

Schwann Cell Proliferation In Vitro Is under Negative Autocrine Control

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Abstract. In healthy adult peripheral nerve, Schwann cells are believed to be generally quiescent. Similarly, cultures of isolated rat sciatic nerve Schwann cells hardly proliferate in serum-supplemented medium. The possibility that Schwann cells negatively regulate their own proliferation was supported by the demonstration that conditioned media from Schwann cell cultures inhibited the proliferation of mitogen-stimulated test cultures. The inhibition could be complete, was dose dependent, and was exhibited when the test Schwann cells were under the influence of different types of mitogens such as cholera toxin, laminin, and living neurons. The inhibition of proliferation was completely reversible and a rapid doubling of cell number resulted when treatment with conditioned medium was withdrawn from mitogen-stimulated Schwann cells. Conditioned medium from cholera toxin-stimulated and immortalized Schwann cell cultures contained less antiproliferative activity than that found in medium from quiescent Schwann cell cultures. However, media conditioned by two actively proliferating rat Schwannoma cell lines were rich

sources of antiproliferative activity for Schwann cells. Unlike the mitogen-stimulated Schwann cells, whose proliferation could be inhibited completely, the immortalized and transformed Schwann cell types were nearly unresponsive to the antiproliferative activity. The antiproliferative activity in Schwann and Schwannoma cell conditioned media was submitted to gel filtration and SDS-PAGE. The activity exists in at least two distinct forms: (a) a high molecular weight complex with an apparent molecular mass >1,000 kD, and (b) a lower molecular weight form having a molecular mass of 55 kD. The active 55-kD form could be derived from the high molecular weight form by gel filtration performed under dissociating conditions. The 55-kD form was further purified to electrophoretic homogeneity. These results suggest that Schwann cells produce an autocrine factor, which we designate as a "neural antiproliferative protein," which completely inhibits the in vitro proliferation of Schwann cells but not that of immortalized Schwann cells or Schwannoma lines.

IN mammalian peripheral nerve Schwann cell (SC)¹ proliferation is highly regulated in that it is observed essentially only during development and after nerve injury. During development, SCs recognize and adhere to axons and then are stimulated to proliferate and eventually populate the entire length of the axon (Webster and Favilla, 1984). In healthy adult peripheral nerve SCs generally do not proliferate, but they can reenter the mitotic cycle during Wallerian degeneration induced by nerve trauma. These same SC behaviors can be reproduced in vitro in that SCs contact and adhere to neuronal processes and then proliferate in response to a neuritic mitogenic signal (Salzer and Bunge, 1980). In mature nerve and in established cocultures SCs eventually cease to proliferate even in the continued presence of neurons. Interestingly, axonal fragments obtained from adult nervous tissue and neuronal cultures are potent mitogens for SCs in vitro (Salzer et al., 1980; DeVries et al., 1983).

1. *Abbreviations used in this paper:* APU, antiproliferative units; BrdU, bromodeoxyuridine; CM, conditioned medium; NAP, neural antiproliferative protein; SC, Schwann cell.

Unlike most other cell types, isolated rodent SCs proliferate very slowly under standard in vitro conditions (Wood, 1976; Manthorpe et al., 1980; Muir et al., 1989a). In addition to the neuritic mitogen, a limited array of soluble agents stimulate the proliferation of cultured SCs (for review see Ratner et al., 1986), such as glial growth factor (Brockes et al., 1979), glial maturation factor (Bosch et al., 1984), transforming growth factor- β (Ridley et al., 1989), laminin, and fibronectin (McGarvey et al., 1984), as well as agents such as cholera toxin and dibutyryl cAMP that raise intracellular cAMP levels (Brockes et al., 1979). In addition, several autocrine mitogens have been postulated for SCs including the SC basal lamina component laminin (McGarvey et al., 1984; Dziadek et al., 1986), glial maturation factor (Bosch et al., 1984; Lim et al., 1988), transforming growth factor- β (Ridley et al., 1989), and an activity reported in medium conditioned by primary and immortalized SCs (Porter et al., 1987). Thus, even though cultured SCs can produce a number of potential autocrine mitogens, they remain virtually quiescent even in high cell density, long-term cultures.

Transplacental induction of rat tumors with the carcinogen

ethylnitrosourea has yielded several Schwannoma cell lines, and the RN22 (Pfeiffer and Wechsler, 1972) and D6P2T (Bansal and Pfeiffer, 1987) clonal lines have been used extensively for the study of SC biology. Transformed SCs expressing the oncogenes SV-40 large T and v-Ha-ras (Ridley et al., 1988) can proliferate in the absence of added mitogens. In addition, Porter and co-workers (1987) describe an immortalization of rat sciatic nerve SCs without the use of viral infection, oncogene transformation, or chemical carcinogens. They found that primary SC cultures, expanded continuously with a combination of glial growth factor and forskolin, eventually yielded a population of cells that continue to proliferate in the absence of exogenous mitogens. Similarly, we have generated by long-term exposure to cholera toxin an immortalized line of rat sciatic nerve SCs that continuously divide (unpublished observation). All of the above SC lines provide *in vitro* models for studies of the regulation of SC proliferation.

The mechanisms underlying the cessation of SC proliferation in the developing nerve and *in vitro* are obscure. A virtually unexplored possibility is that SC proliferation is regulated by inhibitory signals. Here we provide evidence that SC proliferation *in vitro* is under negative autocrine regulation. We developed and used an *in vitro* microplate assay of bromodeoxyuridine (BrdU) incorporation into DNA to quantify and monitor this antiproliferative activity after various treatments and during fractionations. We have isolated from conditioned media (CM) a 55-kD protein, which we have termed neural antiproliferative protein or NAP, that inhibits DNA synthesis by mitogen-stimulated SCs.

Materials and Methods

Cell Culture

Purified populations of quiescent SCs were obtained from dissociated neonatal rat sciatic nerves as previously described (Muir et al., 1989a). Dense (but subconfluent) SC cultures ($3-4 \times 10^6$ cells/100-mm dish) were grown in Dulbecco's modification of Eagle's medium supplemented with penicillin and L-glutamine (1 mM) (DME) containing 2 or 10% calf serum, and CM was collected every 2 d. Loss of normal growth control (immortalization) of secondary SCs was achieved by continuous treatment with 20 ng/ml cholera toxin (Sigma Chemical Co., St. Louis, MO) and passaging 1:6 every 4 d for 2-3 mo. The immortalized SCs were further passaged 1:10 every 5 d for 1-2 mo without cholera toxin. RN22 (Pfeiffer and Wechsler, 1972) and D6P2T Schwannoma cells (Bansal and Pfeiffer, 1987) were cultured in DME containing 10% calf serum. For the collection of serum-free RN22 CM, dense ($3-4 \times 10^6$ cells/100-mm dish) cultures were rinsed twice with PBS to remove serum proteins and the cells were grown in serum-free DME for 2 d before medium collection. Serum-containing and serum-free CM were also collected from rat brain astroglia (prepared according to Rudge et al., 1985), oligodendroglia (McCarthy and de Vellis, 1980), sciatic nerve, and mouse 3T3 fibroblasts. Cells were determined to be free of mycoplasma.

