the glial cells of the PNS, enhance peripheral nerve regeneration by creating a supportive terrain for elongating neurites (for review, see Fawcett and Keynes, 1990) and by secreting both neurotrophic and neurotropic factors (e.g., Bandtlow et al., 1987; Muir et al., 1989; Acheson et al., 1991; Rende et al., 1992). Recent studies have demonstrated that Schwann cell-containing implants of PNS tissue support the regeneration of CNS neurons in many different areas of the CNS (Blakemore, 1977; Weinberg and Raine, 1980; David and Aguayo, 1981; Richardson et al., 1982; Wrathall et al., 1982; Kromer and Cornbrooks, 1985; Politis and Spencer, 1986; Smith and Stevenson, 1988; Campbell et al., 1992). Several studies indicate that cultured purified Schwann cells transplanted into damaged or demyelinated areas of the adult mammalian spinal cord support nerve fiber outgrowth and form myelin sheaths (for a review, see Guénard et al., 1993).

Despite these promising findings, the stimulation of regenerative repair by Schwann cells transplanted into the CNS appears to be limited by factors that are poorly understood. A number of observations suggest that reactive astrocytes, often present after injury or demyelination, exert a negative influence on the ability of Schwann cells to function in the CNS (Raine et al., 1978; Harrison, 1980; Sims and Gilmore, 1983; Blakemore et al., 1986; Blakemore and Crang, 1989). Furthermore, both in areas of experimentally demyelinated spinal cord and in multiple sclerosis plaques, myelination of CNS fibers by Schwann cells is observed only in areas containing few astrocytes (Itaya et al., 1983, 1985). At the PNS–CNS interface in a healthy animal, astrocytes exclude Schwann cells from entering the CNS by an unknown mechanism. After injury astrocytes proliferate rapidly, become hypertrophied, and secrete numer-

Received Aug. 5, 1993; revised Oct. 14, 1993; accepted Oct. 26, 1993.

This work was supported by NIH Grant NS 28059 and National Multiple Sclerosis Society Grant RG 2210-A-2 to P.M.W., and funds from The Miami Project to Cure Paralysis. We are grateful to Dr. John Klose for his help with the statistical analysis; Anna Gomez, Margaret Bates, and Francisco Cruz for excellent technical support; and Robert Camarina for photographic assistance. The laminin, type IV collagen, and 217c antibodies were generous gifts from Dr. C. Gatchalian (University of Oregon), Dr. A. Charonis (University of Minnesota), and Dr. J. de Vellis (University of California at Los Angeles), respectively. Hybridoma cell lines producing the monoclonal antibodies O4 and O1 were a generous gift from Dr. M. Schachner (Zurich, Switzerland).

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ous humoral factors (Bignami and Dahl, 1976; Nieto-Sampedro et al., 1983; Reier and Houle, 1988; Hatten et al., 1991). Such factors include tumor necrosis factor- α (TNF- α), prostaglandins, and transforming growth factor- β (TGF- β) (Robbins et al., 1987; Cockram, 1990), all of which have been shown to have inhibitory influences on target cells and could act to inhibit the function of transplanted Schwann cells. At the present time, very little is known from *in vivo* studies about the nature of interactions that might occur between reactive astrocytes located at a lesion site and transplanted Schwann cells.

Past studies have shown that Schwann cells proliferate in response to a mitogen on neuronal membranes, secrete extracellular matrix (ECM) components, form a complete basal lamina in contact with neurons and ensheath axons, and produce myelin sheaths only when basal lamina is deposited (Wood and Bunge, 1975; Carey et al., 1983; Bunge et al., 1986, 1989; Eldridge et al., 1989; Kleitman et al., 1991). To determine if astrocytes influence normal interactions between neurons and Schwann cells, we have used new techniques for the isolation and purification of Schwann cells, astrocytes, and neurons and have recombined these cells *in vitro*. In the present study, we used these culture methods to evaluate the influence of both astrocytes and astrocyte-conditioned medium on neuron-dependent Schwann cell proliferation and on the myelination of dorsal root ganglion (DRG) axons by Schwann cells. We now report that in our experimental model astrocytes inhibit Schwann cell proliferation and myelination of DRG neurites and slightly decrease basal lamina formation. Myelination was blocked at an early stage in the sequence of events leading to the formation of myelin. Furthermore, our findings suggest that these effects are mediated by a factor(s) released by astrocytes.

Materials and Methods

Preparation of purified cell populations

Neuronal cultures. Pure cultures of dissociated rat embryonic dorsal root ganglion (DRG) neurons were obtained according to techniques developed in our laboratory (Bunge et al., 1983; Kleitman et al., 1991). The pure neuronal cultures were subsequently maintained in Eagle's Minimal Essential Medium (EMEM; GIBCO, Long Island, NY) supplemented with 5% heat-inactivated human placental serum, nerve growth factor (NGF; 50 ng/ml), and glucose (0.4%, w/v) at 35°C with 5% CO₂.

Schwann cell cultures. Schwann cells from the sciatic nerve of embryonic day 21 (E21)–postnatal day 1 (P1) Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA) were purified according to the method of Brookes et al. (1979). Schwann cells were allowed to proliferate in Dulbecco's Modified Eagle's Medium containing 10% heat-inactivated fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT) (D medium) supplemented with pituitary extract (10 μ g/ml; Sigma) and forskolin (2 μ M; Sigma) (Porter et al., 1986). At least 5 d prior to the addition of the Schwann cells to the neuronal cultures, the mitogens were removed from the medium in which the Schwann cells were maintained. This ensured that Schwann cell proliferation observed in the neuron–Schwann cell cocultures was due to axonal contact rather than to residual soluble exogenous mitogens. Schwann cells were grown in a humidified atmosphere at 37°C with 5% CO₂. The purity of the cultures was determined by immunostaining for S-100, a Schwann cell marker, Thy-1.1, a fibroblast marker (Raff et al., 1979), and OX-42, a macrophage marker (Robinson et al., 1986). After labeling, the cultures were mounted on glass slides in Citifluor mountant (University College, London, UK) containing the fluorescent nuclear dye Hoechst 33342 (5 μ M; Sigma). The monoclonal OX-42 antibody was purchased from Bioproducts for Science (Indianapolis, IN) and the hybridoma cell line making the Thy-1.1 antibody was purchased from American Tissue Culture Collection (ATCC). The percentages of S-100-, Thy-1.1-, and OX-42-positive cells were evaluated using a Zeiss Universal microscope adapted for epifluorescence at a final magnification of 400 $\times$. For each immunostain, an average of 500 cells was counted. The cultures were

approximately 98.7% pure Schwann cells (S-100-positive cells); 1.3% of the cells were fibroblasts (Thy-1.1-positive cells), and no macrophages were observed.

