Selective Culture of Mitotically Active Human Schwann Cells from Adult Sural Nerves

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We devised a simple method to isolate mitotically active human Schwann cells from sural nerve biopsy specimens and expand the population in culture. Nerve fascicles were treated with cholera toxin for 7 days in culture before dissociation, which increased the cell yield at least twenty-five–fold over immediated tissue dissociation. Digesting the tissue completely with enzymes in serum-containing medium resulted in the highest cell viability, and released 2 to 6 × 10^4 cells/mg of tissue. Seeding the cells on a poly-L-lysine substrate in a small volume of serum-free medium optimized the plating efficiency. Although Schwann cells comprised 90% of the initial culture population, their numbers declined over time due to a faster mitotic rate of the fibroblasts in the presence of cholera toxin alone. However, treating the cultures with a combination of cholera toxin and forskolin, which act synergistically to elevate cyclic AMP levels, inhibited fibroblast growth without causing Schwann cell toxicity. Adding glial growth factor to the adenyl cyclase activators maximized Schwann cell proliferation, and the population rapidly and selectively expanded. Therefore, it should be possible to generate large numbers of Schwann cells from diseased nerves to study defects in cell function or from normal nerves to study the effects of Schwann cell grafts on neuronal regeneration.

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Schwann cells, the only glial cells in the peripheral nervous system (PNS), support neuronal function in several different ways. The myelin sheath produced by Schwann cells permits the rapid transmission of nerve impulses via saltatory conduction at the nodes of Ranvier [1]. A class of peripheral neuropathies distinguished by segmental demyelination and slowed conduction velocity is believed to result from defects in Schwann cell metabolism [2]. These neuropathies can be either inherited or acquired, yet little is known about their pathogenesis. The ability to generate large numbers of human Schwann cells from biopsy specimens may help to identify the cellular mechanism involved in some of these demyelinating diseases.

In addition to facilitating nerve conduction, Schwann cells enable neurons to regenerate after injury. By secreting neurotrophic factors, they aid neuronal survival and stimulate axonal sprouting [3–5], and by expressing adhesion molecules on their cell surface [6–8], Schwann cells guide regenerating axons through the remaining basal lamina tubes back to their synaptic contacts [9, 10]. Transplanting peripheral nerves into the central nervous system demonstrated that Schwann cells also permit central axons to regenerate [11], and Schwann cell implants are now being explored as a means to facilitate recovery in animal models of Parkinson's disease [12], and peripheral nerve [13–15] and spinal cord injury [16–18]. The results from animal experiments appear promising, but testing the potential benefits of these therapeutic approaches in humans would require large numbers of Schwann cells.

Human Schwann cells have been cultured from adult nerves by successive reexplantation, allowing the Schwann cells to slowly migrate out from the attached explant over several weeks in culture [19–22]. However, without the addition of mitogens to the cultures, few of the cells divided, and the yields obtained with this time-consuming technique were very low [20]. Therefore, we devised a method to isolate mitotically active Schwann cells from human nerves and to expand the population in culture with agents selected to stimulate Schwann cell proliferation while inhibiting fibroblast growth.

Materials and Methods

Cell Isolation

Normal human sural nerve specimens were obtained from patients ranging from 18 to 40 years old who underwent graft repair of a brachial plexus injury. Nerve fascicles were dissected from the epineurium and weighed, and 10 mg of

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tissue/ml of feeding medium (FM) was incubated in plastic dishes for 0 to 14 days in humidified 10% carbon dioxide at 37°C. The FM consisted of Dulbecco's modified Eagle's medium containing 4.5 gm/liter of glucose and supplemented with 10% heat-activated fetal bovine serum and 0.1 μg/ml of cholera toxin (Sigma, St. Louis, MO). The tissue was then dissociated with 80 units/ml of collagenase and 0.8 unit/ml of dispase (Boehringer Mannheim, Indianapolis, IN) in the FM. Approximately 20 mg of tissue/ml of enzyme solution was placed in a capped tube, gassed with 10% carbon dioxide, and rocked gently for 18 hours at 37°C. After complete enzymatic digestion, the cells were washed twice with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline solution (PBS) containing 0.04% ethylenediaminetetraacetic acid (EDTA), washed once with S4 serum-free medium [23], and then resuspended in S4.