SC Proliferation Assay and Immunostaining

Quiescent SC microcultures were established in polyornithine-treated, 96-well plates containing 14,000 cells/6-mm well in 100 μ l of DME + 10% calf serum as described (Muir et al., 1989a). For some assays SCs were seeded in polyornithine-coated wells treated with 50 μ l of a 2 μ g/ml solution of rat L2 yolk sac tumor laminin (prepared as described by Engvall et al., 1983). SC/neuron cocultures were established by adding 14,000 SCs and 2,000 embryonic day 8 ciliary ganglionic motor neurons to each microwell as described (Muir et al., 1989a). For routine assays of antiproliferative activity on mitogen-stimulated SCs, microcultures were seeded in DME + 10% calf serum containing 20 ng/ml cholera toxin. Serially diluted test samples were presented for 72 h and BrdU (Sigma Chemical Co.) was added

to a concentration of 1 μ M during the final 24 h. SC proliferation was assessed by direct cell counting and BrdU incorporation into DNA was measured by an ELISA performed on fixed monolayer microcultures as previously described (Muir et al., 1990). Briefly, after BrdU incorporation the cells were fixed by 70% ethanol and the DNA was denatured by incubation with 2 M HCl for 10 min at 37°C. BrdU-DNA was labeled using monoclonal anti-BrdU antibody (50 μ l/well, 1 μ g/ml; Dako-Patts Corp., Santa Barbara, CA) and bound antibody was detected by peroxidase-conjugated rabbit anti-mouse IgG (50 μ l/well, 2 μ g/ml; Dako-Patts Corp.). The colorimetric substrates *o*-phenylenediamine (0.05%) and H₂O₂ (0.02%) were added in 50 mM phosphate/citrate buffer at pH 5 and the reaction was terminated after 5-20 min by the addition of sulfuric acid. The absorbance was measured at 490 nm by a microplate reader (model MR600; Dynatech Laboratories, Inc., Alexandria, VA) interfaced with a computer. Using cholera toxin-stimulated SCs, the titer of each sample in antiproliferative units (APU) per milliliter was expressed as the sample dilution required to inhibit by 50% the maximal incorporation of BrdU into DNA (BrdU-DNA immunoreactivity). The percentage of cells with BrdU-DNA was determined by immunostaining essentially as described above in the ELISA except that the insoluble chromagen diaminobenzidine-tetrahydrochloride was used. Proliferation assays using immortalized SC and rat Schwannoma cell lines were performed as described above for SCs except that no mitogens were added to the serum-supplemented medium.

Fractionation of CM

Medium conditioned by SCs or RN22 Schwannoma cells was filtered (0.2- μ m pore). The CM was concentrated by ultrafiltration using a 10-kD cut-off filter (model YM10; Amicon Corp., Danvers, MA) and then dialyzed with PBS. The CM concentrate was fractionated by CL4B (Pharmacia Fine Chemicals, Piscataway, NJ) gel filtration (2.5 \times 96-cm column) in PBS. The fractions were assayed for antiproliferative activity on SCs as described above. Active fractions were later concentrated using centricon filtration (10-kD cut-off; Amicon Corp.). An active high molecular weight fraction was made 4 M with urea and refractionated on the CL4B column as described above.

To purify and characterize a lower molecular mass form (55 kD) of the antiproliferative activity, CM was fractionated by ion-exchange chromatography using a DEAE-cellulose (Whatman Inc., Hillsboro, OR) column (2 \times 10 cm) equilibrated with 150 mM NaCl/10 mM sodium phosphate buffer, pH 8.0. 500 ml CM was applied to the column and the bound material was eluted by a linear NaCl gradient (0.15-1.0 M). The active nonbinding effluent was filtered through a 100-kD filter (YM100; Amicon Corp.) and then the filtrate was concentrated by a YM10 filter and dialyzed with PBS. The concentrate was further fractionated by CL4B (Pharmacia Fine Chemicals) gel filtration (2.5 \times 96 cm) in PBS. Molecular weight estimation was also performed using an S200 (superfine; Pharmacia Fine Chemicals) gel filtration column (1.0 \times 150 cm) equilibrated with PBS.

SDS-PAGE

SDS-PAGE was carried out according to the procedure of Laemmli (1970), using 12% acrylamide gels under nonreducing conditions. CM-derived active fractions were electrophoresed at 4°C and then the gel was washed thoroughly with 2.5% Triton X-100 to remove SDS and with PBS to remove the Triton. The gel was then cut horizontally into 2.5-mm slices and the slices were minced. The proteins were eluted by diffusion into PBS, the pieces were removed by centrifugation, and the supernatants were dialyzed with PBS and then assayed for antiproliferative activity. The active samples were rerun on SDS gels and then electroblotted to nitrocellulose sheets according to the methods of Towbin et al. (1979). The sheets were stained for protein using colloidal gold (Aurodyne forte; Janssen Life Sciences Products, Piscataway, NJ).

Metabolic Labeling with [³⁵S]Methionine

Protein synthesis by SCs *in vitro* was quantified by the incorporation of radiolabeled amino acids. Quiescent SCs were cultured in DME (98% methionine-free DME + 2% DME) containing 10% dialyzed calf serum and 10 μ Ci/ml Trans³⁵S-label (ICN Biochemicals, Inc., Irvine, CA) in the absence and presence of CM or fractions containing antiproliferative activity. After 72 h the cultures were washed several times with Hank's balanced salt solution, hydrolyzed with 0.2 N NaOH at 37°C for 30 min, and then mixed with Liquiscint (National Diagnostics, Inc., Somerville, NJ) and counted.

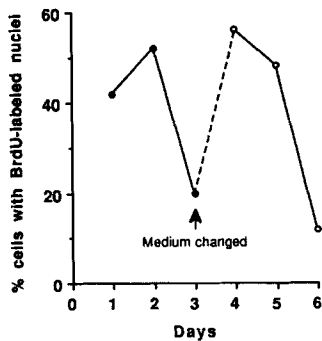


Figure 1. Schwann cell proliferation in response to cholera toxin and medium changes. SC cultures (20,000 cells/cm²) were treated for 3 d with 20 ng/ml cholera toxin in DME containing 10% calf serum without changing the medium and then the medium was removed and the cultures were grown for the next 3 d in 1 part DME containing 10% calf serum and 3 parts saline. Cell proliferation was examined in

replicate cultures by the addition of 1 μ M BrdU to the culture medium for 24-h periods throughout the 6-d experiment. After each 24-h period the assay was terminated by ethanol fixation and the cells were immunostained for BrdU incorporation into DNA as described in Materials and Methods. Data represent the mean of four determinations (50 cells each) per dish and each condition was duplicated in each of two separate experiments. (SD < 8%).

