

Preferential Outgrowth of Central Nervous System Neurites on Astrocytes and Schwann Cells as Compared with Nonglial Cells In Vitro

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ABSTRACT I have compared central nervous system (CNS) neurite outgrowth on glial and nonglial cells. Monolayers of glial cells (astrocytes and Schwann cells) or nonglial cells (e.g., fibroblasts) were prepared and were shown to be >95% pure as judged by cell type-specific markers. These monolayers were then tested for their ability to support neurite outgrowth from various CNS explants. While CNS neurites grew vigorously on the glial cells, most showed little growth on nonglial cell monolayers. Neurites grew singly or in fine fascicles on the glial cells at rates >0.5 mm/d. The neurite outgrowth on astrocytes was investigated in detail. Scanning and transmission electron microscopy showed that the neurites were closely apposed to the astrocyte surface and that the growth cones were well spread with long filopodia. There was no evidence of significant numbers of explant-derived cells migrating onto the monolayers. Two types of experiments indicated that factors associated with the astrocyte surface were primarily responsible for the vigorous neurite outgrowth seen on these cells: (a) Conditioned media from either astrocytes or fibroblasts had no effect on the pattern of outgrowth on fibroblasts and astrocytes, and conditioned media factors from either cell type did not promote neurite outgrowth when bound to polylysine-coated dishes. (b) When growing CNS neurites encountered a boundary between astrocytes and fibroblasts, they stayed on the astrocytes and did not encroach onto the fibroblasts. These experiments strongly suggest that molecules specific to the surfaces of astrocytes make these cells particularly attractive substrates for CNS neurite outgrowth, and they raise the possibility that similar molecules on embryonic glial cells may play a role in guiding axonal growth during normal CNS development.

Growing axons in the developing embryo and regenerating nerve are often in intimate association with glial and other non-neuronal cells, and it has been suggested that such cells play an important part in supporting and directing axonal growth. For example, *in vivo* investigations in the central nervous system (CNS)¹ have indicated that non-neuronal cells may play a role in guiding growing axons or migrating neurons in the developing retina, optic nerve, corpus callosum, and spinal cord (19, 24, 34, 38, 41).

¹ *Abbreviations used in this paper:* anti-MIg-Rd, rhodamine-conjugated anti-mouse Ig; anti-RIg-fl, fluorescein-conjugated anti-rabbit Ig; CM, conditioned media; CNS, central nervous system; GFAP, glial filament acidic protein; MEMH, HEPES-buffered minimal essential medium; NF, neurofilament; FN, fibronectin.

How might non-neuronal cells direct axonal outgrowth? One likely mechanism is by providing a particularly adherent substrate for the migrating growth cone (23). For example, an array of adhesive cells in the midst of a complex tissue containing other, less adherent cells could provide a specific avenue for growing axons. *In vivo* observations have supported the idea that glial or certain non-neuronal cells may be especially attractive substrates for axon growth. For example, regenerating axons in the *Xenopus* optic nerve have been shown to grow preferentially on glial cells compared with the surrounding connective tissue (4). In addition, a recent comparative study of early optic nerve formation has provided strong circumstantial evidence that retinal ganglion cell axons grow preferentially on nonpigmented as compared

with pigmented cells in the optic stalk (39). Further, ultrastructural studies have demonstrated that growth cones often show a strong tendency to migrate in direct association with glial cells (16, 19).

It seems likely that an understanding of the molecular basis of axonal guidance by glial and non-neuronal cells will require *in vitro* analysis. However, while striking interactions between CNS neurites and "non-neuronal cells" have been observed *in vitro* (14, 42), the difficulties in identifying and enriching for these cells have in the past hampered systematic study. More recently, as markers for these cells have become available, studies have indicated that glial cells can have dramatic effects on neuronal morphology (8), maturation (29), and positioning (15). However, there is little information about neurite outgrowth on identified, living cells in culture (26, 30). In the present study, I used a combination of cell type-specific antibodies and highly enriched populations of astrocytes, Schwann cells, and non-neuronal cells to investigate the specificity and character of neurite outgrowth on identified cell types in culture. I show that growth cones from the CNS have a strong preference for migration on glial cells as compared with many types of non-neuronal cells, and provide evidence that molecules associated with the glial cell surface, rather than soluble factors secreted by them, are primarily responsible for this preferential growth. Portions of this work have been presented in preliminary form (10, 11).

MATERIALS AND METHODS

Cell Cultures: All studies were performed with Sprague-Dawley rats obtained from the Imperial Cancer Research Fund animal facility at Mill Hill, London. The date a vaginal plug was detected was counted as embryonic day zero.

Astrocytes were prepared from newborn neocortex by a modification (30) of the method of McCarthy and DeVellis (28). Enzymatically dissociated cells were cultured for 8–10 d, then shaken overnight to remove neurons and oligodendrocytes. The remaining adherent cells were split 1:3 and then pulsed twice for 48 h with 10^{-5} M cytosine arabinoside to kill rapidly dividing leptomeningeal cells. The resulting astrocyte populations (Type I or protoplasmic; reference 33) were >95% pure as judged by staining with an anti-glial filament acidic protein (GFAP) antiserum (31). Leptomeningeal cell contamination was monitored by staining with an antifibronectin antiserum (anti-FN) (3). Staining with a monoclonal antineurofilament (NF) (43) and a monoclonal antigalactocerebroside (35) antibody established that no neurons or oligodendrocytes were present in these cultures.

Sciatic nerve Schwann cells were prepared according to the method of Brockes et al. (7) and were at least 95% pure as judged by staining with mouse anti-Ran-1 (1:100; reference 7). Contaminating fibroblasts were monitored with a mouse monoclonal anti-Thy1.1 (ascites, 1:500; reference 20). Skin fibroblasts were prepared from 2–4 d-old rats. The tissue was chopped into 1-mm³ pieces and incubated in 0.1% collagenase (Type CLS; Worthington Biochemicals, Freehold, NJ) for 45 min and then in 0.125% trypsin for 20 min. The cells were triturated and plated at a density of 50–100,000 cells per 25-cm² tissue culture flask.