Astrocyte cultures. Astrocytes were obtained from the cortex of E21–P1 Sprague–Dawley rats using a modification of the method of McCarthy and de Vellis (1980). The cortical cell suspension was fractionated on an 80% Percoll gradient in 0.25 M sucrose with 0.02 M phosphate buffer at 30,000 $\times$ g for 45 min at 4°C to remove the cellular debris and red blood cells (Rosen et al., 1989). The cell layer found above the red blood cell layer was then collected, filtered through a 15 μ m Nitex filter (Tetko Inc., Briar Crest, NY), and plated into 25 cm² Falcon flasks in D medium. The cells were allowed to proliferate for 8–10 d, and then the cultures were shaken overnight at 150 rpm to remove contaminating oligodendrocytes that were located on top of the astrocyte monolayer (McCarthy and de Vellis, 1980). The cultures were then treated once with fluorodeoxyuridine (2 μ M; Sigma) to eliminate remaining rapidly proliferating meningeal, epithelial, and microglial cells. The purified astrocytes were then replated onto 100 mm Petri dishes, allowed to proliferate in D medium, and subcultured no more than four times (approximately 1 month *in vitro*). The time astrocytes were maintained in culture was kept short to minimize the possibility that the properties of the astrocytes would change with time *in vitro*. The astrocyte cultures were maintained at 37°C in 5% CO₂. The composition of the cultures was evaluated by immunostaining for glial fibrillary acidic protein (GFAP), an astrocyte marker (Bignami et al., 1972); O4, a marker for both oligodendrocyte precursors and oligodendrocytes (Bansal et al., 1989); O1, an oligodendrocyte-specific monoclonal antibody that recognizes galactocerebroside (Sommer and Schachner, 1981; Bansal et al., 1989); A2B5, a marker for oligodendrocyte progenitors and for type 2 astrocytes (Raff et al., 1983); Thy 1.1; and OX-42. Polyclonal GFAP antibody was purchased from Biomedical Technologies Inc. (Stoughton, MA) and the hybridoma cell line making A2B5 antibody was purchased from ATCC. The composition of the cultures was determined by counting as described above. The cultures were 98.5% pure astrocytes (GFAP-positive cells). Of these, 97.1% were type 1 astrocytes (A2B5-negative cells) and 1.4% were type 2 astrocytes (A2B5-positive cells). The cultures also contained 1.3% undifferentiated and differentiated oligodendrocytes (O4- and O1-positive cells), 0.05% fibroblasts, and 0.1% macrophages.

Fibroblast cultures. To determine whether the effects observed with astrocytes could be due to cellular overloading or to the trauma of adding cells to the cultures, fibroblasts that expand in culture in a similar way to astrocytes were added to some neuron–Schwann cell (N-SC) cultures in parallel with astrocytes. Fibroblasts were obtained from the cranial periosteum of E15 Sprague–Dawley rats as reported by Rosen et al. (1989). Fibroblasts were subcultured no more than three times (approximately 1 month *in vitro*) and maintained in EMEM supplemented with 0.4% glucose, NGF (50 ng/ml), and 10% FBS (C medium) in a humidified atmosphere of 5% CO₂ at 35°C. The composition of the cultures was evaluated by immunostaining with the anti-Thy 1.1 and anti-OX-42 antibodies. Cultures were 99.6% fibroblasts and 0.4% macrophages.

Preparation of astrocyte- and fibroblast-conditioned media. O medium [consisting of EMEM supplemented with 0.4% glucose, NGF (50 ng/ml), 1% FBS, insulin (50 μ g/ml; Sigma), putrescine (100 μ M; Sigma), progesterone (20 pM; Sigma), sodium selenite (30 pM; Pfaltz & Bauer, Waterbury, CT), and transferrin (10 μ g/ml; Jackson Immunoresearch Labs, West Grove, PA)] was conditioned by confluent astrocyte or fibroblast monolayers for a 3 d period at 37°C with 5% CO₂ (O-ACM and O-FBCM media, respectively) and filtered through a 0.22 μ m Milipore filter. The conditioned media were aliquoted and stored at –80°C. Myelinating M medium consisting of EMEM supplemented with 0.4% glucose, NGF (50 ng/ml), 15% FBS, and L-ascorbic acid (50 μ g/ml; Sigma) was conditioned overnight by astrocytes, filtered, pooled, and used fresh (M-ACM). Prior to use, all conditioned media were diluted 1:1 (v/v) with fresh medium.

Preparation of the combined cultures. Schwann cells and astrocytes were detached from plates by trypsinization, rinsed, and counted using a hemocytometer. Fibroblasts were collected after gently scraping the dishes, washed, and counted. Prior to seeding, neuronal cultures were rinsed with medium appropriate for either a proliferation or myelination assay (see below) and 50,000 Schwann cells suspended in similar medium were added to produce neuron–Schwann cell (N-SC) cultures. Astrocytes or fibroblasts were added either with the Schwann cells or later as described below to produce neuron–Schwann cell–astrocyte (N-

SC-A) or neuron–Schwann cell–fibroblast (N-SC-Fb) cultures. To test the effects of factors released by astrocytes, some N-SC cultures were fed with medium containing 50% astrocyte-conditioned medium (N-SC-ACM cultures). N-SC cultures treated with fibroblast-conditioned medium (N-SC-FbCM cultures) were used as controls.