Results

Growth Properties of Neonatal Rat Sciatic Nerve SCs

To generate large numbers of cells, we typically expand the primary SCs in the presence of cholera toxin as mitogen (Muir et al., 1989a). We noticed during such cell expansions that the SC number per culture and BrdU incorporation appeared to increase mostly within the first day after each medium change. By 3–4 d after each medium change the cell number stabilized and DNA synthesis decreased until the next medium change caused another rapid burst of proliferation. The decreased rate of proliferation could be due to depletion of mitogen (i.e., cholera toxin) or depletion of medium nutrients (e.g., vitamins, amino acids, etc.), or to the accumulation within the culture medium of substances that inhibit SC proliferation. Similarly, restoring the mitogen and medium or removing CM could result in the burst of proliferation. To address these possibilities we monitored BrdU incorporation over 1-d intervals after selected medium changes. The results are shown in Fig. 1. After treatment with medium containing cholera toxin mitogen the percent of BrdU-labeled cells rose to ~50% but then declined by day 3 to 20% (Fig. 1, closed circles). When the medium was changed either to medium with cholera toxin or to fresh medium alone (data not shown), or even to medium alone diluted to 25% in saline (Fig. 1, open circles), cell proliferation rose again to the 50% level before declining to the 20% level. This propensity of mitogen-stimulated SCs to resume proliferation even in a mitogen-free, nutrient-diluted media suggested that these cells remained under the mitogenic influence of previously applied cholera toxin and under the supportive influence of the media supplement. These data also support the notion that antiproliferative substances may accumulate within SC cultures and that treatments decreasing such an accumulation (i.e., medium changes) will once again permit the cells to respond to mitogens.

SC Response to Mitogens and Inhibition by SC CM

To detect and measure the activity of putative antiprolifera-

tive substances, we used a modification of our ELISA-immunostaining method for quantifying BrdU incorporation (Muir et al., 1990). Replicate SC microcultures were set up in the presence of three distinct SC mitogens, each of which we previously showed to elicit different maximal levels of BrdU incorporation into DNA (Muir et al., 1989a). Compared with untreated SCs, BrdU immunoactivity was increased 35-fold by cholera toxin (20 ng/ml), 2-fold by substratum-bound laminin (20 μ g/ml), and 16-fold by chick ciliary neurons (2,000 cells/well). Each of these mitogen-stimulated SC microcultures was then treated with serial dilutions of CM collected for 2–4 d from dense, virtually quiescent SC cultures. BrdU incorporation into DNA was measured and expressed as the percent of BrdU-DNA immunoactivity achieved by SCs stimulated with a maximal dose of the corresponding mitogen alone. The results are shown in Fig. 2 A. SC CM appeared to possess about the same inhibitory titer (10 APU/ml) against all three mitogens and 90% inhibition was obtained with 50% SC CM. Virtually complete inhibition of BrdU incorporation could be obtained using 10-fold concentrated SC CM at a 10-fold dilution (not shown). Similar results were obtained using SC CM dialyzed with culture medium, indicating that the antiproliferative activity was not due to low molecular weight toxins or medium depletion effects. To test for antiproliferative activity in CM and during CM fractionations we routinely used cholera toxin-stimulated SC microcultures and quantified BrdU incorporation by ELISA. Results from these assays were confirmed by immunostaining the treated microcultures for BrdU and counting the percentage of labeled nuclei by microscopy. In the presence of cholera toxin ~40% of the cells had BrdU-labeled nuclei (Fig. 2 B), and treatment of cholera toxin-stimulated cultures with 50% SC CM reduced this labeling to <4% (Fig. 2 C). SC CM similarly inhibited SC proliferation in response to other mitogens such as dibutyryl cAMP, fibronectin, rat brain axolemmal fragments (DeVries et al., 1983), and peripheral neuron extracts (Salzer et al., 1980a) (results not shown). At very high CM dilutions (1:60–1:120) the extent of BrdU immunoactivity slightly exceeded the maximal BrdU immunoactivity achieved by mitogen alone, suggesting that SC CM might possess an additive mitogenic activity that is masked by the inhibitory activity.

Next we used the BrdU assay to confirm our earlier observation (cf. Fig. 1) that the SC CM-induced inhibition of SC proliferation is reversible. The mitogenic effects of cholera toxin, laminin, and neurons toward untreated or CM-treated SCs over a 4-d period were first examined. The results are shown in Fig. 3 (solid circles). Both cholera toxin (Fig. 3 A) and laminin (Fig. 3 B) increased SC DNA synthesis over the first 2 d, followed by a gradual decline. Neurons increased SC DNA synthesis to reach a maximum by the second day, after which DNA synthesis sharply declined (Fig. 3 C). In the presence of each mitogen, continuous CM treatment suppressed most SC DNA synthesis over the 4-d test period (Fig. 3, solid triangles). To examine the reversibility of the inhibitory activity, SC CM treatment of mitogen-stimulated cultures was discontinued on day 2 and BrdU incorporation was assayed at 24-h intervals thereafter. The data are shown in Fig. 3 (open triangles). A near complete recovery of SC proliferation was observed within 48 h of withdrawal of CM for SCs stimulated by laminin (Fig. 3 B) and within 24 h for those stimulated by neurons (Fig. 3 C) or cholera toxin (Fig.