To determine cell viability, an aliquot of the cell suspension was stained in 100 μM propidium iodide, and then counted in a hemocytometer under a Leitz microscope (Wetzlar, Germany) equipped with epifluorescence. This dye penetrates the membrane of only dead or dying cells and binds to nucleic acids, causing the cell nuclei to fluoresce red when excited at 490 nm. A second aliquot was treated with 50% ethanol to render all cells permeable to the dye, and the total number was counted. The cell suspension was diluted to 4 to 5 × 10^5 cells/ml of S4 medium, and 1 ml was spread on 10-cm plastic dishes coated with 100 μg/ml of poly-L-lysine (MW >300,000, Sigma). Nine milliliters of the FM was added 18 hours after seeding. Plating efficiency was determined by counting the number of both attached and unattached cells 48 hours after seeding. Cells were subcultured when confluent onto poly-L-lysine–coated dishes.

Immunofluorescent Staining

The antibodies and their dilutions used to distinguish between Schwann cells and fibroblasts were as follows: anti–human nerve growth factor (NGF) receptor (HB 8737, American Tissue Culture Collection, Rockville, MD), tissue culture supernatant; anti–bovine S-100 protein (IgG fraction, Dakopatts, Carpinteria, CA), 1:100; anti–bovine galactocerebroside (IgG fraction, Advanced Immunochemicals, Long Beach, CA), 1:500; anti–human glial fibrillary acid protein (IgG fraction, Chemicon, Temecula, CA), 1:100; anti–human laminin (purified IgG, Boehringer Mannheim), 1:20; anti–human fibronectin (purified IgG, Boehringer Mannheim), 1:20; anti–human Thy-1 (gift from Dr Wolfgang Rettig, University of Virginia), tissue culture supernatant [24].

For indirect immunofluorescent staining, cells were seeded on glass coverslips coated with 1 μg/ml of poly-L-lysine. Surface antigens were localized after fixation with 4% formaldehyde in complete Dulbecco's PBS for 10 minutes at 23°C. To localize intracellular antigens, cells were additionally treated with ethanol for 5 minutes at −20°C. Coverslips were incubated with primary antibodies diluted in medium containing 10% serum for 30 minutes at 37°C, washed in three changes of medium, and then incubated with the appropriate fluorescein-conjugated antiimmunoglobulin antibody (affinity purified, Organon Tenika-Cappell), diluted 1:400. After washing and postfixation in 95% ethanol and 5% acetic acid, the coverslips were mounted in AF-1 (Citifluor, London, UK) containing 2 μM Hoechst 33342 and examined with a Leitz fluorescent microscope. Cell number per visual field was determined by counting Hoechst-stained nuclei under ultraviolet (UV) light.

Mitotic Activity

Approximately 1-mm slices of some nerve explants were removed before enzymatic digestion and incubated in 30 μM bromodeoxyuridine (BrdU) for 18 hours. The tissue was fixed in methanol for 15 minutes at 4°C, and the DNA was denatured in 2N hydrochloric acid (HCl) for 1 hour. After neutralization with 0.1 M sodium borate (pH 9), the tissue was incubated for 1 hour with fluorescein-conjugated anti-BrdU (Boehringer Mannheim, 1:5 dilution), and then washed and mounted.

Cells were seeded on glass coverslips and grown in medium containing various Schwann cell mitogens in addition to cholera toxin, including forskolin (Sigma), glial growth factor (GGF) prepared by carboxymethyl cellulose chromatography [25], or 60 μg/ml of bovine axolemma and myelin (provided by Dr Jun Yoshino, Colgate University) [26, 27]. After 48 hours, BrdU was added to a final concentration of 30 μM, and the cells were fixed 18 hours later. Cells were first stained for S-100, and the antigen-antibody complex was cross-linked for 30 minutes with 0.5% carbodiimide in PBS. Coverslips were then processed for BrdU staining.

The proportion of Schwann cells in the S phase was determined by counting the number of cells double-labeled for S-100 and BrdU out of the total S-100–positive population. S-100–negative cells were assumed to be fibroblasts; thus, S-100–negative, BrdU-positive cells were scored as mitotic fibroblasts. The total number of fibroblasts was estimated by subtracting the number of S-100–positive cells from the total number of cells.