Method to determine the effect of astrocytes on Schwann cell proliferation

N-SC cultures established in O medium received either 150,000 or 500,000 astrocytes or fibroblasts at the same time as the addition of Schwann cells or were maintained in medium containing astrocyte- or fibroblast-conditioned medium. Twenty-four hours after the start of the coculture period, N-SC, N-SC-A, N-SC-Fb, N-SC-ACM, and N-SC-FbCM cultures were pulsed for 22 hr with ^3H -thymidine (0.5 $\mu\text{Ci}/\text{ml}$; New England Nuclear, Boston, MA) in O medium and then immunostained and processed for autoradiography. Schwann cells and astrocytes, respectively, were labeled using anti-217c antibody, which recognizes the NGF receptor (Kumar et al., 1990), and anti-GFAP antibody. Cultures were rinsed, blocked with L-15 containing 10% normal goat serum, and incubated for 30 min each at 4°C with anti-217c antibody (1:100) followed by rhodamine-conjugated goat anti-mouse secondary antibody (1:100; Cappel/Organon Teknika, West Chester, PA). The cultures were then fixed for 10 min using 4% paraformaldehyde in 0.1 M phosphate buffer and permeabilized with 0.2% Triton X-100 in fixative and with acetone. The cultures were then incubated with anti-GFAP antibody (1:100) and fluorescein-conjugated goat anti-rabbit antibody (1:50; Cappel/Organon Teknika) for 30 min each at 4°C. After rinsing with buffer and distilled water, the coverslips were dipped into 100% ethanol, air dried, and mounted face up onto glass slides. The cultures were then dipped into NTB-2 Kodak emulsion and kept in a light-tight box for at least 5 d at 4°C. The autoradiograms were developed, fixed, rinsed, air dried, and coverslipped using Citifluor/Hoechst 33342 dye mountant. For each condition in each experiment, cultures with comparable cellular organization and density were analyzed. A minimum of 500 217c-positive cells associated with DRG neurites were evaluated on randomly selected fields from each culture at a final magnification of 400 $\times$. Among these cells, the number of ^3H -thymidine-positive cells was counted. All cells containing at least 10 silver grains over their nucleus were considered ^3H -thymidine positive. The Schwann cell labeling index, expressed as a percentage, was defined as the ratio of the number of ^3H -thymidine-positive and 217c-positive cells over the total number of 217c-positive cells, times 100.

Methods to determine the effect of astrocytes on Schwann cell differentiation/myelination

Effect of astrocytes on Schwann cell differentiation. To study the effect of astrocytes on Schwann cell differentiation, N-SC, N-SC-A, and N-SC-ACM cultures were prepared in M medium. N-SC-A cultures received 100,000 astrocytes together with the Schwann cells and N-SC-ACM cultures were fed with a 1:1 mixture of M:O-ACM medium supplemented with FBS, crude NGF, and L-ascorbic acid to final concentrations of M medium. The experiment was performed three times. At 6 and 8 d after starting myelination, two or three cultures from each condition were immunostained with the differentiation stage-specific antibodies O4 and O1 (Bansal et al., 1989). The O4 antigen is an uncharacterized molecule that begins to be expressed on the surface of neuron-associated Schwann cells prior to myelination, whereas the O1 antigen is galactocerebroside, expressed at the onset of myelination. Thus, O4-positive/O1-positive Schwann cells are more differentiated than O4-positive/O1-negative Schwann cells. The cultures were immunostained using anti-O1 antibody (supernatant; Sommer and Schachner, 1981) and rhodamine-conjugated goat anti-mouse antibody (1:50) followed by anti-O4 antibody (supernatant) and fluorescein-conjugated goat anti-mouse antibody (1:50; Cappel/Organon Teknika), fixed, and mounted on glass slides as described above. The cultures were examined for the presence of O4- and O1-positive Schwann cells. Since O4 was expressed on nearly all Schwann cells in all conditions, the number of O4-positive cells was not counted. The number of O1-positive cells per culture at day 8 was evaluated in three cultures per condition using an eyepiece grid. The numbers of O1-positive cells were averaged, giving the number of O1-positive cells per field.

Effect of astrocytes on Schwann cell myelination. To determine the long-term effects of astrocytes on Schwann cell myelination, different types of cells were recombined with purified neuronal cultures as de-

scribed above, except that Schwann cells were allowed to proliferate on the neurites for 2 weeks prior to adding 100,000 astrocytes or astrocyte-conditioned medium (M-ACM or resupplemented O-ACM) and initiating myelination. Prior to the addition of astrocytes or initiating treatment with astrocyte-conditioned medium, the cultures were maintained in C medium. Myelination was initiated by switching C medium with M medium.

Sudan black staining was performed as reported by Wood and Williams (1984) in cultures maintained in M medium for 2 and 4 weeks, taken from three experiments. Myelination in two cultures per condition per experiment was assessed using an Olympus light microscope at a final magnification of 200 $\times$. The center of the cultures was determined and fields were counted every 2 mm along three lines located 1 mm above, 1 mm below, and at the level of the center of the culture. The amount of myelin in each field was quantified using an eyepiece grid divided into 100 squares, with lines 700 μm apart. Each of these squares was scored 0–5 depending on whether a portion of a number of myelin segments crossed the square. An average of 30 fields per culture selected by stage coordinates was evaluated. The scores were added and averaged over the total number of fields counted per culture, giving an estimate of the average amount of myelin per field.

Immunostaining for extracellular matrix components. To assess whether astrocytes or ACM influenced myelination by affecting the expression of some basal lamina components, 2 weeks postfeeding with M medium, some of the cultures were immunostained with antibodies against laminin and type IV collagen. Living cultures were incubated with either anti-laminin (1:100) or anti-collagen type IV (1:100) antibodies followed by fluorescein goat anti-rabbit antibody (1:50), and then fixed and mounted on glass slides as reported above.

Electron microscopy. Control and astrocyte-seeded cultures myelinated for 2 weeks were processed and embedded in Polybed (Polysciences Inc., Warrington, PA) as described previously (Bunge et al., 1989). Arcs containing large neurite fascicles and Schwann cells were visualized by phase-contrast microscopy, scored, excised, and mounted onto blank plastic blocks. Ultrathin sections were obtained using a Reichart-Jung microtome and stained with Reynold's lead citrate and uranyl acetate. Seven areas from three control cultures and six areas from three experimental cultures taken from three different experiments were examined for the extent of basal lamina formation, and for the extent to which Schwann cells had ensheathed axons and formed 1:1 relationships using a Philips CT-10 transmission electron microscope. An axon was considered ensheathed if at least 50% of its surface was in apposition with Schwann cell cytoplasm. An axon was determined to be in a 1:1 relationship with a Schwann cell when the axon was completely isolated and surrounded by a single Schwann cell. Both myelinated and non-myelinated axons engaged in a 1:1 relationship were counted. The percentage of ensheathed axons and 1:1 relationships were determined for each analyzed culture.