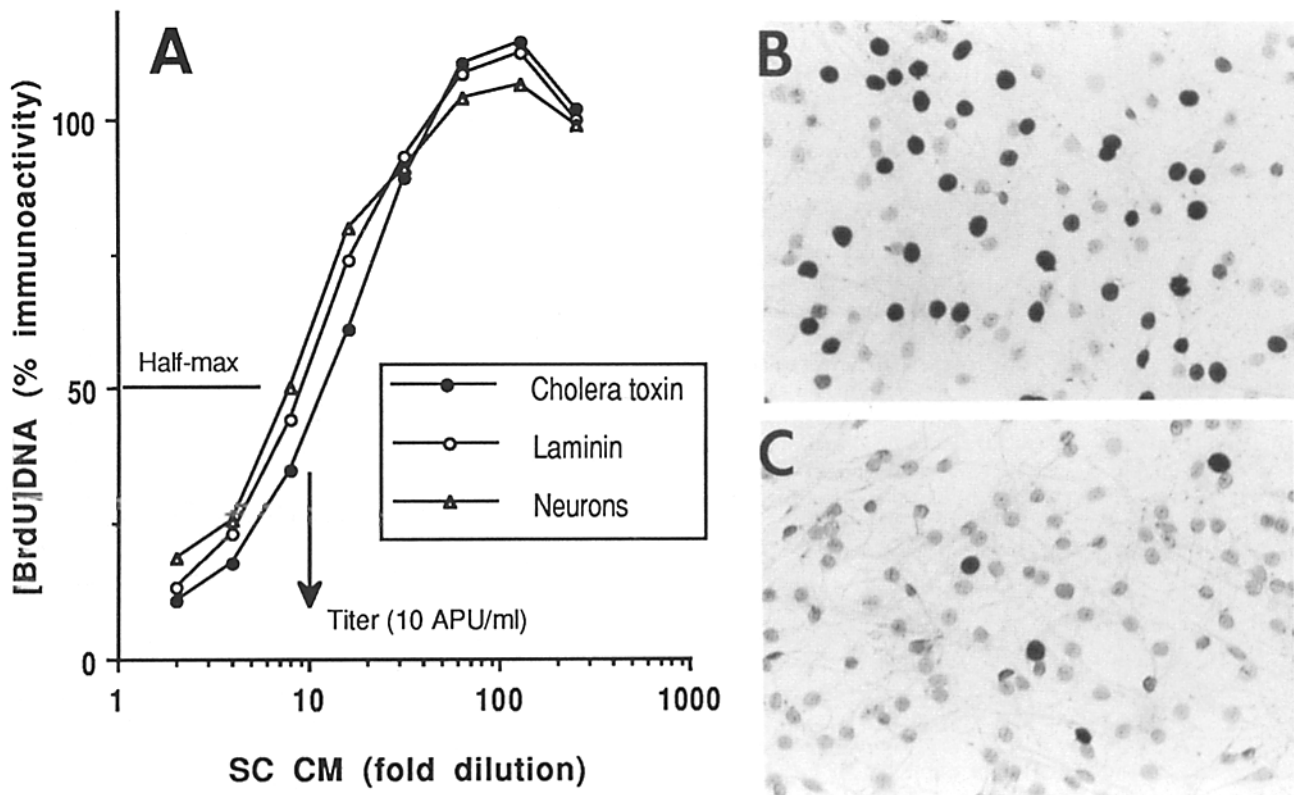


Figure 2. Schwann cell response to mitogens and inhibition by Schwann cell CM. Subconfluent microcultures of rat sciatic nerve SCs (14,000 cells/6-mm-diam well) were grown with 100 μ l DME containing 10% calf serum with one of the following mitogens: soluble cholera toxin (20 ng/ml); polyornithine-treated wells coated with rat laminin (50 μ l/well, 2 μ g/ml), or coculture with ciliary ganglionic neurons (2,000 neurons/well). These mitogen-stimulated SCs were treated for 72 h with serial dilutions of CM from dense SC cultures. SC proliferation was assessed by addition of 1 μ M BrdU to the media during the final 24 h and immunoassay of BrdU incorporation into DNA (A) and immunostaining of BrdU-labeled nuclei (B, C) were performed as described in Materials and Methods. Values for each condition in A were expressed as the percentage of mitogen-stimulated [BrdU]DNA immunoactivity for the corresponding mitogen without SC CM treatment. For cholera toxin-stimulated cultures (B) and cholera toxin-stimulated cultures treated with 50% SC CM (C), BrdU-labeled nuclei were immunostained using diaminobenzidine-tetrahydrochloride and unlabeled nuclei were lightly counterstained with toluidine blue. Data represent the means of quadruplicate determinations from four separate experiments. Cholera toxin and laminin, SD < 6%; neurons, SD < 16%).

3 A). For laminin-stimulated SCs, the kinetics of recovery after withdrawal of the antiproliferative activity appeared similar to the initial BrdU incorporation into DNA in response to laminin. However, cholera toxin- and neuron-treated SCs showed a more rapid burst of DNA synthesis (Fig. 3; compare plots of solid circles with those of open triangles).

Inhibition of cell proliferation might result from direct inhibition or downregulation of DNA synthesis, but may also result from various metabolic impairments that indirectly hinder mitotic activity. In vitro culture medium may accumulate cellular excretions, which then become concentrated during fractionations. Exclusion of low molecular weight metabolites from antiproliferative samples by ultrafiltration, dialysis, or gel filtrations did not diminish the antiproliferative activity (not shown). To examine whether the antiproliferative activity impaired cellular metabolism, SCs and SCs treated with antiproliferative CM and fractions (see below) were cultured for 3 d (the duration of the proliferation assays) in the presence of radiolabeled methionine. If anything, cellular [35 S]methionine incorporation was actually increased slightly (to 105–120% of untreated

controls) by treatment with the antiproliferative activity. Thus we conclude that the antiproliferative factor is not cytotoxic.

Comparison of Different Sources of Antiproliferative Activity

To survey the distribution of antiproliferative activity and to choose a plentiful source for its further characterization, we measured the amount of antiproliferative activity toward SC in selected cell-conditioned media. Different source cultures were set up under identical conditions (culture vessel size, media, volume, cell density) and culture media were conditioned for 2 d before being assayed for antiproliferative activity toward test SC microcultures. The results are shown in Table I. Antiproliferative activity was detectable in all of the SC-related CM (primary, immortalized, transformed cells) but was absent in CM from central glial cells (astroglia, oligodendroglia), brain meningeal, 3T3, or sciatic nerve fibroblast cells. We chose the rat RN22 Schwannoma cells to be our preferred source of antiproliferative activity since these cells (a) produce a reasonably high amount of activity

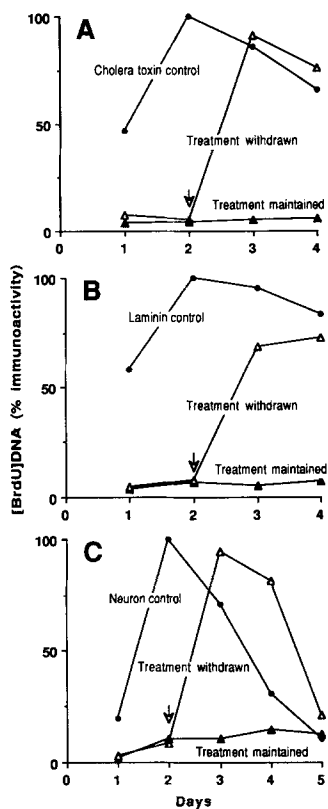


Figure 3. Reversibility of the antiproliferative activity. SC microcultures were treated with cholera toxin (A), laminin (B), or ciliary neurons (C), as described in Fig. 2. BrdU incorporation in response to each mitogen was examined over a 4- or 5-d period (solid circles). A second set of mitogen-stimulated SC cultures was treated with SC CM (a 1:10 dilution of SC CM that was concentrated 10-fold and dialyzed) from the onset of the assays (closed triangles). A third set of replicate cultures was treated with SC CM (as above) from the onset of the assays except that at 2 d the treatment medium was removed and replaced with fresh, unconditioned medium (open triangles). BrdU was added during the final 24 h of each test interval (days 0-1, 0-2, 0-3, and 0-4, and for neurons 0-5) and the assays were terminated by ethanol fixation. [BrdU]DNA was measured by ELISA and immunoreactivity was expressed

as a percent of the maximum value obtained for each mitogen. Data represent the means of two experiments each having quadruplicate determinations. A and B, SD < 8%; C, SD < 14%.

per cell; (b) are more readily available due to a faster rate of doubling; and (c) can be cultured for an extended period in serum-free medium, allowing the activity to be more highly concentrated by ultrafiltration and relatively low in serum contaminants. Most of the following studies were carried out using serum-free RN22 CM. However, certain key experiments performed on the RN22 serum-free CM were replicated using the serum-containing CM from rat nerve SCs.

