

Fibronectin Promotes Rat Schwann Cell Growth and Motility

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ABSTRACT Techniques are now available for culturing well characterized and purified Schwann cells. Therefore, we investigated the role of fibronectin in the adhesion, growth, and migration of cultured rat Schwann cells. Double-immunolabeling shows that, in primary cultures of rat sciatic nerve, Schwann cells (90%) rarely express fibronectin, whereas fibroblasts (10%) exhibit a granular cytoplasmic and fibrillar surface-associated fibronectin. Secondary cultures of purified Schwann cells do not express fibronectin. Exogenous fibronectin has a small effect on promoting the adhesion of Schwann cells to the substrate and does not significantly affect cell morphology, but it produced a surface fibrillar network of fibronectin on the secondary Schwann cells. Tritiated thymidine autoradiography revealed that addition of fibronectin to the medium, even at low concentrations, markedly stimulates Schwann cell proliferation, in both primary and secondary cultures. In addition, when cell migration was measured in a Boyden chamber assay, fibronectin was found to moderately, but clearly, stimulate directed migration or chemotaxis.

Fibronectin, a high molecular weight cell-surface and serum-derived glycoprotein (16, 23, 29, 38), has numerous effects on cells *in vitro*. At concentrations as low as 1–50 $\mu\text{g/ml}$, it can promote the adhesion (22), spreading (23), and migration (3, 9) of fibroblasts and certain other cells, and induce transformed cells to flatten and appear normal (2, 37). Additionally, fibronectin can influence the differentiation of chondrocytes (21, 35) and myoblasts (24). Presumably, many of the activities of fibronectin are operative *in vivo* during tissue formation and in the repair of wounds. Fibronectin is present in various connective tissues, such as the dermis and certain basement membranes (33), and is produced by fibroblasts and certain epithelial cells *in vitro* (16, 23).

While previous studies have suggested that fibronectin is present in the nervous system (12, 19, 20, 31, 34), its role in nervous tissue has not been explored in depth. In the chicken, fibronectin is present along migrating neural crest cells at various stages of development (17), and it can promote adhesion and migration of such cells in culture (10). In rodents, fibronectin is associated with meningeal cells in the central nervous system and with perineurial cells in the peripheral nervous system (19, 31; Kay Fields, personal communication). In addition, neurite outgrowth of retinal cells can be stimulated by fibronectin (1). In cultures, glial cells have been found to synthesize fibronectin and, as in the case of fibroblasts, it is reduced on the cell surface after transformation (20). While a clonal line of Schwann cells has also been reported to synthesize fibronectin (15), the expression of fibronectin by isolated Schwann cells cultures from newborn rat sciatic nerve (6) has not been described. Schwann cells are known to have migratory

properties both *in vivo* and *in vitro* (8, 32). We have investigated the effect of exogenous fibronectin on the growth and migration of primary and secondary cultures of rat Schwann cells.

MATERIALS AND METHODS

Reagents

Type I rat skin collagen was prepared as previously described (4). Cell-surface fibronectin was prepared from urea extracts of third-passage chick embryo fibroblasts (37). The same preparation frozen in small aliquots and stored at -70°C was used for all experiments described in this report. Antibody against fibronectin was the gift of Dr. Jean-Michel Foidart (University of Liege, Belgium). Monoclonal antibody against rat cell-surface antigen Thy 1-1 was obtained from New England Nuclear (Boston, MA). Both fluorescein- and rhodamine-conjugated second antibodies were obtained from N. L. Cappel Laboratories, Inc. (Cochranville, PA) and cholera toxin from Sigma Chemical Co. (St. Louis, MO). Pituitary factor was purified from bovine pituitary glands as described before (6, 8).

Cell Preparation and Cultures

For primary Schwann cell cultures, sciatic nerve from 2- to 3-d-old rats was dissected, dissociated with a solution of trypsin and collagenase type III (Worthington Biochemicals Corp, Freehold, NJ), and passed through a no. 23 hypodermic needle (6). The resulting single cells suspension was cultured in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) (Microbiological Associates, Walkersville, MD) (8). After 2 d, these cultures were treated for 48 h with cytosine arabinoside (Ara C at 10^{-5} M) to prevent overgrowth by fibroblasts. The resulting culture contained <10% fibroblasts. For subculturing, Schwann cells were trypsinized and purified by immune-mediated killing with anti-Thy 1-1 monoclonal IgM antibody and rabbit complement. Two successive killings eliminated all fibroblasts as described previously (6, 8). Purification was evaluated by immunofluorescence with Thy 1-1 antigen.