Expansion of Schwann Cell Cultures

Sural nerves were dissociated as described, and the cells were allowed to attach and spread for 2 days. The cultures were then treated with either 0.1 to 1.0 μg/ml of cholera toxin, 1 to 10 μM forskolin, or various concentrations of cholera toxin and forskolin combined. Additional cultures were treated with a combination of adenylate cyclase activators plus 5 μg/ml of GGF. When the cultures reached confluence, the cells were seeded onto coverslips, and the Schwann cells and fibroblasts were identified morphologically or by indirect immunofluorescent staining. The effect of each treatment was evaluated for at least three passages.

Results

Cell Isolation

Immediately dissociating human sural nerve specimens released 700 to 800 viable cells/mg of tissue. In addition, the plating efficiency was low and the Schwann cells appeared to remain quiescent or had such a delayed response to mitogens that fibroblasts overgrew the cultures (not shown). However, maintaining the nerve in explant culture for 7 days in the presence of cholera toxin increased the cell yield at least twenty-

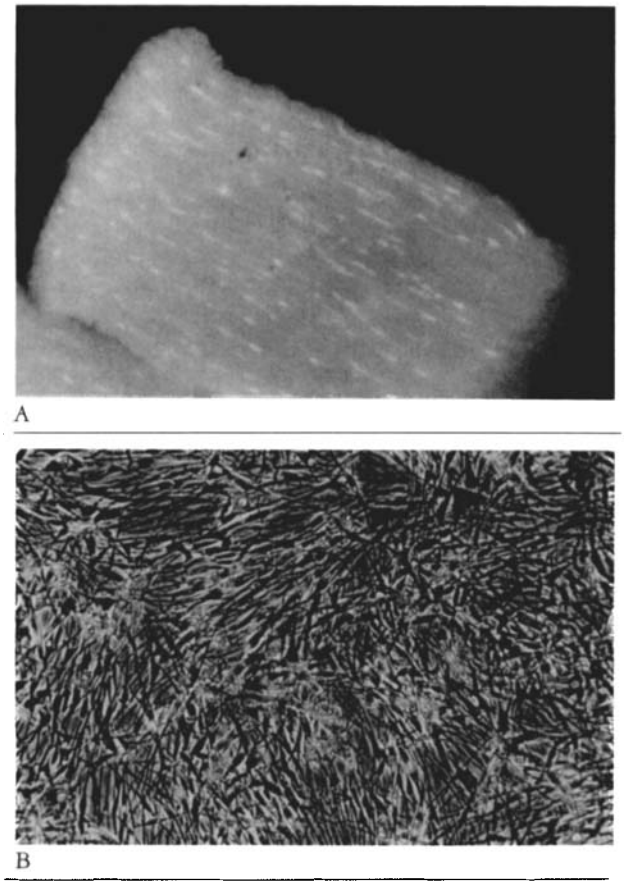

Fig 1. Isolation of cells from sural nerve specimens. Nerve explants were treated for 7 days in culture with 0.1 μg/ml of cholera toxin before enzymatic dissociation. (A) Bromodeoxyuridine labeling for 18 hours before dissociation showed that cells were dividing within the explants (×102 before 31% reduction). (B) Phase micrograph of a live primary culture 4 days after plating (×205 before 30% reduction). Most cells displayed the characteristic bipolar morphology of Schwann cells.

five–fold. The Schwann cells began to divide within the explant by 3 days in vitro but their mitotic activity reached a plateau between 7 and 10 days (Fig 1A).

Approximately 85% of the cells remained viable after the cell isolation procedure, which consistently yielded 2 to 6 × 10^4 cells/mg of nerve. Removing serum from the medium during seeding was found to increase attachment by approximately 30%, and reducing the volume, which increases the rate of attachment, improved the plating efficiency further. Attachment to various substrates including plastic, laminin, fibronectin, type I collagen, extracellular matrix from endothelial cells [28] or EHS sarcoma cells (Matrigel, Collaborative Research, Bedford, MA), and poly-L-lysine was tested. Attachment to poly-L-lysine was found to be superior (data not shown). A maximal plating efficiency of 85% was achieved by seeding in 1 ml of serum-free medium onto poly-L-lysine.

After attachment, the cultures were maintained in serum-containing medium supplemented with cholera toxin to stimulate Schwann cell growth. Four days after plating, most cells displayed the bipolar morphology typical of primary rat Schwann cells, and many contained phase-bright, cytoplasmic inclusions of myelin fragments, as shown in Figure 1B.