Comparison of Antiproliferative Activity toward Normal and Transformed SCs

Table I also shows that cellular antiproliferative activity output into the media is apparently lower when the SCs are actively proliferating, such as under cholera toxin stimulation (SC + CTx) or after immortalization (immortal SC). However, transformed SCs such as the Schwannoma cell lines (RN22, D6P2T) actively proliferate (i.e., have a very short doubling time) and still produce a high amount of antiproliferative activity. This suggests that the Schwannoma cells have lost their responsiveness to inhibition by the antiproliferative activity. To compare the responsiveness of the different SC types, we presented concentrated serum-free RN22 CM to cholera toxin-stimulated primary SCs, immortalized SCs, and the Schwannoma cells, all under otherwise identical culture conditions. The results are shown in Fig. 4. DNA synthesis by cholera toxin-stimulated SCs could be completely inhibited, whereas that by immortalized SCs, RN22, and D6P2T Schwannoma cells could be inhibited by

Table I. Antiproliferative Activity toward Schwann Cells: Comparison of Conditioned Media from Different Sources

Source	Mitotic cycle	APU/ml per 10 ⁶ cells
SC	d	
	16	5.1
SC + CTx	2-3	1.9
Immortal SC	2	1.2
RN22 (+serum)	0.8	6.8
RN22 (serum-free)	1-2	2.6
D6P2T	0.7	7.3
Astroglia	ND	<0.1
Oligodendroglia	ND	<0.1
Meningeal cells	ND	<0.1
Fibroblasts (nerve)	ND	<0.1
3T3 fibroblasts	ND	<0.1

Cells in logarithmic growth were detached and dissociated by trypsin/EDTA and then 2×10^6 cells were seeded in 75-cm² tissue culture dishes. The cultures were grown in DME supplemented with 10% calf serum and after 24 h the cells were counted (by phase microscopy) and the medium was replaced with 10 ml of DME supplemented with 2% calf serum (or DME only for serum-free RN22). The cultures were grown for 48 h and then the cells were counted and the CM was collected. SC + CTx cells were treated with 20 ng/ml of cholera toxin for 2 d before and 24 h after seeding in the 100-mm dishes. The effects of cholera toxin on SC proliferation persist for at least 2 d after its removal from SC cultures. During the 48-h CM collection period, SC + CTx medium did not contain cholera toxin, although a high rate of cell division continued. The CMs were assayed for the ability to inhibit incorporation of BrdU into DNA by mitogen-stimulated SCs as described in Materials and Methods. The titer of each CM was expressed as antiproliferative units per milliliter of CM and normalized to an average cell number (the mean of initial and ending cell numbers) present during the 48-h CM collection period. Data represent the means of eight determinations from four separate CM collection experiments (SD < 10%).

only ~40, 30, and 20%, respectively. Similar results were obtained for each cell type using CM from normal SCs (not shown). These findings indicate that transformed SCs are, at best, only partially responsive to inhibition by the antiproliferative activity, and the more rapidly proliferating cells are the least responsive.

Isolation and Partial Characterization of the Antiproliferative Factor

We submitted SC CM and serum-free RN22 CM to several biochemical fractionation steps and monitored the recovery

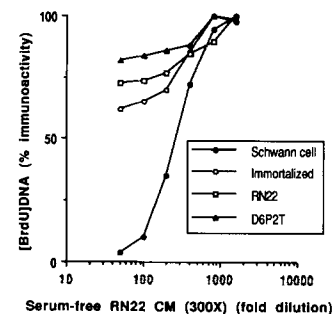


Figure 4. Responsiveness of transformed SC types to the antiproliferative activity. SCs treated with cholera toxin (20 ng/ml), immortalized SCs, and RN22 and D6P2T Schwannoma cells were cultured in microwells (14,000 cells/well) and treated for 72 h with serial dilutions of serum-free RN22 CM (concentrated 300-fold). BrdU was added to the microcultures for the final 24 h and [BrdU]DNA was measured by ELISA. Data represent immunoreactivity expressed as a percent of the maximum value obtained for each actively proliferating cell type. SD < 4%.

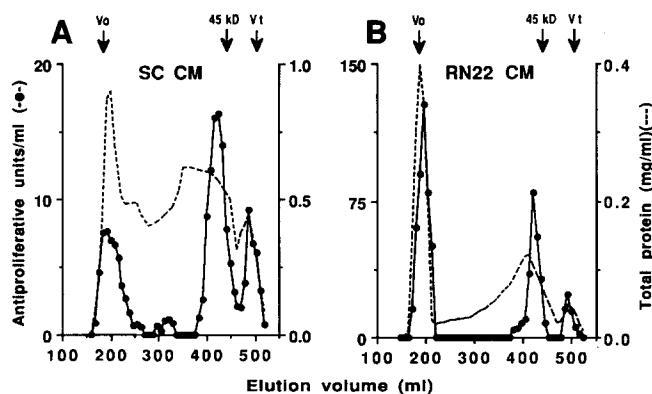


Figure 5. Fractionation of SC CM and serum-free RN22 CM by CL4B gel filtration. In *A*, 150 ml of SC CM was concentrated 30-fold by ultrafiltration (10 kD cut-off), dialyzed with PBS, and then eluted from a CL4B gel filtration column in the same buffer. *B*, Similarly, 1,500 ml of serum-free RN22 CM was concentrated 300-fold by ultrafiltration, dialyzed, and analyzed by CL4B gel filtration. 8-ml fractions were collected and assayed for antiproliferative activity. The titers were expressed in antiproliferative units/milliliter as described in Materials and Methods. All titrations were performed in duplicate and four determinations were made from two separate fractionations. *A* and *B*, SD < 5%. Several molecular weight markers were used and the elution of ovalbumin (45 kD) is shown. *V*_o indicates the high molecular weight (>1,000 kD) void volume and *V*_t indicates the total column volume.

of antiproliferative activity in the derived fractions. The CMs were first concentrated by ultrafiltration and then fractionated by CL4B gel filtration. The elution profile for SC CM (30× concentrated) is shown in Fig. 5 *A* and for serum-free RN22 CM (300× concentrated) in Fig. 5 *B*. For both CMs, three distinct peaks of activity eluted from the column in regions corresponding in molecular masses of >1,000 kD (void volume, *V*_o), 45–55 kD, and <30 kD (total volume, *V*_t). For a sample of SC CM containing 1,500 APU (10 APU/mg; 5 ml of 30× concentrated CM), ~25% of the recovered activity eluted in the high molecular weight void volume, 50% in the 45–55-kD fractions, and 20% in the total volume frac-

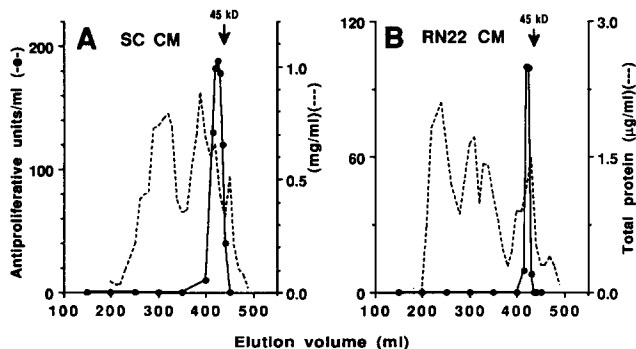


Figure 6. Isolation of the 55-kD antiproliferative factor. SC CM (*A*) and serum-free RN22 CM (*B*) were applied to a DEAE ion exchange column and the flow-through was submitted to ultrafiltration using a 100-kD cut-off filter (to remove any residual high molecular weight material). The filtrate was concentrated and then examined by CL4B gel filtration. The elution of antiproliferative activity was determined as described in Fig. 5.