Neuronal morphology assay

To determine the influence of astrocytes or ACM on neuronal health and viability, the effect of astrocytes and ACM on the size and morphology (eccentric or pycnotic nuclei, presence of chromatolytic response) of the neuronal cell bodies was assessed. Neuronal cultures were either seeded with 100,000 astrocytes (N-A cultures) and maintained in M medium or fed with M:M-ACM (1:1) medium (N-ACM cultures). Neuronal cultures maintained in M medium were used as controls. The media were changed every 2 d for 4 weeks. Neuronal morphology was assessed by toluidine blue staining. The cultures were fixed, rinsed in 0.1 M PBS at pH 7.4, soaked for 30 sec in stain consisting of 1% each of methylene blue, toluidine blue, and borax in water, rinsed in buffer, dehydrated in ethanol, dipped in xylene, and mounted in DPX on glass slides. The morphology of the neurons was examined and the size of the neuronal soma of 80–100 neurons per culture determined using an Olympus light microscope at a final magnification of 400 $\times$; two cultures per condition were evaluated.

Preliminary characterization of the astrocyte factor(s)

Preliminary experiments to determine the heat stability of the factor(s) were performed. Astrocyte-conditioned medium was heated at either 56°C or 100°C for 30 min prior to testing. Activity of the astrocyte factor(s) in the heated conditioned media was then evaluated using the proliferation assay.

In view of the evidence that astrocytes secrete factors with transform-

Table 1. Inhibition of Schwann cell proliferation by astrocytes and astrocyte-conditioned medium

Condition	150,000 astrocytes per culture		500,000 astrocytes per culture	
	Schwann cell L.I. (%)	Inhibition (%)	Schwann cell L.I. (%)	Inhibition (%)
Control	73.6 ± 18.4		63.0 ± 5.2	
Astrocytes	48.0 ± 21.2	34.7	16.8 ± 5.4*	74.6
Fibroblasts	69.7 ± 19.8	5.3	56.3 ± 4.8	10.0
O-ACM	NA	NA	51.4 ± 7.9	19.2
O-FbCM	NA	NA	68.2 ± 4.4	0

Proliferating Schwann cells were counted on whole-mounted cultures processed for autoradiography (see Materials and Methods). The Schwann cell labeling index (L.I.) was defined as the ratio of ³H-thymidine-positive Schwann cells over the total number of 217c-positive Schwann cells. Values shown are means ± SEM of determinations on cultures from two (150,000 astrocytes) and seven (500,000 astrocytes) experiments, respectively. One-way ANOVA followed by Tukey posttest comparisons was used to test significance between pairs of means.

* Statistical significance ($p < 0.05$) between control and any other condition.

ing growth factor- β (TGF- β) activity (Constam et al., 1992), and that TGF- β is known to have effects on cellular proliferation and differentiation (for review, see Barnard et al., 1990), fresh O-ACM was also tested for TGF- β -like activity. Conditioned medium was heated at 80°C for 8 min to activate latent TGF- β that could be present in the medium. The conditioned medium was first assayed for TGF- β -induced inhibition of proliferation of CCL-64 mink lung epithelial (Mv1Lu) cells (Danielpour et al., 1989) with or without heating. The heated conditioned medium was then tested in N-SC cultures; a blocking experiment was also performed by adding a monoclonal antibody to TGF- β 1 (10 μ g/ml; R & D Systems, Minneapolis, MN) to heated conditioned medium. It has been reported that astrocytes can be stimulated by lipopolysaccharide to secrete a factor with tumor necrosis factor- α (TNF- α) activity (Robbins et al., 1987; Lieberman et al., 1989) that has been shown to have detrimental effects on oligodendrocytes (Robbins et al., 1987; Selmaj et al., 1991). An assay for TNF- α activity in astrocyte-conditioned medium was established using WEHI 164 cells, which are killed by picomolar amounts of TNF- α (Vanderslice and Collins, 1991). For this assay, WEHI 164 cells growing in Optimum medium (GIBCO) supplemented with 10% FBS were resuspended and plated onto collagen-coated Aclar coverslips at 25,000 cells per coverslip. Twenty-four hours later, the WEHI 164 cells were treated with ACM diluted 1:1 with Optimum:FBS medium. After 24 hr, the WEHI 164 cells were stained with trypan blue and the number of viable cells determined. WEHI 164 cells were purchased from ATCC.

Statistical analysis

Data are presented as mean ± SEM. One-way analysis of variance (between conditions) followed by Tukey HSD posttest comparisons between mean individual cells was used. Statistical analysis was performed using a commercially available software program (StatSoft Inc., Tulsa, OK). Statistical significance was accepted for $p < 0.05$.

Results

The cultures used in this study consisted of dissociated purified sensory DRG neurons to which purified Schwann cells were added. The neuronal somata were scattered within an area in the center of the cultures, with their bare neurites growing radially toward the periphery. Whether Schwann cells were added to the cultures alone or together with astrocytes or fibroblasts, Schwann cells were always aligned along the neurites (Fig. 1A), whereas astrocytes and fibroblasts formed a flat monolayer on the entire surface of the coverslips. By electron microscopy, it was possible to observe that astrocytes and fibroblasts had formed a monolayer underneath the neurons.

Table 2. Inhibition of myelination by astrocytes and M-ACM

Condition	2 weeks		4 weeks	
	Amount of myelin per field	Inhibition (%)	Amount of myelin per field	Inhibition (%)
Control	179 ± 17		174 ± 35	
Astrocytes	40 ± 29*	77.6	58 ± 38*	67.0
M-ACM	136 ± 2	24.0	123 ± 13	29.3

Myelin segments were counted on Sudan black-stained whole-mounted cultures 2 and 4 weeks after switching to M medium (see Materials and Methods). M-ACM was used, diluted 1:1 (v/v) with fresh M medium. Values shown are means and SEM of determinations on duplicate or triplicate cultures in each group. One-way ANOVA followed by Tukey posttest comparisons was used to test significance between pairs of means.

* Statistical significance ($p < 0.05$) between control and astrocyte or ACM groups.