Immunofluorescent Staining

Human Schwann cells were identified after 4 days in culture by their positive staining for galactocerebroside, S-100, and the NGF receptor (Fig 2A–C). By 12 days in culture, galactocerebroside staining was not detected, but staining for S-100 and the NGF receptor persisted for at least eight passages (39 days). The matrix molecules, laminin and fibronectin, were found on the surface of Schwann cells and fibroblasts, respectively (not shown). However, distinguishing Schwann cells from fibroblasts was difficult because of the punctated staining pattern. Moreover, fibronectin may not be a reliable fibroblast marker because it has been detected on the surface of some rat Schwann cells in culture by double-staining [29]. We found that human fibroblasts were more easily identified with an antibody to the surface Thy-1 antigen (Fig 2D).

S-100–positive staining was then used to determine the percentage of Schwann cells in the total cell population after various times in culture. In primary cultures at day 4, 90% of the cells were S-100 positive (Fig 3). Schwann cells still predominated at day 12 after two passages, but after this point, the percentage of fibroblasts markedly increased. After eight passages at day 39, only 27% of the population was comprised of Schwann cells.

Response to Mitogens

In a mixed population of Schwann cells and fibroblasts, it was difficult to determine which cell type was responding based on morphology and BrdU labeling alone. Therefore, the cultures were double-stained for S-100 to identify Schwann cells and for BrdU to identify mitotic cells (Fig 4A, B). The response of the two cell populations to various mitogens is shown in the Table.

Since Schwann cells could not be maintained in culture without the use of mitogens, cholera toxin was always included in the medium. When treated with cholera toxin alone, 28% of the Schwann cells and 43% of the fibroblasts entered the S phase of the cell cycle during the 18-hour labeling period. The mitotic activity of the Schwann cells was increased further, without affecting the mitotic rate of the fibroblasts, by combining cholera toxin with either axonal membrane fragments or GGF. The addition of myelin fragments did alter the mitotic rate of either cell type. However, the combination of cholera toxin, forskolin, and GGF