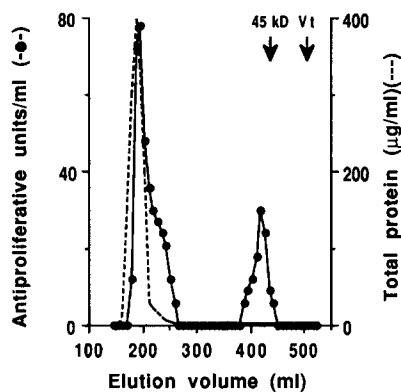


Figure 7. Dissociation of the 55-kD antiproliferative factor from the high molecular weight complexed form. The high molecular weight void volume fractions (180–204 ml) from CL4B gel filtration of serum-free RN22 CM (cf. Fig. 5 *B*) were pooled, concentrated, and then dialyzed with 4 M urea in PBS. The dissociated sample was submitted a second time to CL4B gel filtration and was eluted with PBS. 8-ml fractions were collected and assayed for antiproliferative activity. Fractions containing urea, which eluted in the column *V*_t, were dialyzed with PBS before they were assayed. The titers were expressed in antiproliferative units/milliliter as described in Materials and Methods. All titrations were performed in duplicate and four determinations were made from two separate fractionations. SD < 8%. Several molecular weight markers were used and the elution of ovalbumin (45 kD) is shown. *V*_o indicates the high molecular weight (>1,000 kD) void volume and *V*_t indicates the total column volume.

tions; 5% of the activity was unaccounted for. The highest specific activity of 28 APU/mg total protein was found in fractions eluting in ~420 ml, which corresponded to a molecular mass between 45 and 55 kD (Fig. 5 *A*). For a serum-free RN22 CM sample containing 7,500 APU (380 APU/mg; 5 ml of 300× concentrated CM), 50% of the recovered activity eluted in the high molecular mass void volume, 30% in the fractions corresponding to 45–55 kD, and 5% in the total volume fractions; 20% of the activity was unaccounted for. The highest specific activity of 800 APU/mg was found in fractions eluting in ~420 ml, which corresponded to a molecular mass between 45 and 55 kD.

We attempted to isolate the 55-kD antiproliferative factor by DEAE ion exchange fractionation of SC CM (10 APU/ml, 7 APU/mg). Most of the inhibitory activity flowed through a DEAE column at pH 8, and the total inhibitory activity of the nonbinding effluent was increased almost twofold and the specific activity more than 14-fold. In addition to this increased recovery of flow-through activity, ~25% of the applied activity bound to and could be recovered from the column. Gel filtration of the recovered DEAE-binding material indicated that only the high molecular weight form of the activity binds to DEAE (data not shown). The DEAE-binding activity was eluted by low ionic condition (<0.25 M NaCl), indicating that this activity was not attributed to proteoglycans, which typically are eluted by stronger ionic conditions. The DEAE flow-through was submitted to ultrafiltration using a 100-kD cut-off filter (to remove any residual high molecular weight material) and then the filtrate was submitted to CL4B gel filtration. The results are shown in Fig. 6 *A*. Antiproliferative activity emerged as one sharp peak in a region corresponding to a molecular mass of ~55

kD. About 60% of the original activity in the crude SC CM was recovered in the peak fractions (elution volume = 416–432 ml) with a 28-fold purification (200 APU/mg).

Serum-free rat RN22 Schwannoma cell CM was submitted to the same fractionation steps just described. The starting serum-free RN22 CM contained about one-half the titer of the antiproliferative activity in SC medium, but >50 times the specific activity (380 APU/mg). The titer of the DEAE nonbinding fraction was slightly greater than that of the loaded serum-free RN22 CM even though >40% of that activity bound to and could be eluted from the DEAE column. The DEAE nonbinding fraction was passed through a 100-kD cut-off filter, concentrated, and then examined by CL4B gel filtration. The results are shown in Fig. 6 B. The antiproliferative activity eluted with 416–424 ml in a single sharp peak which had a specific activity >40,000 APU/mg, representing 20% recovery and 100-fold purification compared with the crude CM.

Next we examined the properties of the high molecular weight activity from the first CL4B fractionation (cf. Fig. 5). The void volume (V_0) fractions (180–204 ml; 3,000 APU total, 200 APU/mg) obtained from RN22 CM shown in Fig. 5 B were brought to 4 M urea and further fractionated by a second CL4B gel filtration column equilibrated with PBS. The results are shown in Fig. 7. The eluted fractions were assayed for antiproliferative activity and fractions containing urea, which eluted with \sim 500 ml (V_t), were dialyzed with PBS before they were assayed. Activity eluted in two major peaks having molecular masses similar to those observed for CM in the first gel filtration step (cf. Fig. 5 B). Only the high molecular mass peak was observed if the sample was submitted to gel filtration without urea dissociation (results not shown). Apparently the high molecular weight material could be at least partially dissociated by urea to release the 55-kD form of the activity. The 55-kD fraction (416–424 ml) had a specific activity of 300,000 APU/mg, which corresponded to an 800-fold purification. Interestingly, the total activity recovered from the CL4B column (including both the 1,000- and 55-kD peaks) was 1.5 times greater than the loaded activity, suggesting either that NAP was activated by dissociative conditions or that an inhibitor was removed. The purified 55-kD factor, termed NAP, elicited 50% inhibition of total BrdU incorporation at 65 pM. Treatment of mitogen-stimulated SCs with 3.5 ng/ml of purified NAP reduced the percentage of BrdU-labeled nuclei from 40 (\pm 5) to 20 (\pm 4) and 20 ng/ml essentially reduced the percentage to zero.

The high molecular weight fraction from gel filtration of the serum-free RN22 CM was also examined by SDS-PAGE and gel slices were extracted and assayed for antiproliferative activity. The results are shown in Fig. 8. Antiproliferative activity was essentially extracted from a single gel slice corresponding to the molecular weight region 55 kD. While the high molecular weight form of the activity apparently did not enter the gel, the dissociative conditions (SDS) released a significant amount of the active 55-kD NAP. The active material that eluted from the preparative SDS gel was examined by analytical SDS-PAGE, electroblotting, and colloidal gold staining. The results showed that this preparation of the 55-kD antiproliferative factor was electrophoretically pure (Fig. 9). The activity of this purified 55-kD NAP was greatly diminished by heat (90°C, 20 min), NaOH hydrolysis, and digestion with pronase. These properties suggest that

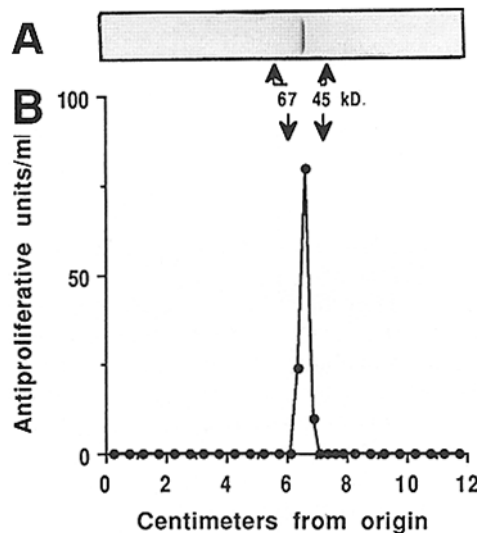


Figure 8. Purification of the 55-kD antiproliferative factor by SDS-PAGE. The high molecular weight void volume fractions (180–204 ml) from CL4B gel filtration of serum-free RN22 CM (cf. Fig. 5 B) were pooled and concentrated. The sample was dissociated with SDS and electrophoresed under nonreducing conditions on a preparative 8% acrylamide gel. The gel was washed to remove SDS and then cut horizontally into 2.5-mm slices. The proteins were eluted from the slices and then assayed for antiproliferative activity. The active fractions were rerun on analytic SDS gels and then electroblotted to nitrocellulose sheets that were stained for protein using colloidal gold. Molecular weight markers were albumin (67 kD) and ovalbumin (45 kD).

the antiproliferative activity toward SCs resides in a 55-kD protein.