The cells were passaged at least twice before use. Cultures that contained >95% fibroblasts as judged by staining with an anti-FN antibody were then used as test monolayers. Leptomeningeal cells were prepared by a similar protocol using meninges from newborn brain as the starting material. The cells obtained were <5% GFAP+ and >95% Ran-2+/fibronectin+ (3, 32).

All cells were fed three times weekly with Dulbecco's modified minimum essential medium supplemented with 10% fetal calf serum (Gibco Laboratories, Grand Island, NY), 6 g/liter glucose, 50 U/ml of penicillin-streptomycin, and 2.5 µg/ml of fungizone. Cells were removed from the dish with 0.5 mg/ml trypsin in 1 mM EDTA. Polylysine-coated dishes were prepared by overnight incubation in 5 µg/ml polylysine (Sigma Chemical Co., St. Louis, MO) in water. The dishes were rinsed three times in sterile distilled water and air-dried before use.

Preparation of Monolayers: Cells to be tested as monolayers were plated onto uncoated or polylysine-coated 35-mm Nunc (Nunc Labware, Roskilde, Denmark) or Falcon culture dishes (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, CA) in plastic cloning rings. Astrocytes and fibro-

blasts were plated at 5,000 or 20,000 cells per 4- or 8-mm (i.d.) rings, respectively, and Schwann cells at 20,000 cells per 4-mm ring. Two or three rings were set up per dish. The cells were allowed to attach for 24 h and then the rings were removed and the dishes washed with HEPES-buffered minimal essential medium (MEMH). The resulting islands of cells were then incubated for at least 1–3 d before use. In some experiments 5,000 astrocytes were plated inside a 4-mm ring and 5,000 fibroblasts or astrocytes were plated outside. After the cells had settled down, the rings were removed and the cells were allowed to migrate to fill the intervening 1-mm gap and thus form a continuous monolayer. The integrity of the astrocyte-fibroblast border was monitored in several experiments by double labeling with anti-FN and anti-GFAP (see below). The amount of intermingling of the two cell populations was found to be minimal over the time course of these experiments. Before use, the monolayers were normally irradiated with 2,000 rad from a ⁶⁰Co source to inhibit further cell division.

Explants: Pregnant rats were killed with an overdose of ether or a blow to the head and the embryos were removed aseptically. All dissections were carried out in MEMH. For retinal cultures, eyes were removed and the neural retina was dissected free of other ocular tissue. The retinas were then placed in the culture dish and cut into two or four pieces, each of which contained part of the optic disk. The pieces were placed vitreal side down on top of the preformed cell monolayers in 600–700 µl of medium.

Spinal cords were dissected from E15 rats and freed of meninges and dorsal root ganglia. Cerebral cortex, hippocampus, cerebellum, and spinal cord (hemisected) were dissected free of meninges and cut into 1-mm³ pieces and placed on the monolayers.

The extent of neurite outgrowth was routinely measured on living cultures with phase-contrast optics. Some living explants were scored first by phase-contrast microscopy and then immediately fixed and remeasured after staining with an anti-NF antibody (see below). The outgrowth measured by both means agreed, indicating that scoring by phase-contrast microscopy was valid.

Conditioned Media (CM): CM was obtained from confluent cultures of astrocytes or fibroblasts ($1-3 \times 10^5$ cells/75 cm² flask) incubated for 4–7 d in complete media. For studies on soluble factors, the CM was diluted 1:1 with fresh medium before use. For testing substrate-bound factors, undiluted CM was incubated on polylysine-coated Nunc dishes overnight at 4°C in a 5% CO₂ atmosphere. The dishes were washed twice in MEMH and the explants were then added in fresh or conditioned media. Neurite outgrowth was measured 24 h later.

Immunofluorescence and Immunoperoxidase Labeling: The antibodies used in this study have been previously described. For immunoperoxidase labeling, cultures were fixed in methanol and incubated with a mouse monoclonal anti-NF antibody (RT97 ascites, 1:50; reference 43), then incubated with an affinity-purified horseradish peroxidase-coupled goat anti-mouse immunoglobulin (1:40, Tago Inc., Burlingame, CA). The cells were then postfixed with 1% glutaraldehyde (Fluka A. G., Basel, Switzerland) in 0.1 M PO₄, pH 7.4, for 5 min at room temperature and incubated with 0.5 mg/ml diaminobenzidine in 50 mM Tris-HCl, pH 7.6, with 0.01% H₂O₂. The reaction was stopped by rinsing in the same buffer without diaminobenzidine or H₂O₂.

For simultaneous localization of astrocytes and neurites, cultures were rinsed twice with MEMH, fixed for 5 min in methanol at –20°C, rehydrated, and incubated with rabbit anti-GFAP (1:1000; reference 31) and anti-NF antibody. The dishes were rinsed three times and incubated with a fluorescein-conjugated sheep anti-rabbit immunoglobulin (sheep anti-Rlg-fl, 1:100; Wellcome Laboratories, Kent, England or Sigma Chemical Co.) and a rhodamine-conjugated goat anti-mouse Ig (goat anti-MIg-Rd, 1:80; Cappel Laboratories, Inc., Cochranville, PA). The sheep anti-Rlg-fl and goat anti-MIg-Rd were preabsorbed with Affigel-bound (Bio-Rad Laboratories, Richmond, CA) MIg or Rlg, respectively, to remove cross-reacting antibodies. To localize fibroblasts and neurites simultaneously, I labeled living cultures with a rabbit anti-FN antiserum (1:100; reference 3), fixed them in methanol, and then labeled them with anti-NF and appropriate conjugates as above. To label neurons, type II (fibrous) astrocytes, and immature glial cells, I stained cultures with the mouse monoclonal antibody A₂B₃ as described (1:50 ascites; reference 33). All antibodies were diluted in MEMH with 20% fetal calf serum and 0.02% sodium azide. Cultures were mounted either in glycerol or in glycerol with a reagent to inhibit fluorescein bleaching (Citifluor; Department of Chemistry, City of London University).