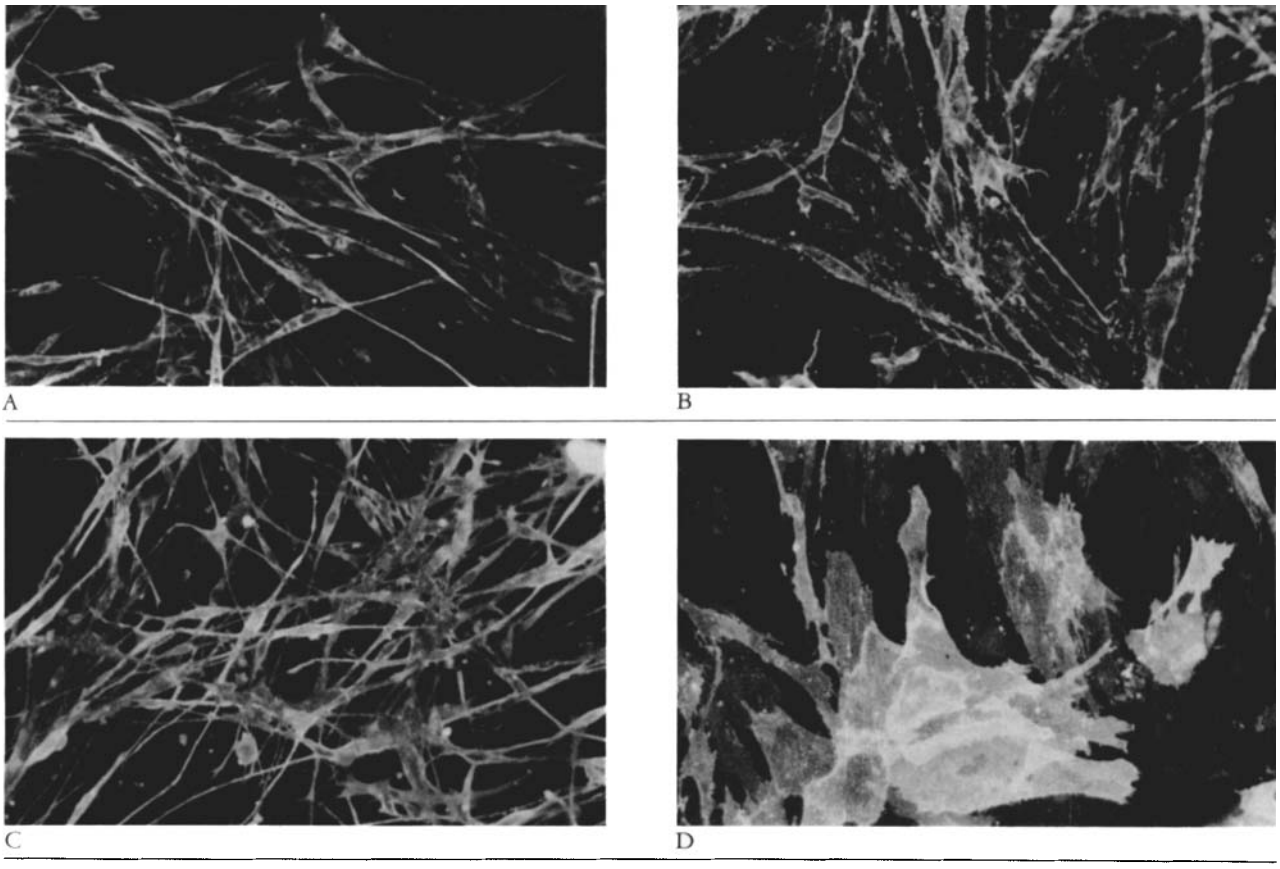

Fig 2. Identification of cell types in culture. Schwann cells were identified by indirect immunofluorescent staining. Staining for (A) galactocerebroside, (B) nerve growth factor (NGF) receptors, and (C) S-100 was evident at day 4 (primary culture). Galactocerebroside was no longer detectable at day 12, but staining for NGF receptors and S-100 persisted for at least 39 days. (D) By day 18, the number of fibroblasts detected by staining for the Thy-1 antigen had markedly increased. (A–D, ×205 before 31% reduction.)

stimulated the mitotic rate of the Schwann cells to 45% while reducing that of the fibroblasts to 9%.

Expansion of Schwann Cell Cultures

Activators of adenyl cyclase such as cholera toxin and forskolin reportedly have opposite effects on the proliferation of Schwann cells and fibroblasts [30, 31]. We found that the fibroblasts continued to proliferate when treated with up to 1 μg/ml of cholera toxin. Cholera toxin receptors (GM1 ganglioside) are concentrated in Schwann cell membranes [32, 33], and we found most of the bound toxin associated with Schwann cells by immunofluorescent staining (not shown). Forskolin, at 10 μM, blocked fibroblast growth but this concentration was toxic to human Schwann cells. However, 0.1 μg/ml of cholera toxin plus 1 μM forskolin inhibited fibroblast growth without adversely affecting the Schwann cells. The addition

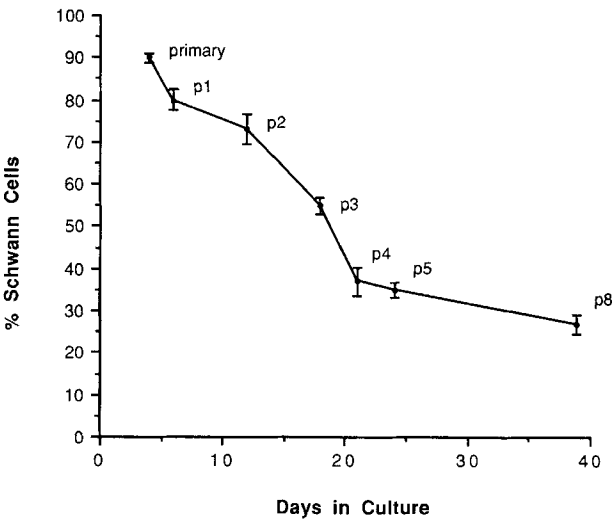

Fig 3. Decline of the Schwann cell population over time. Cells were seeded onto coverslips at each passage (p), *and the Schwann cells were identified by S-100–positive staining. The total cell number was determined by nuclear staining with Hoechst dye 33342. Values represent the mean of four to eight fields ± standard error of mean, and over 200 cells/time point were counted.*