Discussion

Our *in vitro* observations indicate that SCs contribute significantly to the regulation of their own proliferation by releasing an autocrine antiproliferative factor that can override stimulation by neurons and all other tested mitogens. The antiproliferative activity in CM occurs in two molecular forms separable during fractionations, a 55-kD form and a high molecular weight form with which part of the 55-kD form may be associated. Although it was possible to sequester the 55-kD NAP from solution using immobilized high molecular weight material (V_0 , Fig. 7) (unpublished observation), we have not yet determined the nature of this association or to which component in the high molecular weight material the 55-kD NAP was bound. Even after dissociation and separation of the 55-kD fraction (cf. Fig. 7) much of the antiproliferative activity continued to be expressed in a high molecular mass form. Possibly the complex was only partially dissociated in 4 M urea, or alternatively, the high molecular mass complexes might contain two different antiproliferative factors, a high molecular weight substance and the 55-kD NAP. Some characterized components of the high molecular weight fraction are known to be mitogenic for cultured SCs (e.g., laminin and fibronectin). Proteoglycans are possible candidates for being high molecular weight inhibitors and recently Yamaguchi and Ruoslahti (1988) demonstrated that expression of proteoglycan can inhibit the proliferation of Chinese hamster ovary cells. However, previously character-

ized SC-derived proteoglycans (see Muir et al., 1989b) did not possess significant antiproliferative activity for SCs. Eccleston and co-workers (1989) have demonstrated that type I collagen matrices can decrease the rate of DNA synthesis in SCs. While type I collagen had partial inhibitory effects on SC proliferation in our assays, type I collagen was not detected in antiproliferative fractions of SC CM.

The high molecular weight complex did not lose activity after dissociation and separation of the 55-kD activity (cf. Fig. 7), suggesting that the 55-kD NAP might not be active when complexed. It is interesting to speculate that the 55-kD NAP exists in a precursor form or that it might be activated by dissociation from an inhibitor. Presently, limited quantities of the 55-kD NAP have hampered detailed biochemical analysis, although recently we have found that adult (but not neonatal) sciatic nerve extracts are a rich source of antiproliferative activity. This observation suggests that the NAP may be involved in nerve development.

The control of SC proliferation has been most thoroughly studied using *in vitro* methods. Recently a number of mitogens have been described for SCs which may act through an autocrine pathway including laminin, glial maturation factor, and transforming growth factor- β (Bosch et al., 1984; Ratner et al., 1986; Lim et al., 1988; Ridley et al., 1989). Autocrine SC mitogens might be expected to accumulate within the culture medium, and upon reaching a critical concentration should stimulate proliferation. Other reported SC mitogens such as fibronectin are present in culture medium supplemented with serum and should also be able to stimulate SC proliferation. The present study derived from our recurring observation that SC responses to mitogens would gradually decay between medium changes (cf. Fig. 1) and in particular that SCs would proliferate only temporarily when placed in coculture with neurons (cf. Fig. 3 C). We examined in more detail the reason for this lack of SC proliferation *in vitro* and found that medium conditioned by SC cultures would inhibit the proliferation of mitogen-stimulated SCs. The inhibition could be virtually complete, was dose dependent and reversible, and was exhibited when the test SCs were under the influence of several mitogens (e.g., laminin, cholera toxin, neurons in coculture) thought to have different mechanisms of action.

Although several growth inhibitors have recently been characterized, little is known about their modes of action. Steck and co-workers (1979) found that medium conditioned by contact-inhibited (quiescent) 3T3 fibroblast cultures inhibited DNA synthesis by subconfluent fibroblast cells. In this study SC CM was collected from dense but subconfluent cultures. However, SCs extend very long processes that course together, making it difficult to eliminate the possibility that NAP is produced in response to cell-cell contact or culture density. This possibility seems unlikely because, in contrast to fibroblasts, SCs produce an antiproliferative activity at all densities. Also, even at very high density, SCs continue to divide slowly and remain responsive to exogenous mitogens.

Purified SCs can be established in large numbers by treatments with readily available mitogens. The cultures can be expanded for many weeks, and soon after discontinuing treatment with mitogens normal growth control returns and the SCs revert to a nearly quiescent state. After months in culture, or even after being stored frozen, SCs remain

responsive to exogenous mitogens and can proliferate and differentiate when in coculture with neurons (Porter et al., 1986). We examined whether proliferating SCs produced more or less of the antiproliferative activity compared with quiescent SCs. CMs from cholera toxin-stimulated and immortalized SCs and from two rat Schwannoma cell lines were all found to contain antiproliferative activity for SCs. However, the titer of cholera toxin-stimulated SC CM was <50% of unstimulated SC CM and immortalized SC CM had even less activity (cf. Table I). Considering that the immortalization of SCs was achieved by long-term exposure to cholera toxin, it is possible that elevated cAMP results in a progressive decrease in production. Also, it is possible that mitogen stimulation or proliferation in general reduces production of the antiproliferative activity. In contradistinction, RN22 and D6P2T Schwannoma cells divide even more rapidly than the immortalized SCs, but unlike the immortalized SC CM, Schwannoma CMs had a relatively high titer of antiproliferative activity for SCs. Apparently Schwannoma cells can proliferate even though considerable antiproliferative activity accumulates in their culture medium. The Schwannoma lines were induced by carcinogens and differ in several ways from the immortalized cells. While both retain many characteristics of primary SCs in culture, the immortalized cells can also retain the ability to differentiate and produce myelin in response to neurons (Porter et al., 1987).