Cultures stained for immunofluorescence were examined on a Zeiss microscope equipped for phase-contrast and epifluorescent illumination with appropriate dichroic mirrors and barrier filters for rhodamine and fluorescein. Photographs were taken on Kodak Tri-X or Ektachrome 400 film at 400 ASA. Living and peroxidase-stained cultures were examined on a Leitz orthoplan inverted microscope with phase-contrast or dark-field optics and photographed on Ilford PAN-F film.

Electron Microscopy: For transmission electron microscopy cultures grown on plastic dishes were gently rinsed twice in prewarmed (37°C) buffer (0.12 M sucrose, 0.5 mM CaCl₂, and 0.075 M phosphate, pH 7.3). The cultures

were then fixed in warm 2% glutaraldehyde in the same buffer for 30 min at room temperature, rinsed with 0.1 M phosphate buffer (pH 7.3), and postfixed for 15 min on ice in 1% OsO₄ in the same buffer. The dishes were rinsed several times with buffer and then with distilled water, stained en bloc with 1% aqueous uranyl acetate for 1 h in the dark, dehydrated through alcohols, and embedded in araldite. After polymerization, the blocks were viewed in the light microscope and areas of interest were cut out and remounted in the desired orientation, usually normal to the plane of the dish and the axis of neurite outgrowth. Thin sections were stained with uranyl acetate and lead citrate and viewed on a JEOL 100CX electron microscope at 80 kV.

Cultures for scanning electron microscopy were grown on polylysine-coated glass coverslips and fixed for 30 min in glutaraldehyde as above, and then for an additional 24–48 h at 4°C. They were then rinsed in phosphate buffer and postfixed for 30 min at room temperature in 1% OsO₄ in phosphate buffer, dehydrated through alcohols, and critical-point-dried with liquid CO₂. The samples were then coated with 15 nm of gold-palladium and viewed in a Cambridge S4-10 scanning electron microscope at 15 kV.

RESULTS

Retinal Neurites Grow on Astrocytes But Not Fibroblasts

The outgrowth of neurites from E15 retina onto astrocyte monolayers was dramatic (Fig. 1). Growth began within 6 h of plating and proceeded at a rate between 0.6 and 1 mm/d (see Table I). The outgrowth pattern was radial and the bulk of the outgrowth emanated from the site of the optic disk. The neurites formed fine fascicles measuring 2–3- μ m diam on these monolayers. Larger bundles of neurites were rarely observed when neurites were in contact with the astrocytes. Fig. 1 also shows a culture that has been double stained with anti-GFAP and anti-NF antibodies to allow a fuller appreciation of the underlying astrocyte monolayer and the extent of neurite outgrowth. Although the fascicles of neurites grew in a generally radial fashion, on a finer level it could be seen that the course of these fascicles was crooked (see also Figs. 4 and 6c). This figure also shows that the underlying monolayer was not markedly perturbed by the neurites at these early times.

Scanning electron microscopy of retinal neurites growing on astrocyte monolayers revealed the predominance of single neurites and small fascicles in the outgrowth zone (Fig. 2a). Scanning micrographs also showed the close adherence of the neurites to the surface of the monolayer. At the outgrowth front, the neurites were tipped by prominent growth cones. These growth cones often had filopodia that were 10–15- μ m long. Broad, thin lamellapodia were also a common feature of these organelles (Fig. 2).

In thin sections, neurites were readily identified by their size (0.2–0.6 μ m) and their characteristic microtubules (Fig. 3). Bundles of 10-nm glial filaments were prominent in the astrocytes that formed the monolayer. The neurites were often flattened when they were in apposition to the astrocytes. Fig. 3 also shows the intimate association between the dorsal surface of the astrocytes and the neurites. The small fascicles of neurites were in close apposition to the monolayer. There was no indication of ensheathment by glial elements.

Frequent monitoring of living cultures by phase-contrast microscopy failed to reveal any explant-derived cells migrating onto the astrocyte monolayers either before or in concert with the front of the growing neurites. Furthermore, when cultures were stained with the monoclonal antibody A₂B₅, which labels neurons, glial precursor cells, and type II (fibrous) astrocytes (33), only two elements were stained: the neurites and a few dark process-bearing cells found within 100–200 μ m of the explant border (not shown).

In striking contrast to the lush outgrowth on astrocytes, there was very little growth of retinal neurites onto monolayers of skin fibroblasts (Fig. 1b) even after 5–6 d (the longest period examined). This lack of growth was not due to neuronal cell death since fascicles of neurites were seen at the periphery of the explant (Fig. 1b), and explants grown on fibroblasts for 24 h and then transplanted onto astrocytes showed profuse outgrowth within the next 24 h (not shown). Several other non-neural cell types—fibroblasts derived from sciatic nerve, lung and skeletal muscle, the epithelial line PTK-1, the fibroblastic lines 3T3, and Rat-1 and leptomeningeal cells—gave results similar to those seen with skin fibroblasts.

Neurites from Other CNS Regions

The only CNS tissue tested that showed appreciable neurite growth on fibroblasts was E15 spinal cord. As illustrated in Fig. 4, spinal cord showed extensive neurite outgrowth on astrocytes, and small but consistent growth onto the fibroblasts. Neurite growth on fibroblasts was much slower than that seen on astrocytes and was mostly in the form of a few fascicles.

Explants from E15 cerebral cortex gave qualitatively the same results as the retinal explants: vigorous neurite outgrowth on astrocytes (Fig. 4c), but little on skin fibroblasts. However, the pattern of outgrowth on astrocytes was more irregular than that seen with retina. There was no readily defined outgrowth front as the length of neurites from the same explant varied by several hundred microns after 48 h. Explants of E20 cerebellum gave results similar to those seen with the cerebral cortex. However, the situation was more complex, since large numbers of phase-dark process-bearing cells (presumably granule cell precursors) migrated onto the monolayers behind the neurite outgrowth front (Fig. 4d). Explants of E20 hippocampus gave results similar to those seen with cerebellum (not shown).