Astrocytes block Schwann cell proliferation

To determine the effects of astrocytes on neuron-induced Schwann cell proliferation (Salzer et al., 1980), Schwann cells were added to the neuronal cultures alone or together with astrocytes or fibroblasts. The proliferation assay was performed in defined medium. Analysis of cultures obtained from seven experiments indicated that more Schwann cells were labeled with tritiated thymidine in N-SC cultures than in N-SC-A cultures (Fig. 1A–C,G–I); the difference was statistically significant. Astrocytes inhibited Schwann cell proliferation in a dose-dependent manner (Table 1, Fig. 1A,B,D,E). For similar seeding densities (500,000 cells per culture), astrocytes inhibited proliferation by 74.6% whereas fibroblasts decreased Schwann cell proliferation by 10%. Astrocytes seeded at a lower density (150,000 cells per culture) inhibited proliferation by 34.7%. The fact that fibroblasts only slightly affected the Schwann cell labeling index indicates that the effect on Schwann cell proliferation was a specific property of the astrocytes. A lower Schwann cell labeling index was also consistently observed in N-SC-ACM cultures than in N-SC cultures (Table 1, Fig. 1B,C,E,F). An effect of the astrocyte-conditioned medium could not, however, be definitively demonstrated (Table 1). Schwann cell proliferation was not affected by fibroblast-conditioned medium (Table 1).

Astrocytes and astrocyte-conditioned medium block Schwann cell myelination

Myelination was evaluated on Sudan black-stained cultures. Thin myelin segments were scattered over the cultures; the Schwann cell nucleus was located at the midpoint of these segments, protruding from the myelin sheath. Myelination in N-SC cultures was compared to that in N-SC-A and N-SC-ACM cultures 2 weeks after switching to M medium. Quantitative analysis indicated a 73.5% average decrease (69.4%, 77.6%) in the formation of myelin in astrocyte-containing cultures when compared to control cultures (Tables 2, 3; Fig. 2A,B); the inhibition of myelination induced by astrocytes was statistically significant when compared to control cultures. This inhibition appears to be partially mediated by a diffusible factor(s) released by the astrocytes since myelination was also decreased in cultures exposed to astrocyte-conditioned medium (Tables 2, 3; Fig. 2C). When serum-containing medium was used (Table 2) the decrease in myelination was not statistically significant, whereas when the experiment was performed using low serum-containing

Figure 1. Schwann cell proliferation in N-SC (*A, D, G, J*), N-SC-A (*B, E, H, K*), and N-SC-ACM (*C, F, I, L*) cultures. Phase-contrast (*A, D*) and fluorescence micrographs show immunostaining for 217c (*G*) and GFAP (*J*) in an N-SC culture processed for autoradiography. Schwann cells are lying along axons (*A*) with the position of their nucleus marked with the Hoechst nuclear dye (*D*, asterisks). Schwann cells (*G*, arrows), 217c positive, are GFAP negative (*J*, arrows) and show silver grains above their nuclei when dividing (*A*, arrows; ^3H -thymidine positive). In cultures containing astrocytes, astrocytes occupy a flat monolayer that is indistinct by phase microscopy (*B, E*); astrocytes are recognizable by their large Hoechst-stained nuclei (*E*, double arrow) and by light immunostaining for GFAP (*K*, double arrow). The morphological appearance of N-SC-ACM cultures under phase-contrast microscopy (*C, F*) resembles that in N-SC cultures (*A, D*). Note the decrease in the number of ^3H -thymidine-positive cells in N-SC-A (*B*) cultures as compared to N-SC (*A*) cultures. Scale bar, 50 μm .

Table 3. Inhibition of myelination by astrocytes and O-ACM

Condition	Amount of myelin per culture	Inhibition (%)
Control	203 ± 22	
Astrocytes	62 ± 36*	69.4
O-ACM	54 ± 10*	73.4

Myelin segments were counted on Sudan black-stained whole-mounted cultures 2 weeks after switching to M medium (see Materials and Methods). O-ACM supplemented with FBS, NGF, and L-ascorbic acid to the final concentrations of M medium was used, diluted 1:1 (v/v) with fresh M medium. Values shown are means and SEM of determinations on duplicate or triplicate cultures in each group. One-way ANOVA followed by Tukey posttest comparisons was used to test significance between pairs of means.

* Statistical significance ($p < 0.05$) between control and astrocyte or ACM groups.

ing astrocyte-conditioned medium, the inhibitory effect of the astrocyte factor(s) increased significantly (Table 3).

An assessment of myelination in cultures maintained for 4 weeks indicated that the inhibition of myelination induced by astrocytes was slightly reduced as compared to 2 weeks (Table 2); this decrease in inhibition with time was not, however, statistically significant. Furthermore, maintaining the cultures for 4 weeks did not reduce the inhibition of myelination produced by the factor(s) present in astrocyte-conditioned medium (Table 2). Thus, the inhibitory influence of astrocytes and their conditioned medium did not decrease with time. Segments of degrading myelin were very rarely seen. In a single experiment, we determined if astrocytes and astrocyte-conditioned medium would induce myelin degradation in already myelinated N-SC cultures. Two weeks after adding astrocytes or starting astrocyte-conditioned medium treatment, the amount of degrading myelin, seen as short rows of Sudan black-stained droplets, was comparable in N-SC-A, N-SC-ACM, and N-SC cultures (data not shown). Thus, we conclude that astrocytes or astrocyte-produced factors do not cause myelin breakdown.

Astrocytes inhibit O1 expression

To evaluate at what stage of Schwann cell differentiation astrocytes exerted their effects, N-SC, N-SC-A, and N-SC-ACM cultures were immunostained for the differentiation molecules O4 and O1 6 and 8 d after switching to M medium. Within 6 d Schwann cells expressed O4 in all culture groups, whereas O1-positive Schwann cells were observed only in the control N-SC cultures, mostly in the outer part of the cultures (data not shown). After 8 d, the intensity of the O4 staining had increased and was similar in all cultures although the labeling was slightly patchy in the presence of astrocytes (Fig. 3*A,C,E*). The number of O1-positive Schwann cells increased in the control cultures (Fig. 3*B*), whereas relatively fewer O1-positive Schwann cells were present in the N-SC-A (Fig. 3*D*) and N-SC-ACM (Fig. 3*F*) cultures. N-SC-A cultures showed the fewest O1-positive Schwann cells. Quantitative analysis at day 8 (data not shown) indicated that astrocytes and astrocyte-conditioned medium inhibited the expression of O1 by 99.1% and 62.7%, respectively. The length of the myelin segments was also shorter in N-SC-A than in N-SC cultures (Fig. 3*B,D*). Astrocytes thus appear to inhibit the expression of the O1 antigen in Schwann cells; this suggests that astrocytes interfere with the differentiation of the Schwann cells at a step prior to the onset of myelination.