Subcultures completely devoid of Thy 1-1 positive cells were considered pure Schwann cells. Doubling times of purified Schwann cells were determined as follows: secondary cells were plated on 35-mm Petri dishes at a density of 7×10^4 cells/dish and grown in DMEM-FCS or DMEM-FCS supplemented with various concentrations of fibronectin. Cells were trypsinized and counted in suspension with a Coulter counter (Coulter Electronics, Inc., Hialeah, FL) daily. Data did not differ by >10% and were averaged from duplicates.

Cell growth was also assayed by autoradiography. Stable primary and purified secondary cultures grown on glass cover slips were treated for 72 h with various concentrations of mitogens. Tritiated thymidine ($5 \mu\text{Ci/ml}$) was added to the medium from 48 to 72 h after culture. In primary cultures, only cells with Schwann cell phenotype were counted. Stimulation index (SI, percent of labeled cells in stimulated cultures per percent of labeled cells in unstimulated cultures) was evaluated for each condition.

Immunofluorescence

For double-labeling, living cells grown on cover slips were first incubated with monoclonal antibody against rat cell-surface antigen (Thy 1-1) for 30 min and subsequently treated with an anti-mouse IgG coupled to fluorescein. The cover slips were then fixed in 2% formaldehyde in 0.1 M phosphate buffer, pH 7.4, and incubated with goat antiserum against fibronectin for 30 min. After incubation with anti-goat IgG coupled to rhodamine, the cover slips were examined with both rhodamine and fluorescence filters in a Zeiss photoscope III equipped with epifluorescence. For labeling of intracellular fibronectin, the cover slips were first fixed in acetone and treated as described above.

Cell Adhesion

Trypsinized cells were plated on collagen-coated bacteriological plastic dishes (14) in the presence of Eagle's minimal essential medium containing 200 μg of bovine serum albumin (BSA)/ml and fibronectin where indicated. After 1 h, the unattached cells were removed and the attached cells were trypsinized and counted in an electronic cell counter.

Migration Test

The migratory response of both primary and subcultured Schwann cells was

tested in Boyden blind well chambers (Neuro Probe Inc., Bethesda, MD, model 025-187) (25). Since Schwann cells, like fibroblasts (25), do not attach to the polycarbonate filters (Nuclepore Corp., Pleasanton, CA), the filters were pre-coated with either gelatin (10–20 mg/l) (10) or fibronectin (20 $\mu\text{g/ml}$).

Schwann cells were detached from the tissue culture flask with 0.1% (wt/vol) trypsin (Worthington Biochemicals Corp., Freehold, NJ) and suspended in DMEM containing 1 mg/ml of BSA (Reheis Chemical Co., Phoenix, Ariz.). The cell suspension (0.2 ml at 3×10^5 cells/ml) was introduced in the top compartment. Attractant solutions (0.030 ml) were introduced into the bottom wells of the Boyden chambers and the filters were placed above them. The chambers were incubated for 4 h at 37°C in a humidified atmosphere of air with 5% CO_2 . The filters were finally stained with Dipp-Quick (Harleco, Inc., Puerto Rico) and mounted on slides. Cells that did not migrate through the filter were wiped off the upper surface with a cotton swab. Migrated cells were counted randomly in 15 consecutive fields at $\times 400$. Assays were run in triplicate or quadruplicate. At different time intervals, membranes were fixed and processed for scanning electron microscopy as described before (8) and examined in an ETEC Autoscan.