The outgrowth characteristics of the CNS explants on astrocytes and fibroblasts were independent of the nature of the artificial substrate on which the monolayers were growing: similar results were obtained if the culture dishes were coated with polylysine or collagen. In addition, similar neurite outgrowth on astrocytes was seen if serum-free medium N1 was used (6). However, the cell monolayers had to be alive to promote neurite outgrowth: astrocyte or fibroblast monolayers fixed for 5 min with 0.01–1% glutaraldehyde, 0.5–4% formaldehyde, methanol, or 70–100% ethanol at room temperature or –20°C, or heat (56°C for 30 min) would not support neurite outgrowth, even in the presence of astrocyte CM.

Growth of Retinal Explants on Schwann Cells

Retinal neurites grew vigorously on monolayers of highly enriched sciatic nerve Schwann cells. As in the case of astrocytes, the neurites routinely extended over 500 μ m from the explant border within 24 h after explanting. Furthermore, where the neurites were in contact with underlying Schwann cells the growth was in the form of fine fascicles (Fig. 5). However, unlike astrocytes, the Schwann cells underwent extensive migration when contacted by the growing neurites, the cells often clearing away from the area around the explant, lining up along the neurites, and piling up near the outgrowth front. Because of this behavior, reliable quantitation of growth rates on these cells was not feasible.

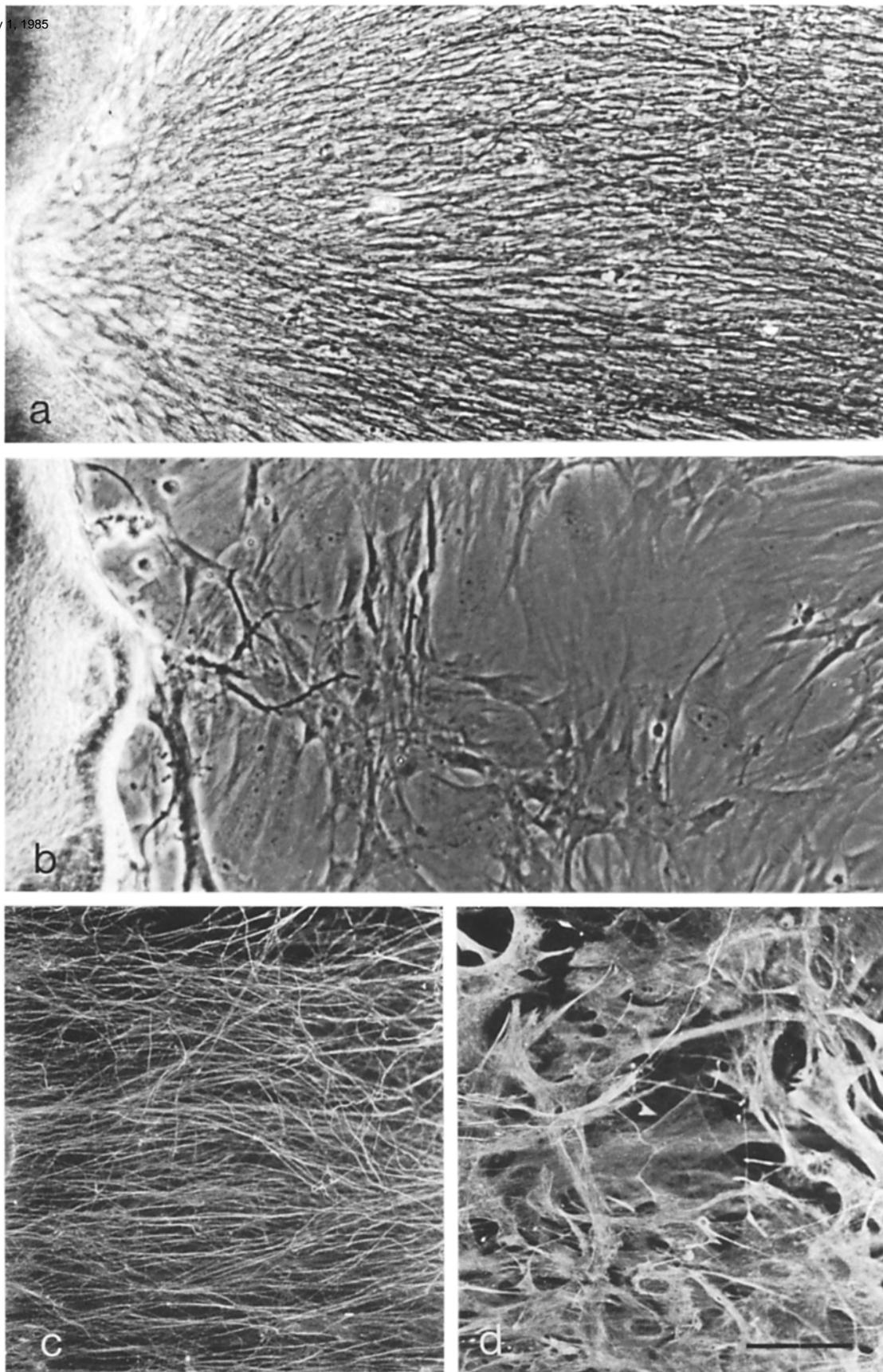


FIGURE 1 Retinal neurites grow vigorously on astrocytes but not fibroblasts. E15 retinal explants were placed on preformed monolayers of astrocytes (*a*, *c*, and *d*) or fibroblasts (*b*) and cultured for 48 h. *a* and *b* were labeled with a mouse monoclonal anti-NF antibody followed by a horseradish peroxidase-coupled goat anti-mouse IgG second antibody, processed with diaminobenzidine as described in Materials and Methods and viewed with phase-contrast optics. The optic disk region of the explants (*left*) is shown in both fields. *c* and *d* show a field from a culture similar to that in *a*, but which has been double labeled with mouse anti-NF and rabbit anti-GFAP followed by goat anti-MIg-Rd and goat anti-RIg-fl and viewed with rhodamine (*c*) or fluorescein (*d*) optics to visualize the neurites and underlying astrocyte monolayers, respectively. Bar, 100 μm ; $\times 175$.