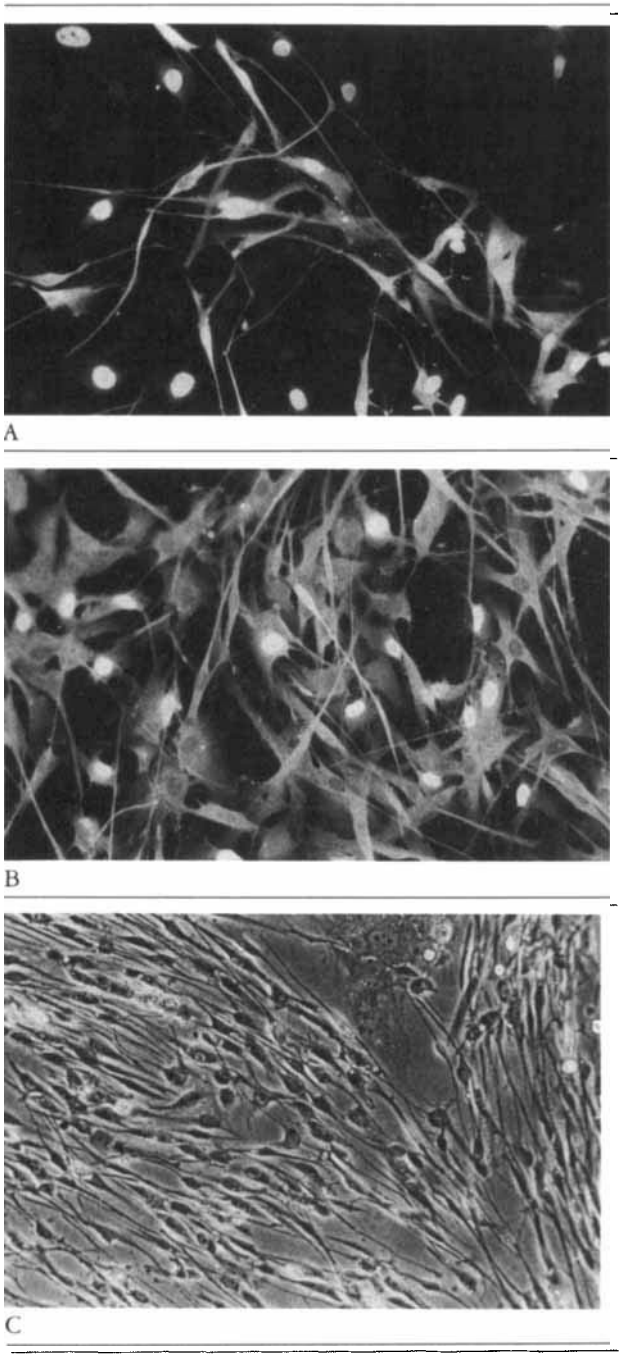

Fig 4. Selective expansion of the Schwann cell population. Cells were double-labeled for S-100 (stains the Schwann cell cytoplasm) and bromodeoxyuridine (stains the nucleus of cells in the S phase). (A) Cultures were maintained for three passages on cholera toxin alone. Fibroblasts eventually overgrew the cultures because they divided faster than Schwann cells. (B) Cultures were maintained for three passages on a combination of cholera toxin, forskolin, and glial growth factor. Schwann cells proliferated rapidly while fibroblast growth was inhibited, and the number of fibroblasts decreased with each passage. (C) Schwann cells comprised 98% of the cultures, as shown by phase microscopy. (A–C, ×205 before 31% reduction.)

of 5 μg/ml of GGF to this combination of adenyl cyclase activators resulted in the rapid and selective proliferation of Schwann cells, and the fibroblasts were diluted out with each subculture (see Table; Fig 4A, B). Cultures highly enriched in Schwann cells (98%) were obtained after three passages (Fig 4C).

Discussion

The objective of this study was to maximize the recovery of adult human Schwann cells from peripheral nerve specimens and to optimize their mitotic activity in culture. We devised a rapid, simple, and effective method incorporating standard techniques, and discuss the most crucial aspects of the procedure below. First, removing the nerve fascicles from the epineurial sheath eliminated one source of contaminating fibroblasts. Second, preculturing the nerve fascicles in the presence of cholera toxin for 7 days before dissociation increased the cell yield fivefold. Degenerating the axonal and myelin membranes within the explant stimulated Schwann cell proliferation [34–36], which was augmented by activating the cyclic AMP pathway with cholera toxin [37, 38]. Third, the tissue was completely digested with enzymes in serum-containing medium to maintain the highest viability. Finally, optimal plating efficiency was achieved by seeding the cells on a poly-L-lysine substrate in a small volume of serum-free medium.