RESULTS

We first investigated whether cultured rat Schwann cells contained fibronectin using two different immunofluorescent probes (Fig. 1). As expected in primary Schwann cells, contaminating fibroblasts (10%) identified by the presence of the Thy 1-1 antigen (Fig. 1a) all expressed fibronectin on the cell surface, within the cytoplasm (Figs. 1b and 2a), and in the footprints left by the cells on the substrate (Fig. 2a, inset). Unlike the fibroblasts, however, only a few Schwann cells (<10% of total Schwann cell number) stained with antibodies against fibronectin. This staining was focal and seemed to be membrane-associated (Fig. 2b). Since we observed no fibronectin staining in secondary Schwann cells (Fig. 3a), it is possible that the fibronectin present on some of the primary Schwann cells originated from the fibroblasts. In contrast, we found a dense fibrillar fibronectin network on Schwann cells

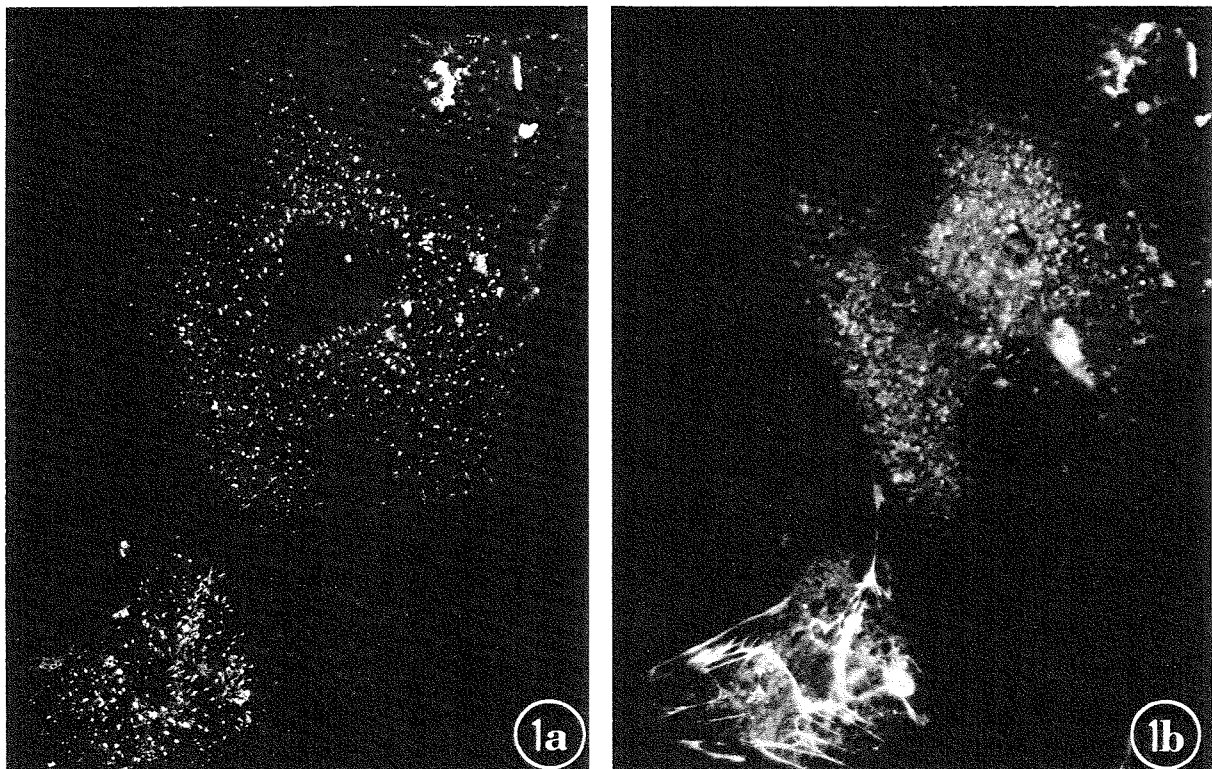


FIGURE 1 Double labeling of Thy 1-1 antigen with fluorescein-conjugate (a) and of fibronectin with rhodamine-conjugate (b) in fibroblasts contaminating primary rat Schwann cell cultures. a shows the typical punctuate distribution of Thy 1-1 antigen on two large fibroblasts. b shows the fibronectin labeling in these same fibroblasts; the right one has cytoplasmic (granular) fibronectin, while the left one shows surface fibrillar fibronectin. $\times 550$.

within 1 d after the addition of 20 $\mu\text{g}/\text{ml}$ of fibronectin to the culture (Fig. 3 *b*). While the overall morphology of the individual Schwann cell did not change, the cells treated with fibronectin had a tendency to cluster and this response was fibronectin dose-dependent.