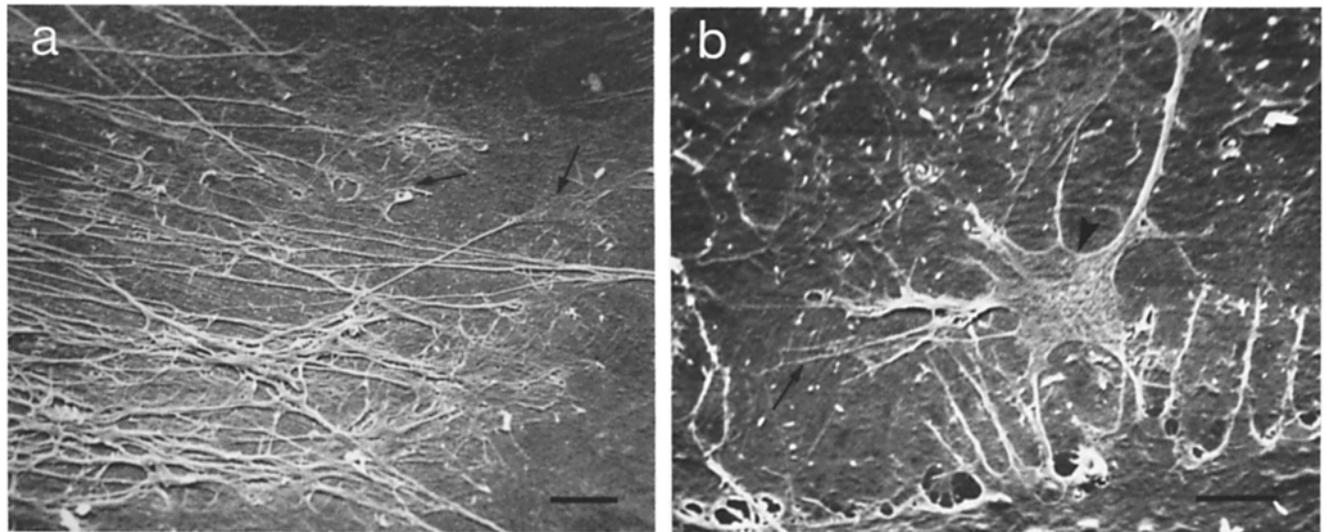


FIGURE 2 Scanning electron micrographs of retinal neurite outgrowth on astrocyte monolayers. (a) Overview of outgrowth front. Note the fine neurite fascicles, single neurites, and broad, flattened growth cones (arrows). Bar, 10 μm . $\times 900$. (b) Higher-power view of growth cone on astrocyte monolayer. Note prominent lamellapodium (arrowhead) and long filopodia (e.g., long arrow). Bar, 5 μm . $\times 2,200$.

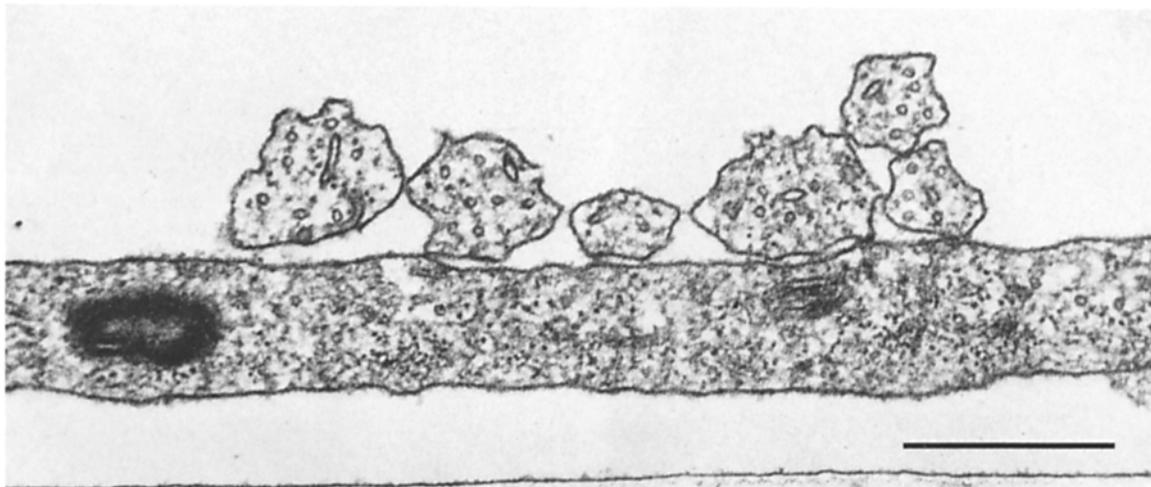


FIGURE 3 Transmission electron micrograph of retinal neurites on an astrocyte monolayer. Note the prominent microtubules in the neurites, and the abundant 10-nm glial filaments (seen in cross section) in the underlying astrocyte. Note also the close apposition of the neurite and glial cell membranes. Bar, 1 μm . $\times 28,000$.

Retinal Neurite Outgrowth Is Dependent on Explant Age

The vigorous and rapid outgrowth of retinal neurites onto astrocytes or Schwann cells was only observed when explants were taken from E14 to E16 embryos. If explants were taken from E13 animals, there was an 18–24 h delay in the onset of neurite outgrowth. Explants from E18 animals showed a much reduced neurite outgrowth, and explants from newborn animals showed only fine neurites that grew only 100–200 μm in 48 h. Neurons from these older explants migrated onto the monolayers and survived for at least 2 wk, but few long neurites were observed.