Figure 2. Myelination in N-SC (*A*), N-SC-A (*B*), and N-SC-ACM (*C*) cultures: Sudan black-stained areas of myelination in cultures of comparable organization and density grown in myelinating medium for 2 weeks. Long, thin myelin segments are stained in black (arrows). Note the difference in the number of myelin segments in control cultures (*A*) versus cultures containing astrocytes (*B*) or maintained in medium containing ACM (*C*). The number of myelin sheaths is strikingly reduced in cultures containing astrocytes. Scale bar, 100 μ m.

Figure 3. Expression of the O4 and O1 antigens in N-SC, N-SC-A, and N-SC-ACM cultures 8 d after switching to myelinating medium: fluorescence micrographs of N-SC (*A, B*), N-SC-A (*C, D*), and N-SC-ACM (*E, F*) cultures immunostained for O4 (*A, C, E*) and O1 (*B, D, F*). In all types of cultures, Schwann cells are O4 positive (*A, C, E*), although the staining is slightly patchy in the presence of astrocytes (*C*). Note the apparent decrease in the length of O1-positive Schwann cells in cultures containing astrocytes and the decrease in the number of O1-positive Schwann cells in N-SC-A and N-SC-ACM cultures. Scale bar, 100 μ m.

Astrocytes and astrocyte-conditioned medium do not affect the expression of laminin and type IV collagen

The possibility that astrocytes affected myelination by influencing the deposition by Schwann cells of the basal lamina molecules collagen type IV and laminin was evaluated in N-SC,

N-SC-A, and N-SC-ACM cultures 2 weeks after switching to M medium. There was no difference between culture groups in the intensity and pattern of immunostaining for collagen type IV or laminin. Both nonmyelinating and myelinating Schwann cells (Fig. 4*A, B, E, F*) displayed type IV collagen and laminin on their surface in the presence of astrocytes (Fig. 4*C, D, G, H*). Data are

Figure 4. Comparison of the expression of collagen type IV and laminin in N-SC and N-SC-A cultures. Phase-contrast (*A, E*) and fluorescence (*C, G*) micrographs show immunostaining with anti-collagen type IV (*C*) and anti-laminin (*G*) antibodies of an N-SC culture 2 weeks after switching in myelinating medium. By phase contrast, Schwann cells and myelinated axons (*arrows*) can be observed. In N-SC-A cultures [phase contrast (*B, F*), and fluorescence with anti-collagen type IV (*D*) and anti-laminin (*H*) antibodies], astrocytes underlying the Schwann cells and axons are not visible. By immunofluorescence, type IV collagen (*C, D*) and laminin (*G, H*) form a sheath surrounding the axons. The intensity of the type IV collagen and laminin staining in astrocyte-containing cultures is similar to that observed in controls. Scale bar, 100 μm .

Table 4. Effect of astrocytes on basal lamina formation by Schwann cells

Condition	Number of Schwann cells examined	Schwann cells with basal lamina (%)			
		Complete	Patchy	None	Total
Control	166	58.8	41.2	0	100
Astrocytes	140	17.5*	67.2	15.3	84.7

Electron micrographs were examined to determine the amount of basal lamina deposited on the Schwann cell surface. A Schwann cell was considered to have basal lamina if a patchy or complete basal lamina was seen on the surface of the Schwann cell not in apposition to the axons. One-way ANOVA followed by Tukey posttest comparisons was used to test significance between pairs of means.

* Statistical significance ($p < 0.05$) between control and astrocyte groups.

not shown for the N-SC-ACM cultures. Thus, astrocytes and astrocyte-conditioned medium did not block the expression of two of the major components of Schwann cell basal lamina.

Astrocytes decrease axonal ensheathment, the segregation of large axons into a 1:1 relationship, and basal lamina formation

The effects of astrocytes on the axonal–Schwann cell relationship and basal lamina formation were studied by electron microscopy. Although the expression of type IV collagen and laminin by Schwann cells was not affected by astrocytes, ultrastructural analysis revealed slight differences in the amount of basal lamina deposition around the abaxonal surface of Schwann cells between control and astrocyte-containing cultures. A basal lamina was present around all axon–Schwann cell units in N-SC cultures (Fig. 5*A*, Table 4), being continuous in 58.8% of the units. In contrast, only 84.7% of the units in N-SC-A cultures displayed a basal lamina (Fig. 5*B,C*; Table 4). In 17.5% of the units examined, the basal lamina was complete, whereas in the remaining units the basal lamina was patchy and discontinuous. This slight decrease in basal lamina deposition in the presence of astrocytes is likely to reflect the fact that since less myelin is formed in N-SC-A than in N-SC cultures, basal lamina deposition which is proportional to myelination is decreased.

Differences in the state of axonal ensheathment between N-SC and N-SC-A cultures were striking after 2 weeks in M medium. In control cultures, Schwann cell processes had grown into the axon bundles. Bifurcating processes had subdivided the axonal fascicles, engulfing some of the axons (Fig. 5*A*). Most axons were ensheathed in groups; only 12.1% of them were singly ensheathed (Table 5). In N-SC-A cultures, the invasion of the axonal fascicles by Schwann cell processes was reduced and less advanced than in control cultures; Schwann cells extended a few short processes and occasionally a long process. These long processes were less bifurcated and engulfed larger groups of axons (Fig. 5*B,C*) in astrocyte-containing cultures than in control cultures. Axons with little interaction with Schwann cells were seen “trapped” between neighboring Schwann cells. Single axons surrounded by Schwann cell processes were occasionally observed but represented only 5.9% of the axons, a statistically significant reduction when compared to control cultures (Table 5). The percentage of axons engaged in 1:1 relationships was also significantly decreased in the presence of astrocytes (Table 5). Interestingly, astrocyte processes were rarely seen invading axon bundles. Although astrocytes reduced the extension of Schwann cell processes and thus interactions between axons and

Table 5. Effect of astrocytes on axonal ensheathment and segregation of large axons into 1:1 relationships by Schwann cells

Condition	Number of axons examined	1:1 relationships (%)			Ensheathed axons (%)
		MA	non-MA	Total	
Control	7,607	3.4	1.2	4.6 ^a	12.1
Astrocytes	11,014	0.2*	0.5*	0.7*	2.9*

Electron micrographs were examined for profiles indicating that single axonal ensheathment or a 1:1 relationship had been established. An axon was considered singly ensheathed when at least 50% of its surface was surrounded by Schwann cell cytoplasm that separated it from other axons ensheathed by the same Schwann cell. A 1:1 relationship was considered to be established if a Schwann cell was interacting with a single axon, completely ensheathing it, and if basal lamina was present around the axon–Schwann cell unit. One-way ANOVA followed by Tukey posttest comparisons was used to test significance between pairs of means.