Fibronectin not only bound to the Schwann cells but also stimulated their growth. We observed that pure Schwann cells incubated with fibronectin had a doubling time of 3 d, while untreated cells had a doubling time of 7 d. We evaluated the stimulation index by autoradiography: both primary and pure secondary Schwann cells increased their cell number severalfold in response to fibronectin in a time- (data not shown) and dose-dependent manner (Fig. 4). Primary cells responded to concentrations as low as 5 $\mu\text{g}/\text{ml}$, with maximal activity at 10 $\mu\text{g}/\text{ml}$, and a threefold increase in cell number over controls was observed. Second-passage cells required 20 $\mu\text{g}/\text{ml}$ for maximal cell proliferation and the cell number increased greater than fourfold. This is the first demonstration of fibronectin promoting cell growth. Soluble collagen (20 $\mu\text{g}/\text{ml}$), which is known to stimulate fibronectin binding to fibroblasts (28), slightly exacerbated the effect of fibronectin (data not shown).

Schwann cells do not normally proliferate well in culture unless specific mitogens are present. The effect of 20 $\mu\text{g}/\text{ml}$ of fibronectin on Schwann cell growth was compared to that of various other mitogens (Fig. 5). Primary cells maintained in

DMEM-FCS supplemented with fibronectin divide threefold more than untreated cells, whereas, as previously described, when stimulated with cAMP they divide only two and a half times more (27). Secondary cells treated with fibronectin divided fourfold more than the control cells. This stimulation is superior to 6 $\mu\text{g}/\text{ml}$ of bovine pituitary extract or 1 $\mu\text{g}/\text{ml}$ of cholera toxin but remains less effective than their combination, which is routinely used at the same concentrations to grow Schwann cells in our laboratory. Primary Schwann cells do not divide in defined medium but have a stimulation index of 2.2 when this medium is supplemented with 20 $\mu\text{g}/\text{ml}$ of fibronectin.

We then investigated whether fibronectin could influence Schwann cell adhesion and migration. Fibronectin had a small (~25%) but significant effect on cell adhesion to collagen (data not shown) and clearly stimulated both primary and secondary Schwann cell migration in a dose-dependent manner (more than a threefold increase at 5 $\mu\text{g}/\text{ml}$) (Table I). The checkerboard analysis (Table I) shows that the cells migrate in a chemotactic manner to a gradient of fibronectin. In this test, filters were pretreated with fibronectin (20 $\mu\text{g}/\text{ml}$) (to ensure maximal cell adhesion) which elevates the random migration of the cells in the absence of added fibronectin in the Boyden chamber. The cells migrate maximally towards a positive gradient of fibronectin (top row) while less motility is observed when equal concentrations are present on both sides of the