Role of the Glial Cell Surface in Neurite Outgrowth

To determine whether the dramatic differences in the capability of glial and non-neural cells to support CNS neurite

outgrowth reflected cell-cell interactions mediated by surface-bound or soluble molecules, I carried out two types of experiments. First, growing retinal neurites were confronted with a choice of astrocytes or fibroblasts in the same culture dish. Second, the effects of soluble and substrate-bound CM factors from either astrocytes or fibroblasts were tested for their effect on retinal neurite outgrowth. Astrocytes were used for these experiments as they form more complete and stable monolayers than the Schwann cells, thus allowing for more reliable quantitation.

Choice Experiments

In the choice experiments, continuous monolayers of astrocytes and fibroblasts were set up as described in Materials and Methods. The well-defined border between the adjacent astrocytes and fibroblasts (e.g., Fig. 7) was stable for 2–3 d. When retina explants were plated onto the astrocyte side of the monolayer, the neurites grew in their characteristic radial,

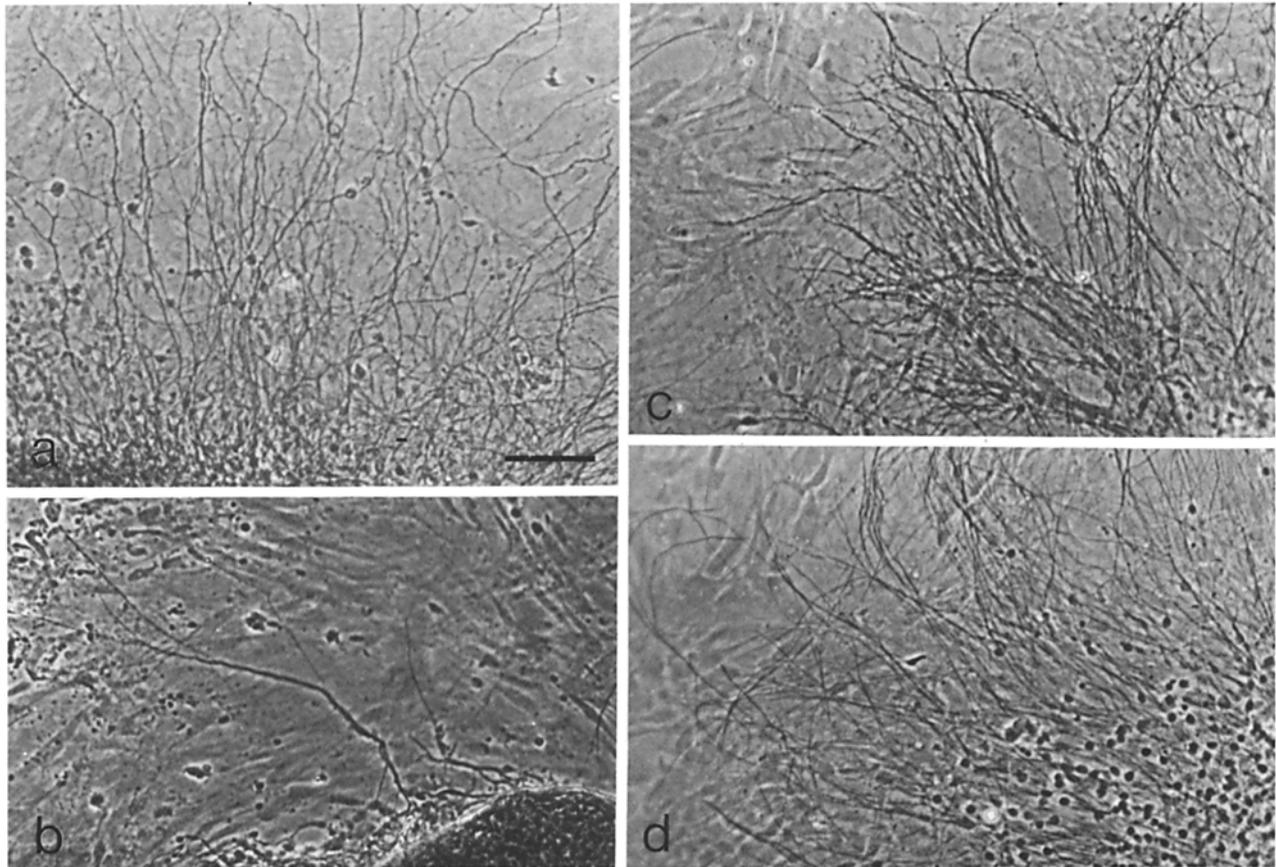


FIGURE 4 Outgrowth of neurites from other CNS regions on astrocytes or fibroblasts. Explants were cultured for 40–48 h and the neurites were visualized with horseradish peroxidase as in Fig. 1, a and b. E15 spinal cord explants showed extensive neurite outgrowth on astrocytes (a) and a small but consistent outgrowth on fibroblasts (b). Explants from E15 cerebral cortex (c) and E20 cerebellum (d) show vigorous neurite outgrowth on astrocyte monolayers. Neither of these explants displayed appreciable neurite growth on fibroblast monolayers. Phase-contrast optics. Bar, 100 μm . $\times 120$.

finely fasciculated manner. However, as seen in Figs. 6 and 7, when these neurites encountered the fibroblast border they stopped or turned and often formed bundles that ran parallel to the astrocyte-fibroblast frontier. This turning or stopping behavior was not observed when retinal neurites crossed an astrocyte-astrocyte border. The growth of neurites on the nonborder regions of the monolayer was not affected. Fig. 7 shows a region of the border where the fibroblasts were not confluent; note that the neurites grew neither on the fibroblasts nor on the polylysine-coated substratum, but stayed on the astrocytes. Retinal explants placed on the fibroblast side of the frontier exhibited little neurite outgrowth, even when they were placed within 500 μm of the astrocyte monolayer.

The choice experiments strongly suggested that the predominant influence of the astrocytes on the growing neurites was localized, and not due to soluble factors. To test this possibility further, I performed CM experiments. The explants were set up as described but grown in the presence of 50% fresh media and 50% CM from either fibroblasts or astrocytes. The results summarized in Table I show that there was no outgrowth of retinal neurites on fibroblast monolayers, even in the presence of astrocyte CM. In addition, CM from fibroblasts did not inhibit neurite outgrowth from retinal explants grown on astrocyte monolayers. Moreover, the degree of fasciculation was not appreciably affected by the CM.