^a 1:1 relationships including nonmyelinated (non-MA) and myelinated (MA) axons are combined.

* Statistical significance ($p < 0.05$) between control and astrocyte groups.

Schwann cells were diminished, the astrocytes did not physically interfere with the contact between Schwann cells and axons (Fig. 5*B,C*). Except in one culture where astrocytes separated axons and Schwann cells into nerve microfascicles, astrocytes always formed a carpet underneath the axons and their associated Schwann cells. This suggests that the effect of astrocytes on Schwann cell function is mediated by soluble factors.

Astrocytes and astrocyte-conditioned medium do not induce neuronal morphological changes

To assess if the effects of astrocytes or astrocyte-conditioned medium on myelination could be due to an indirect harmful influence on the neurons rather than on the Schwann cells themselves, the morphology of the neuronal somata of DRG neurons in neuron, N-A, and N-ACM cultures was examined after maintenance in M medium for 4 weeks. In all groups of cultures, the cell bodies were round in shape, with a centered nucleus (data not shown). There was no significant difference in the size of the cell bodies among the groups ($70.4 \pm 0.7 \mu\text{m}$ in neuronal cultures, $74.1 \pm 1.1 \mu\text{m}$ in N-A cultures, and $64.4 \pm 1.0 \mu\text{m}$ in N-ACM cultures). Thus, neither astrocytes nor astrocyte-conditioned medium induced chromatolysis under the culture conditions tested.

Astrocyte-conditioned medium does not show detectable TGF- β and TNF- α activities

Preliminary experiments (data not shown) to characterize further the astrocyte inhibitory factor(s) indicate that the factor(s) is heat stable; that is, its activity was retained after heating at 56°C or 100°C for 30 min.

To test for TGF- β -like activity, serum-free astrocyte-conditioned medium was heated at 80°C for 8 min, a treatment known to activate latent TGF- β . Heated astrocyte-conditioned medium did not inhibit Mv1Lu cell proliferation as would be expected if TGF- β were present, nor did heating further strengthen the inhibitory effect of the astrocyte factor(s) on Schwann cell proliferation on DRG neurites. Furthermore, the astrocyte inhibitory effects were not blocked by anti-TGF- β 1 antibody. TNF- α -like activity in astrocyte-conditioned medium was evaluated using the WEHI 164 cell survival assay, capable of detecting picogram quantities of TNF- α . No WEHI 164 cell death was observed after treatment with medium generated by astrocytes. Thus, no detectable TGF- β - or TNF- α -like activity was found

Figure 5. Electron micrographs of N-SC (*A*) and N-SC-A (*B*, *C*) cultures 2 weeks after switching to myelinating medium. In control cultures (*A*) most axons are ensheathed (*star*) or segregated into myelinated 1:1 relationships (*double asterisks*). Myelin has formed and most Schwann cell (SC)–axon units are surrounded by a continuous basal lamina. In the presence of astrocytes (*B*), axon–Schwann cell interactions are more limited (*arrowheads*); ensheathment (*star*) and 1:1 relationships (*asterisk*) are decreased. Astrocyte processes (*As*) are mostly localized on the side of the culture facing the substrate (*upper side of B*). At a higher magnification (*C*), axons neighboring astrocyte processes (*As*) are only partially surrounded by Schwann cell processes (*arrowheads*). Scale bars: *A* and *B*, 5 μ m; *C*, 0.5 μ m.

in the astrocyte-conditioned medium generated under our tissue culture conditions.

Discussion

The present study shows that cortical astrocytes inhibit Schwann cell proliferation and differentiation *in vitro*. Under the tissue culture conditions tested, Schwann cell division induced by DRG neurons and the formation of myelin sheaths were both inhibited by astrocytes. Furthermore, the expression of galactocerebroside, one of the earliest events in the myelination process (Wood et al., 1990), was inhibited by astrocytes. The segregation of large axons by Schwann cell processes into 1:1 relationships that precede myelination was also inhibited by astrocytes and basal lamina deposition was slightly decreased. These results indicate that astrocytes interfere with axon–Schwann cell interactions that are critical in the differentiation of Schwann cells into myelin-forming cells.

Soluble factors released by cortical astrocytes are partially responsible for the inhibition of Schwann cell proliferation and myelination. For instance, Schwann cell division was decreased, and O1 expression and myelination were inhibited by a factor(s) present in astrocyte-conditioned medium. Astrocytes release numerous factors that could affect Schwann cell function, including TGF- β and TNF- α (Lieberman et al., 1989; Constam et al., 1992). Under our experimental conditions, astrocyte-conditioned medium did not contain demonstrable TGF- β – or TNF- α –like activity.

The mechanisms by which astrocytes inhibit Schwann cell proliferation are unclear. Axonal surface molecules are known to stimulate Schwann cell proliferation (Wood and Bunge, 1975; Salzer et al., 1980). A factor(s) released by astrocytes could have interfered with the interaction between the axonal mitogen and its receptor on the Schwann cell surface. The possibility that Schwann cell division was blocked because of a lack of initial association or adhesion with neurites is unlikely since Schwann cells were seeded on the neuronal cultures 1 d prior to adding the astrocytes or their conditioned medium, allowing the establishment of Schwann cell–neuronal contact. An alternative possibility is that the astrocyte factor blocks some step in the intracellular signaling pathway that mediates the mitogenic response. Decrease in Schwann cell proliferation has also been noticed at demyelinated CNS sites as Schwann cells migrate into lesion sites (Blakemore, 1975; Raine et al., 1978; Gilmore et al., 1982; Sims and Gilmore, 1983, 1989; Harrison, 1985; Dusart et al., 1989). Sims and Gilmore (1989) attributed this decrease in Schwann cell proliferation to an association with large-caliber demyelinated axons that caused the invading Schwann cells to stop dividing and prematurely differentiate. These studies did not consider the possibility that other CNS cellular and/or humoral elements could have affected Schwann cell proliferation. Our data support the latter hypothesis and indicate that the decrease in Schwann cell proliferation seen in *in vivo* studies could also be due to proliferation inhibitory factors released by astrocytes present near or within CNS lesions.