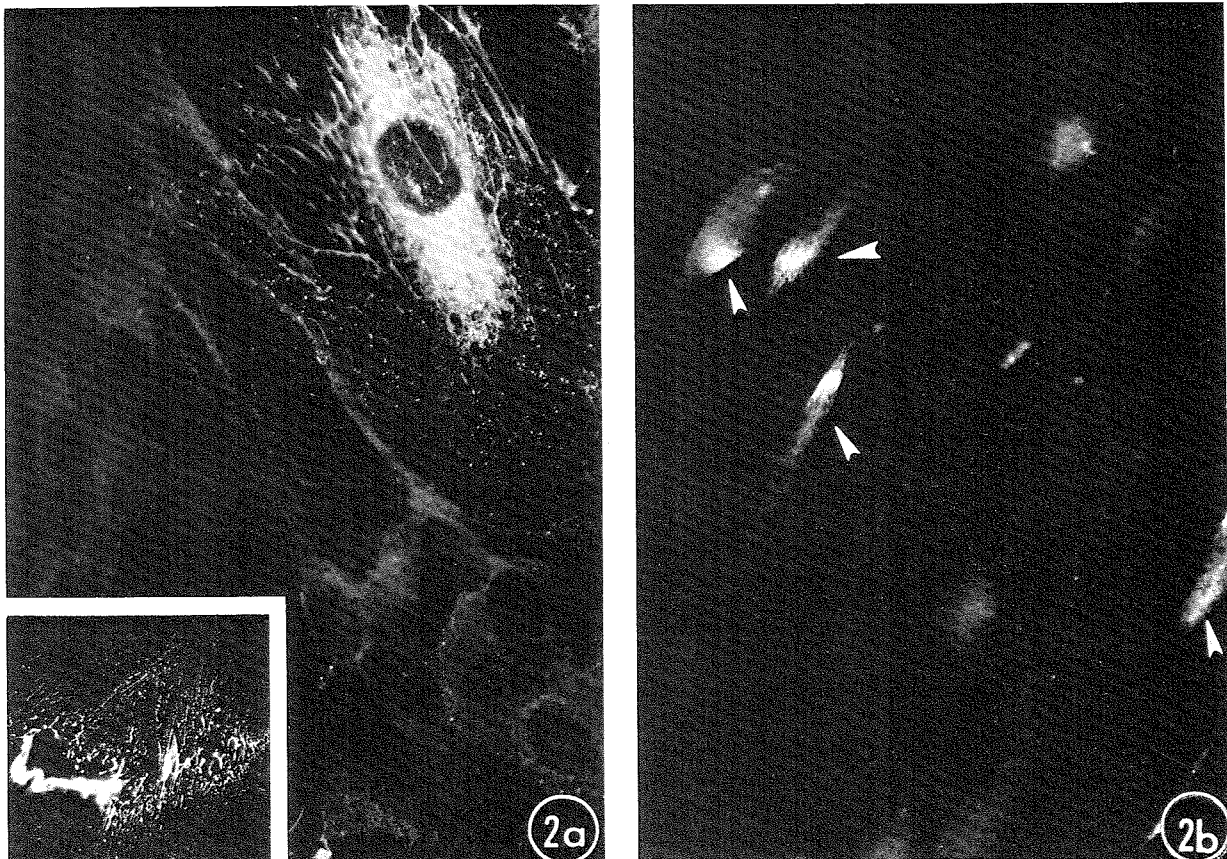


FIGURE 2 Immunofluorescence staining of endogenous fibronectin in primary rat Schwann cells. In *a*, after acetone fixation, one fibroblast stained with antifibronectin antibody shows bright perinuclear fibronectin as well as fibrillar surface fibronectin, whereas a large group of Schwann cells remain unstained. Acetone extraction has altered the usual spindle shape of the Schwann cells. $\times 380$. *Inset*: after living cell incubation, fibroblasts sometimes detach and leave fibronectin fibrils on the glass, forming footprints at the level of the surface adhesive sites. $\times 150$. *b* shows scattered spindle shaped Schwann cells after formaldehyde fixation. Three cells in the upper left corner and one at the right show a focal staining for fibronectin (arrowheads) which seems to be membrane-associated. The same Schwann cells did not stain with Thy 1-1 antibody. $\times 530$.