The ability of CM factors to promote neurite outgrowth when bound to polylysine-coated dishes was also tested. Such

factors from astrocytes, fibroblasts, or PTK-1 cells would not promote outgrowth from retinal explants. Moreover, if astrocytes were removed from the dishes by scraping or with 1 mM EDTA or EGTA before the addition of the explants, the material remaining on the dish did not promote retinal neurite outgrowth, even in the presence of astrocyte CM.

DISCUSSION

The results reported here demonstrate that CNS neurites grow preferentially on astrocytes and Schwann cells as compared with a range of non-glial cells *in vitro*. Several observations suggest that molecules associated with the glial cell surface, rather than soluble secreted molecules, are responsible for the ability of glial cells to promote CNS neurite outgrowth. First, the pattern of neurite growth on the glial cells was similar to that seen when neurites are grown on very adherent artificial substrates (17, 22): the growth cones were flattened and had long filopodia, the neurites grew in fine fascicles, and though the overall growth pattern was radial, the neurites traced crooked paths (Figs. 2, 4, and 6*b*). Second, when growing neurites encountered boundaries between astrocytes and fibroblasts, they abruptly altered their growth behavior. Neurites from the same explants that did not encounter the nonglial cells, or neurites that crossed astrocyte-astrocyte borders, showed no change in their growth characteristics. Third, CM experiments failed to demonstrate that astrocytes secrete

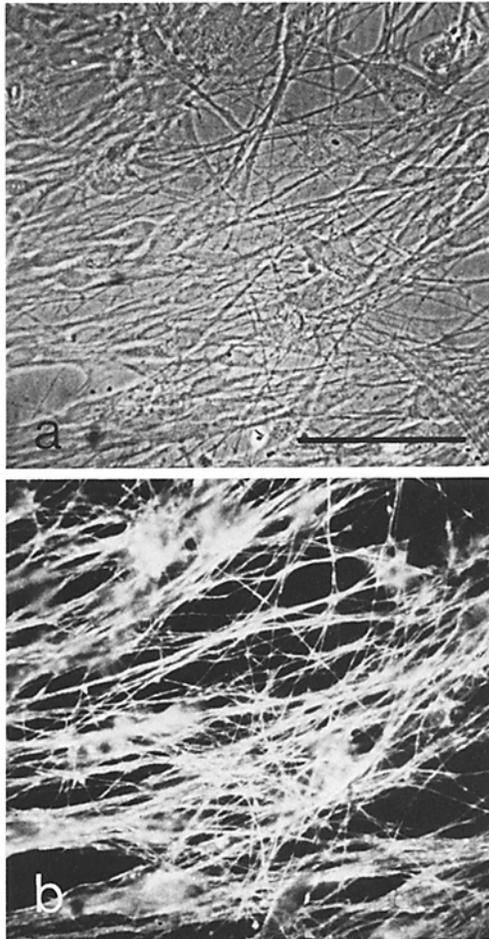


FIGURE 5 Retinal neurite outgrowth on sciatic nerve Schwann cells. A portion of the neurite outgrowth zone in a culture labeled with mouse anti-NF followed by goat anti-MIg-Rd is shown viewed with phase-contrast (a) and fluorescence (b) optics. The Schwann cells do not form a confluent monolayer and several gaps are evident. The neurite outgrowth is less regular than that seen on astrocytes (Fig. 1). Bar, 50 μm ; $\times 450$.

molecules that make nonglial cells or polylysine-coated surfaces attractive for CNS neurite outgrowth. Table I shows that the rate of neurite outgrowth was characteristic of the cells upon which they were growing, and independent of the media. Taken together, these results strongly suggest that cell surface-associated molecules are necessary for CNS neurite growth on glial cells. Similar conclusions have also been reached in studies using dissociated CNS neurons in vitro (12, 30). However, our results do not exclude the possibility that soluble factors secreted by glial cells also contribute to the ability of these cells to promote CNS neurite outgrowth, since killed astrocyte or fibroblast monolayers did not support neurite outgrowth from CNS explants.

What is the nature of the glial cell surface molecules that are so attractive to growth cones? In principle, they could be intrinsic plasma membrane molecules or cell-bound components of the extracellular matrix. Since the cultured astrocytes used here do not express either fibronectin (3) or laminin on their surface (reference 23 and unpublished observations), it is unlikely that these matrix molecules are involved. In addition, since the effects of glial cells are seen in serum-free medium, it does not appear that they are mediated by a cell-

bound serum component. Furthermore, astrocytes did not secrete molecules into the culture medium that could promote retinal neurite outgrowth when bound to artificial substrates. Other studies have also noted that while such molecules are potent stimulators of peripheral neurite outgrowth, intrinsic CNS neurites are not responsive to such substrate-bound factors (references 9, 21, and 27 and J. Fallon, manuscript submitted). Although the neural cell adhesion molecule (N-CAM) has been found to mediate adhesion between neurons or between neurons and muscle (36, 37), it has not been reported to play a role in mediating neuron-glial interactions (14). But, recent studies have indicated that N-CAM-like molecules may be present on some populations of glial cells (2, 36); therefore, a possible role of this molecule in the phenomena reported here should not be ruled out. A different cell adhesion molecule that could be involved in neuron-glial adhesion has recently been identified using an *in vitro* binding assay ("Ng-CAM"; reference 13). However, Ng-CAM appears to be present on the surface of neurons but not glial cells. It seems likely then that the glial cell surface components that mediate these neurite-glial cell interactions may be novel extracellular matrix or intrinsic plasma membrane molecules.