The possibility that loss of growth control could be due to the loss of responsiveness to the antiproliferative activity was examined. For these experiments concentrated serum-free RN22 CM was tested for its ability to inhibit the proliferation of cholera toxin-stimulated SCs, immortalized SCs, and Schwannoma cells. Although RN22 CM completely inhibited mitogen-stimulated SCs, DNA synthesis by the transformed cells was only partially inhibited (cf. Fig. 4). Similar results were obtained in identical experiments by using the purified 55-kD NAP instead of the RN22 CM. Thus, the loss of responsiveness to NAP could explain the loss of growth control by immortalized and transformed SC lines. Taken together, these observations suggest that mitogen-stimulated SC cultures may produce somewhat less antiproliferative activity but remain under negative autocrine control. Also, it seems plausible that immortalization (loss of normal growth control) by long-term mitogen stimulation might involve the selection of subclones that produce less and respond less to the antiproliferative activity. In this context, subcloning of transformed cell lines would select for cells that are unresponsive to, but produce, antiproliferative activity; these cells would soon dominate over more responsive cells. Consistent with this prediction, the two clonal Schwannoma lines we studied are constitutive producers of NAP, yet are nearly unresponsive to its effects. These observations raise challenging questions about the mechanism of autocrine growth regulation by NAP and its possible role in aberrant SC cell proliferation. Further study is required to determine whether autocrine growth control by NAP influences SC differentiation and their reentry into the mitotic cycle during nerve regeneration.

This work was supported by National Science Foundation grant NS-17034 and National Institutes of Health grant 26349.

Received for publication 4 July 1990 and in revised form 24 August 1990.

References

- Bansal, R., and S. E. Pfeiffer. 1987. Regulated galactolipid synthesis and cell surface expression in Schwann cell line D6P2T. *J. Neurochem.* 49:1902-1911.
- Bosch, E. P., J. G. Assouline, J. F. Miller, and R. Lim. 1984. Glial maturation factor promotes proliferation and morphological expression of rat Schwann cells. *Brain Res.* 304:311-319.
- Brockes, J. P., P. Fields, and M. C. Raff. 1979. Studies on cultured rat Schwann cells. I. Establishment of purified populations from cultures of peripheral nerve. *Brain Res.* 165:105-118.
- DeVries, G. H., L. N. Minier, and B. L. Lewis. 1983. Further studies on the mitogenic response of cultured Schwann cells to rat CNS axolemma-enriched fractions. *Dev. Brain Res.* 285:87-94.
- Dziadek, M., D. Edgar, M. Paulsson, R. Timpl, and R. Fleischermajar. 1986. Basement membrane proteins produced by Schwann cells and in neurofibromatosis. *Ann. NY Acad. Sci.* 486:248-259.
- Eccleston, P. A., R. Mirsky, and K. R. Jessen. 1989. Type I collagen preparations inhibit DNA synthesis in glial cells of the peripheral nervous system. *Exp. Cell Res.* 182:173-185.
- Engvall, E., T. Krusius, V. Wewer, and E. Ruoslahti. 1983. Laminin from rat yolk sac tumor: isolation, partial characterization and comparison with mouse laminin. *Arch. Biochem. Biophys.* 222:649-656.
- Laemmli, U. K. 1970. Cleavage of structural protein during the assembly of the head of bacteriophage T-4. *Nature (Lond.)* 227:680-685.
- Lim, R., D. J. Hicklin, T. C. Ryken, J. F. Miller, and E. P. Bosch. 1988. Endogenous immunoreactive glial maturation factor-like molecule in cultured rat Schwann cells. *Dev. Brain Res.* 40:277-284.
- Manthorpe, M., S. D. Skaper, and S. Varon. 1980. Purification of mouse Schwann cells using neurite-induced proliferative in serum-free monolayer cultures. *Brain Res.* 196:467-482.
- McCarthy, K. D., and J. de Vellis. 1980. Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. *J. Cell Biol.* 85:890-902.
- McGarvey, M. L., Baron-Van Evercooren, H. K. Kleinman, and M. Dubois-Dalcq. 1984. Synthesis and effects of basement membrane components in cultured rat Schwann cells. *Dev. Biol.* 105:18-28.
- Muir, D., C. Gennrich, S. Varon, and M. Manthorpe. 1989a. Rat sciatic nerve Schwann cell microcultures: responses to mitogens and production of trophic and neurite-promoting factors. *Neurochem. Res.* 14:1003-1012.
- Muir, D., E. Engvall, S. Varon, and M. Manthorpe. 1989b. Schwannoma cell-derived inhibitor of the neurite-promoting activity of laminin. *J. Cell Biol.* 109:2353-2362.
- Muir, D., S. Varon, and M. Manthorpe. 1990. An enzyme-linked immunosorbent assay for bromodeoxyuridine incorporation using fixed microcultures. *Anal. Biochem.* 185:377-382.
- Pfeiffer, S. E., and W. Wechsler. 1972. Biochemically differentiated neoplastic clone of Schwann cells. *Proc. Natl. Acad. Sci. USA.* 69:2885-2889.
- Porter, S., M. B. Clark, L. Glaser, and R. P. Bunge. 1986. Schwann cells stimulated to proliferate in the absence of neurons retain full functional capability. *J. Neurosci.* 6:3070-3078.
- Porter, S., L. Glaser, and R. P. Bunge. 1987. Release of autocrine growth factor by primary and immortalized Schwann cells. *Proc. Natl. Acad. Sci. USA.* 84:7768-7772.
- Ratner, N., R. P. Bunge, and L. Glaser. 1986. Schwann cell purification *in vitro*: an overview. *Ann. NY Acad. Sci.* 486:170-181.
- Ridley, A. J., H. F. Paterson, M. Noble, and H. Land. 1988. Ras-mediated cell cycle arrest is altered by nuclear oncogenes to induce Schwann cell transformation. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:1635-1645.
- Ridley, A. J., J. B. Davis, P. Stroobant, and H. Land. 1989. Transforming growth factors-beta 1 and beta 2 are mitogens for rat Schwann cells. *J. Cell Biol.* 34:19-24.
- Rudge, J. S., M. Manthorpe, and S. Varon. 1985. The output of neuronotrophic and neurite-promoting agents from rat brain astroglial cells: a microculture method for screening potential regulatory molecules. *Dev. Brain Res.* 19:161-172.
- Salzer, J. L., and R. P. Bunge. 1980. Studies of Schwann cell proliferation. I. An analysis in tissue culture of proliferation during development, Wallerian degeneration, and direct injury. *J. Cell Biol.* 84:739-752.
- Salzer, J. L., R. P. Bunge, and L. Glaser. 1980. Studies of Schwann cell proliferation. III. Evidence for the surface localization of the neurite mitogen. *J. Cell Biol.* 84:767-778.
- Steck, P. A., P. G. Voss, and J. L. Wang. 1979. Growth control in cultured 3T3 fibroblasts. Assays of cell proliferation and demonstration of a growth inhibitory activity. *J. Cell Biol.* 83:562-575.
- Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and applications. *Proc. Natl. Acad. Sci. USA.* 76:4350-4354.
- Webster, H. de F., and J. T. Favilla. 1984. Development of peripheral nerve fibers. In *Peripheral Neuropathy*. P. J. Dyck, P. K. Thomas, E. H. Lambert, and R. P. Bunge, editors. W. B. Saunders Co., Philadelphia. 329-359.
- Wood, P. 1976. Separation of functional Schwann cells and neurons from peripheral nerve tissue. *Brain Res.* 115:361-375.
- Yamaguchi, Y., and E. Ruoslahti. 1988. Expression of human proteoglycan in Chinese hamster ovary cells inhibits cell proliferation. *Nature (Lond.)* 336:244-246.