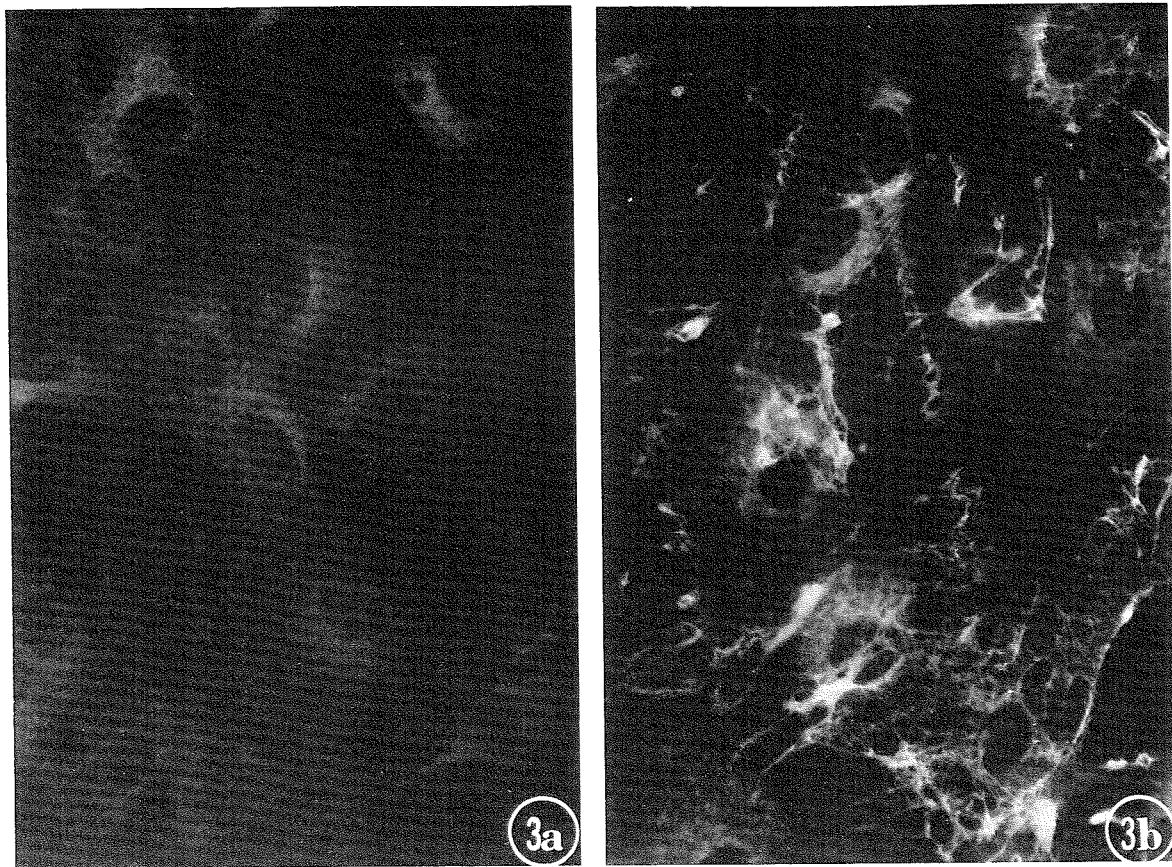
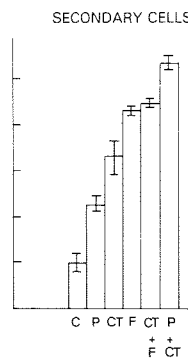
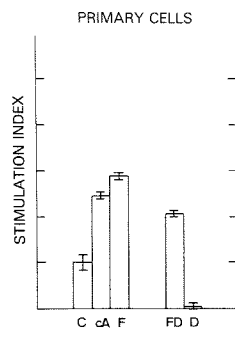
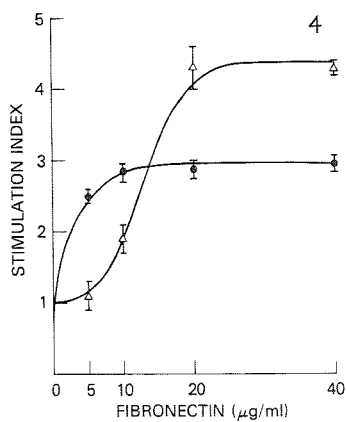


FIGURE 3 Immunolabeling of exogenous fibronectin in pure secondary rat Schwann cells. Cells were treated with cholera toxin and exhibit the typical flat phenotype induced by this mitogen. (a) A large group of cells before treatment with fibronectin. There is no endogenous fibronectin stain in secondary cells. (b) The same cells now covered with fibrillar fibronectin on their surface after growth for 3 d in the presence of exogenous fibronectin ($20 \mu\text{g/ml}$). $\times 380$.



FIGURES 4 and 5 Stimulation index of primary and purified secondary Schwann cells as measured by radioautography. Each value is the average of duplicates \pm their SD (as indicated). Fig. 4: Effect of the various concentration of chicken fibronectin on primary (●) and secondary (▲) Schwann cell growth. Fig. 5: Comparison of fibronectin with other mitogens. Cells are grown in serum medium (C). cAMP (cA) was added at a concentration of 10^{-5} M and fibronectin (F) at $20 \mu\text{g/ml}$. The right part of the primary cells histogram shows results with cells grown in defined medium without (D) or with fibronectin (FD). In the secondary Schwann cell histogram, pituitary factor (P) was added at a concentration of $6 \mu\text{g/ml}$, cholera toxin (CT) at a concentration of $1 \mu\text{g/ml}$ and fibronectin (F) at $20 \mu\text{g/ml}$ as for primary cells. When two mitogenic factors were added, they were used at the same concentrations as indicated above.