The mechanisms by which cortical astrocytes, or soluble factors released by astrocytes, interfere with myelination are also unclear. The insertion of Schwann cell processes into axonal fascicles, the engulfment of axons, and segregation of axons into 1:1 relationships with Schwann cell processes are decreased in the presence of astrocytes. Since these steps must occur before myelination can begin, our observations suggest that Schwann cell differentiation is inhibited by astrocytes at a stage prior to

the initiation of myelination. This hypothesis is supported by the observation that in the presence of astrocytes the expression of O1, but not that of O4, was inhibited. Furthermore, the expression of the ECM molecules laminin and collagen type IV by Schwann cells was not influenced by astrocytes. Basal lamina formation, an event linked to axonal engulfment, segregation, and a prerequisite in the formation of myelin (Eldridge et al., 1987), was only slightly decreased. We do not believe that the decrease in basal lamina formation in the presence of astrocytes explains the inhibition of myelination. Adding laminin to myelinating medium (Eldridge et al., 1989) increased both axonal ensheathment and basal lamina deposition but did not improve myelination in the presence of astrocytes (V. Guenard and P. M. Wood, unpublished observation). Astrocytes thus seem to interfere with the normal course of axon–Schwann cell interactions that lead to full Schwann cell maturation.

This pattern of inhibition of Schwann cell differentiation resembles that observed in the presence of antibodies to the neural cell adhesion molecule (CAM) L1 (Wood et al., 1990). Recent observations suggest that CAM-mediated events may involve the induction of gene expression in the responsive cell (Schuch et al., 1989). An explanation for the inhibition of Schwann cell differentiation by cortical astrocytes could be that they release factors that block CAM-mediated shape changes necessary for Schwann cell process extension. Alternatively, the astrocyte factor could block intracellular pathways of gene induction triggered by Schwann cell receptor binding to axonal or basal lamina ligands. For example, the expression of the gene coding for myelin-associated glycoprotein, which is believed to be involved in the segregation of axons (Owens et al., 1990), could be blocked by the astrocyte factor. Astrocytes of similar origin have been demonstrated to release a soluble factor(s) with similar effects on oligodendrocytes associated with DRG neurites in culture, decreasing both their proliferation and ability to myelinate (Rosen et al., 1989). Interestingly, anti-L1 antibody also blocks oligodendrocyte myelination of sensory axons in culture (J. Archelos and P. M. Wood, unpublished observations). The fact that oligodendrocyte myelination is blocked suggests that the astrocyte factor inhibits interactions specifically involved in differentiation toward myelination.

The fact that the observed effects of cortical astrocytes on Schwann cell proliferation or myelination are on specific Schwann cell functions and are not due to generalized cytotoxicity is demonstrated by several of our observations. First, the astrocyte inhibitory factor(s) did not affect the expression of O4, one of the earliest stages of Schwann cell differentiation, nor did it influence the expression of laminin and collagen type IV, two of the major components of basal lamina involved in myelinating processes. In addition, preexisting myelin was only slightly degraded, if at all, by astrocytes and astrocyte-conditioned medium. The findings that the observed effects represent a cell-specific property of astrocytes was shown by comparing the influence of fibroblasts and their released factor(s) using similar tissue culture conditions. Fibroblasts were chosen for this comparison because they exhibit a pattern of growth much like that of astrocytes in culture. Both fibroblasts and fibroblast-conditioned medium failed to inhibit Schwann cell proliferation. Furthermore, indirect harmful effects of astrocytes and their released factor(s) on neuronal health or physical blockage of Schwann cell–axonal interactions by astrocyte processes cannot be responsible for the astrocyte effects. No evidence of chromatolytic or axonal morphological changes was observed in our

cultures, and at the ultrastructural level, all cultures but one showed astrocytes forming a carpet underneath the neurons and Schwann cells; astrocyte processes did not appear to intermingle directly with Schwann cell processes and neurites. Thus, the astrocyte effects are not due to physical interference with Schwann cell process extension or to physical blocking by astrocyte processes of access of Schwann cell process to axonal ligands.

Our finding that astrocytes inhibit Schwann cell myelination *in vitro* correlates with the reported evidence that reactive (i.e., GFAP-positive) astrocytes limit Schwann cell remyelination *in vivo* in the demyelinated or damaged CNS (Ghatak et al., 1973; Itoyama et al., 1983, 1985). Astrocytes also seem to limit host Schwann cell penetration in the cord (Blakemore, 1975; Sims and Gilmore, 1983; Harrison, 1985). In their transplantation studies using CNS-derived glial cell cultures, Blakemore and Crang (1989) demonstrated that the extent of remyelination by endogenous Schwann cells depends on the presence of type 1 astrocytes in the injected cell suspension. Schwann cell remyelination exceeded on oligodendrocyte remyelination only when cultures deficient in type 1 astrocytes were transplanted. Baron-Van Evercooren et al. (1992) reported that Schwann cells transplanted into the brain of newborn shiverer mice are excluded by astrocytes in areas containing endogenous oligodendrocytes; astrocytes limited Schwann cell migration and prevented the formation of myelin by the grafted Schwann cells. Sims and Gilmore (1983) and Blakemore et al. (1986) also noted that more CNS fibers are myelinated by Schwann cells in areas containing few astrocytes. Recent studies completed in our laboratory have shown that cortical astrocytes transplanted together with purified populations of Schwann cells in semipermeable guidance channels (Guénard et al., 1992) decrease the ability of Schwann cells both to enhance peripheral nerve regeneration and form myelin (Guénard et al., 1994). These results further strengthen the interpretation that the results of the present study may be extrapolated to the *in vivo* situation. The possibility that Schwann cells decrease the astrocytic reaction observed by others also exists but has not yet been elucidated.

Even though these results apply to astrocytes obtained from cortical tissue, there is no current evidence that astrocytes isolated from other areas of the brain or the spinal cord would behave differently in this system.

We conclude that purified cortical astrocytes inhibit both the neuron-dependent proliferation of Schwann cells and myelination of axons by Schwann cells *in vitro*, some of these effects being mediated by a soluble factor(s) secreted by astrocytes. These findings should be considered prior to designing strategies based on Schwann cell transplantation to repair the injured or demyelinated CNS, a method with future clinical application (Bunge, 1975). Decreasing the extent of the astrocytic reaction and/or inhibiting the activity of astrocyte inhibitory factor(s) may improve the effectiveness of the Schwann cell transplants. Characterization of the astrocyte inhibitory factor(s) is thus important and is currently underway. It is our hope that these studies will lead to an improved understanding of some of the cellular responses observed following demyelination.

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