Human Schwann cells were previously cultured from adult nerve specimens by successive reexplantation [19–22], but the yields obtained with this technique were very low. High yields of either rat [39] or human Schwann cells [40] were obtained by enzymatic dissociation with fetal and newborn tissues, which have a higher cell density and less connective tissue. However, only 600 cells/mg were obtained from adult rat nerves by enzymatic digestion [30], and we initially obtained similar cell yields from adult human nerves. However, preculturing the explant in the presence of cholera toxin stimulated the Schwann cells to detach from their myelin sheaths and undergo mitosis, which dramatically increased the cell yield. A 2-cm sural nerve biopsy specimen (100 mg of nerve) produced about 2×10^6 Schwann cells, and their number continued to expand in culture.

The cell composition of the cultures was determined over time by staining with antibodies to cell-specific antigens. We found that like rat Schwann cells in culture, human Schwann cells transiently expressed galactocerebroside [41, 42] yet continued to express S-100 protein, NGF receptors, and laminin in long-term cultures as previously reported [40, 43, 44]. Schwann cells comprised at least 90% of the initial culture population, and many mitotic human Schwann cells were detected. However, the proportion of fibroblasts increased with each subculture because in the presence

Effect of Mitogens on Cell Proliferation[a]

Treatment	Nuclear Incorporation of BrdU	
	Schwann Cells (%)	Fibroblasts (%)
Cholera toxin (0.1 μg/ml)	28 ± 1.0	43 ± 2.4
Cholera toxin + myelin (60 μg/ml)	30 ± 0.9	51 ± 1.9
Cholera toxin + axolemma (60 μg/ml)	36 ± 1.3[b]	46 ± 1.7
Cholera toxin + GGF (5 μg/ml)	37 ± 1.9[b]	35 ± 1.2
Cholera toxin + GGF + forskolin (1 μM)	45 ± 3.2[b]	9 ± 0.7[b]

[a]Cells grown on glass coverslips were treated for 48 hr and then labeled with 30 μM BrdU for an additional 18 hr. The percentage of mitotic Schwann cells and fibroblasts was determined by double-staining for S-100 and BrdU, as depicted in Fig 4A and B. Over 400 cells in each treatment group were counted. Values represent the mean of 4–8 fields ± SEM.
[b]Significantly different from cholera toxin alone ($p > 0.01$, Student's t test).

BrdU = bromodeoxyuridine; GGF = glial growth factor.

of cholera toxin alone, their rate of proliferation was faster than that of the Schwann cells. Adding a second mitogen for human Schwann cells, either GGF or axolemma [20, 45], increased the mitotic rate of the Schwann cells to approximately that of the fibroblasts.

These results suggest that it may be possible to maintain cultures highly enriched in human Schwann cells by treatment with a combination of mitogens immediately after isolation. Moreover, activation of adenyl cyclase, which stimulates Schwann cell mitosis [30], has blocked the response of fibroblasts to growth factors [31]. We found that cholera toxin and low concentrations of forskolin, which act synergistically to elevate cyclic AMP levels [46], were required to inhibit fibroblast growth without causing Schwann cell toxicity. Adding GGF to the adenyl cyclase activators potentiated the growth rate of the human Schwann cells, as has been shown for rat Schwann cells [38, 47], and virtually pure cultures were obtained within a few passages. However, this combination of mitogens could prove to be problematic since prolonged treatment has led to the transformation of rodent Schwann cells [48], which formed tumors when injected back into nerves [49].

Although human Schwann cells can be isolated from fetal [40, 43, 44] and autopsy tissue [45], the ability to generate large numbers of Schwann cells from biopsy specimens would offer unique advantages. With our procedure, relatively large numbers of Schwann cells can be obtained from a sural nerve biopsy specimen within a few weeks. Therefore, it may soon be possible to study biochemical defects in patients with demyelinating neuropathies of genetic or unknown causes while the patient is still living. Also, if human Schwann cell cultures can be expanded without transformation or loss of their functional properties, it may be possible to transplant autologous Schwann cells for clinical experimentation, thus eliminating autoimmune-mediated graft rejection.

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