filter (diagonal) or when fibronectin is present only in the upper chamber (left column). When filters were not pretreated with fibronectin, random migration was considerably reduced, and the overall migration towards fibronectin ($20 \mu\text{g/ml}$) showed a much larger increase in the response in a dose- as well as in a time-dependent manner (Fig. 6) and up to 40-fold. These data indicate that fibronectin is a real chemoattractant for Schwann cells since they migrate directionally towards fibronectin.

DISCUSSION

We report here that the majority of rat Schwann cells will not retain fibronectin on the cell surface unless it is added exoge-

nously. While the added fibronectin had little effect on cell morphology, we observed a large stimulation in cell number. The mitogenic effect of fibronectin was time- and dose-dependent. Exogenous fibronectin appeared to have a stronger mitogenic effect on secondary cells than on primary cells, perhaps due to the presence of endogenously produced fibronectin originating from the fibroblasts contaminating the primary cultures. Fibronectin was slightly more mitogenic than

TABLE I
Effect of Various Concentrations of Fibronectin on Purified Secondary Schwann Cell Migration *

Fibronectin in upper compartment		Fibronectin in lower compartment			
$\mu\text{g/ml}$			$\mu\text{g/ml}$		
0	234 \pm 51	428 \pm 68	484 \pm 75	735 \pm 14	710 \pm 21
0.3	181 \pm 11	265 \pm 53	507 \pm 27	665 \pm 32	604 \pm 29
1.2	128 \pm 39	369 \pm 41	361 \pm 26	494 \pm 43	524 \pm 47
4.7	131 \pm 54	193 \pm 42	346 \pm 12	463 \pm 33	474 \pm 57
12.9	163 \pm 51	236 \pm 50	325 \pm 24	442 \pm 12	419 \pm 24

* The number of Schwann cell migrating through each filter was quantified. Each value is the average of triplicates \pm their SD.

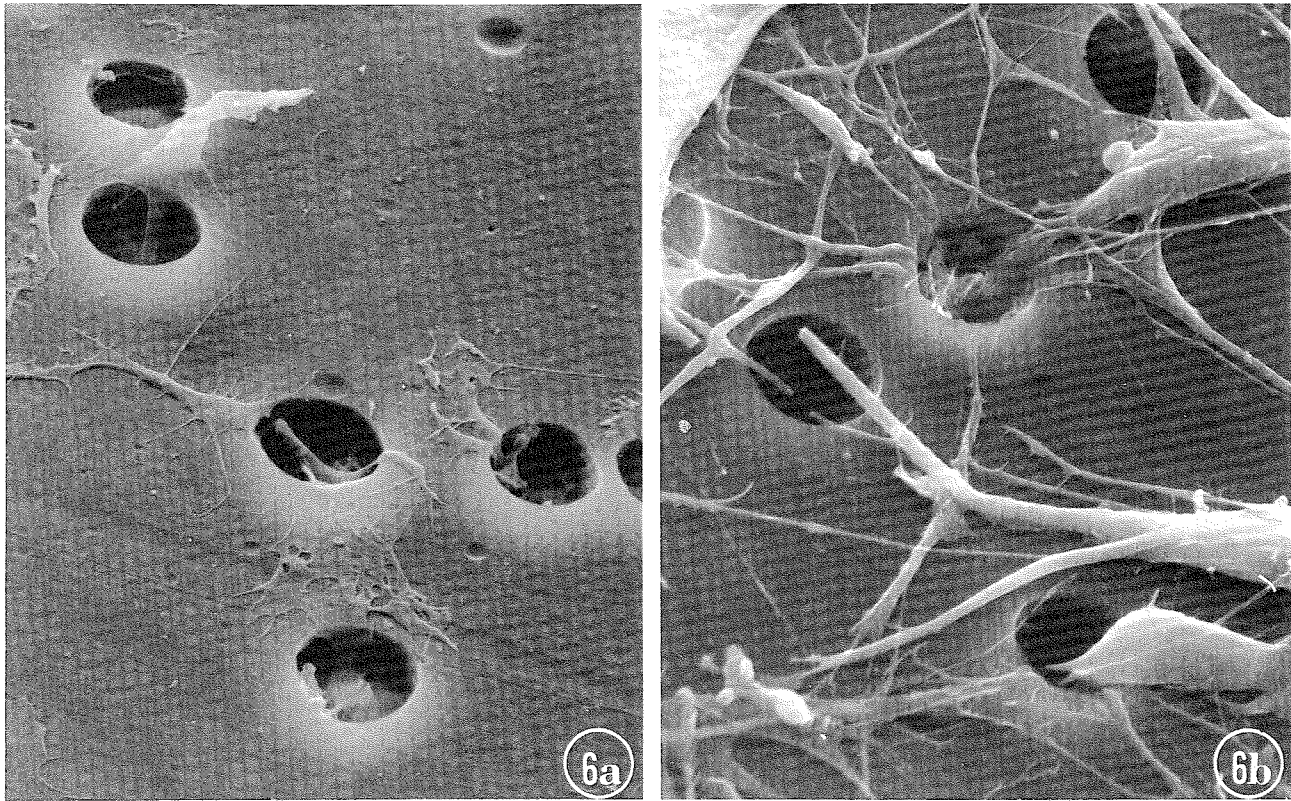


FIGURE 6 Scanning electron micrographs of the underside of the filter in the Boyden chamber assay. Pure Schwann cells added in the upper chamber, migrated toward fibronectin (20 $\mu\text{g/ml}$) present in the lower chamber. In a after 30 min, no cells are present but processes are seen emerging through the filter holes ($\times 2,030$). In b, after 4 h, many Schwann cells are identified on the lower part of the filter as a result of fibronectin-induced chemotaxis. $\times 2,160$.

low concentrations of pituitary factor or cholera toxin which is routinely used to stimulate Schwann cell growth (8). However, other studies have shown that highly purified pituitary extract or 500 $\mu\text{g/ml}$ of crude pituitary factor, alone or in combination with 10 $\mu\text{g/ml}$ of cholera toxin, had a more potent effect on mitosis, producing respectively a ten-, five-, and sixfold stimulation of Schwann cell growth (7, 26).

Treatment with both fibronectin and cholera toxin did not result in an enhanced Schwann cell mitosis over that seen with fibronectin alone. Both fibronectin (13) and cholera toxin (6) can bind to gangliosides, and it is thought that these glycolipids are the cell-surface receptors for these molecules. It is thus possible that fibronectin and cholera toxin are competing for the same receptors on the Schwann cell surface and that therefore these two mitogens are not able to have additive effects.

Fibronectin has not previously been reported to be a mitogen for any cells. Neuroblastoma cells (5) and rat follicular cells

(18) appear to require fibronectin for growth in defined medium. Since many cells must adhere before they can divide, it is likely that fibronectin is acting in the defined medium as an attachment glycoprotein. In the case of the primary Schwann cells, however, the cells are already adherent and fibronectin does not significantly cause them to flatten further. This suggests that, for Schwann cells, fibronectin may be a true mitogen.

The effect of a variety of mitogens has also been studied on Schwann cells isolated from dorsal root ganglia explants. In this different tissue culture system, cAMP and cholera toxin had variable effects on Schwann cell proliferation (36). However, it was shown that neurites and axolemma fractions were the best mitogens for these Schwann cell cultures, increasing tritiated thymidine incorporation from 10- to 20-fold (30, 36). In these organotypic cultures, the background of Schwann cell mitosis was much lower than in our purified Schwann cell cultures and thus comparison between these results and ours is difficult.

Fibronectin also appears to be a chemoattractant for Schwann cells. It is active at concentrations similar to those reported for fibroblasts (9), smooth muscle cells (11), and neural crest cells (10). Fibronectin is both chemotactic and chemokinetic for these cells (3). Although the directed migration of Schwann cells was stimulated somewhat less than twofold over that seen in the chemokinetic studies, fibronectin is clearly a chemoattractant for these cells as defined by the checkerboard analysis. Although our observations were done on Schwann cells grown in the absence of neurites, the role of fibronectin might be of biological importance in the repair of nerve injury.

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