These *in vitro* findings should be reconciled with the well-established *in vivo* observations that peripheral but not central glia are capable of supporting regeneration of adult CNS or peripheral nervous system neurons (1). Differences in the maturity of the astrocytes being studied seems unlikely to account for the different results obtained *in vitro* and *in vivo* since astrocytes from adult brain are also capable of promoting neurite outgrowth from these embryonic CNS explants (J. Fallon and R. M. Lindsay, unpublished observations). It seems more likely then that the "inhibition" of extended, directed axon regrowth observed *in vivo* is due to the unfavorable geometry of the astrocyte scar rather than to some inhibitory property of the astrocyte surface, and that mature CNS neurons, while clearly capable of regeneration (1), may have different growth requirements than their embryonic counterparts. The observation that axons from grafted embryonic CNS neurons can grow through mature host CNS tissue (5) is consistent with this latter view.

The experiments presented here provide evidence for a role of the glial surface in directing CNS neurite outgrowth *in vitro*. They suggest that appropriately arrayed glial or non-neural cells may offer not only a favorable three-dimensional environment for growing axons (18, 40, 41), but also a preferred substrate pathway. In addition, the predilection for neurite outgrowth on glial cell substrates is not limited to neurons of CNS origin: peripheral nervous system neurites also show a strong preference for growth on glial cells (reference 11, and J. Fallon, submitted for publication). These peripheral neurites, however, do show significant differences in their response to nonglial cell substrates. Finally, the system presented here could form the basis of a quantitative assay for the biochemical identification of the molecules that mediate neurite-glial interaction.

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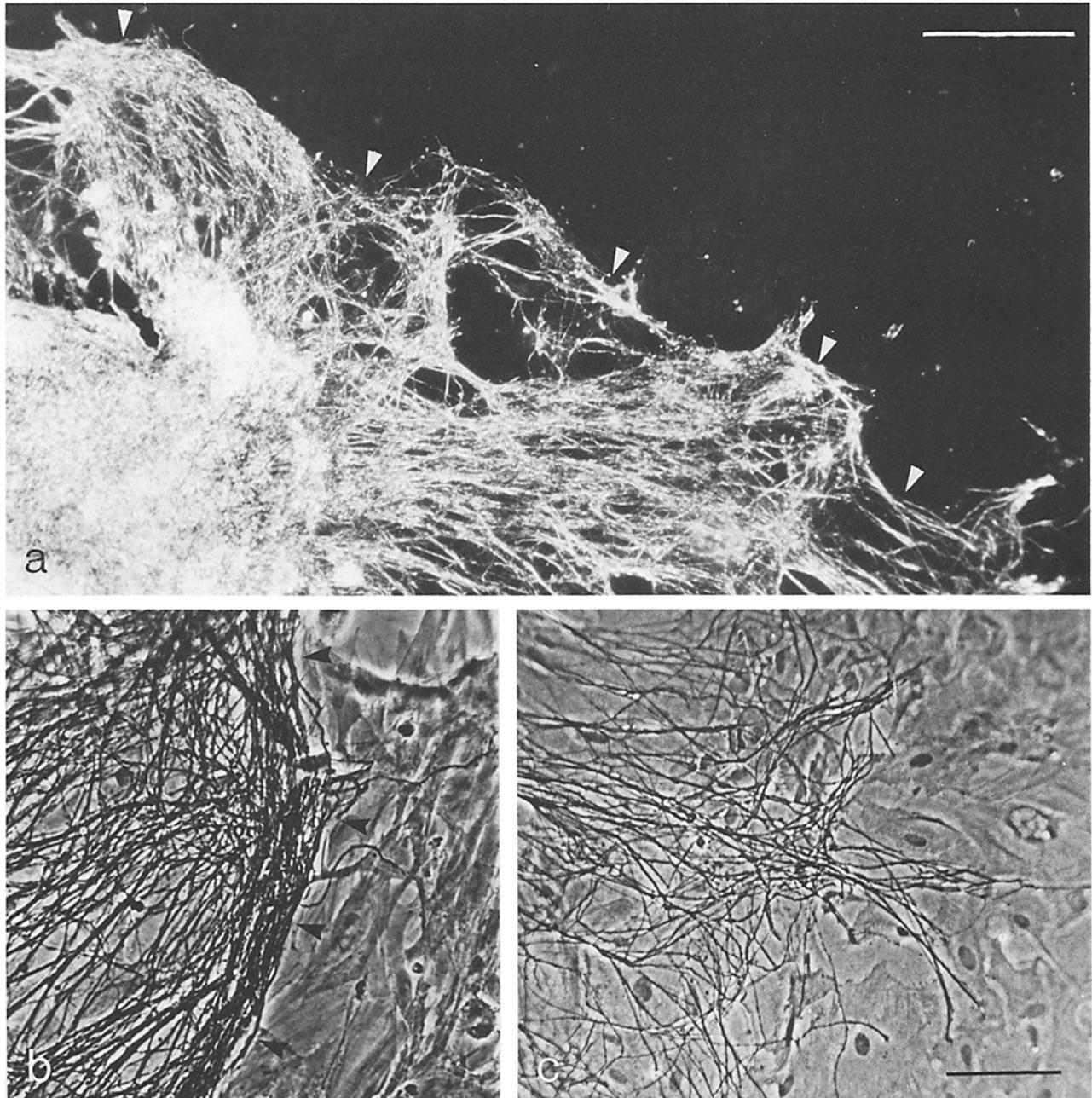


FIGURE 6 Behavior of retinal neurites confronted with a choice of astrocytes and fibroblasts. Adjacent monolayers of astrocytes and fibroblasts were constructed as described in Materials and Methods. Retinal explants were plated on the astrocyte side and the neurites were observed as they encountered the fibroblast frontier. (a) Low-power dark-field view of a culture labeled with mouse anti-NF followed by horseradish peroxidase-coupled goat anti-MiG. The region of the border between astrocytes (*lower left*) and fibroblasts (*upper right*) is indicated by arrowheads. Bar, 0.25 mm. $\times 93$. (b and c) Phase-contrast images of the outgrowth front of the same explant at border (b) and nonborder (c) regions. Note that the neurites stop or turn at the border (arrowheads) between the astrocytes (*left*) and the fibroblasts (*right*) and do not encroach on to the fibroblasts. Bar, 100 μm . $\times 140$.

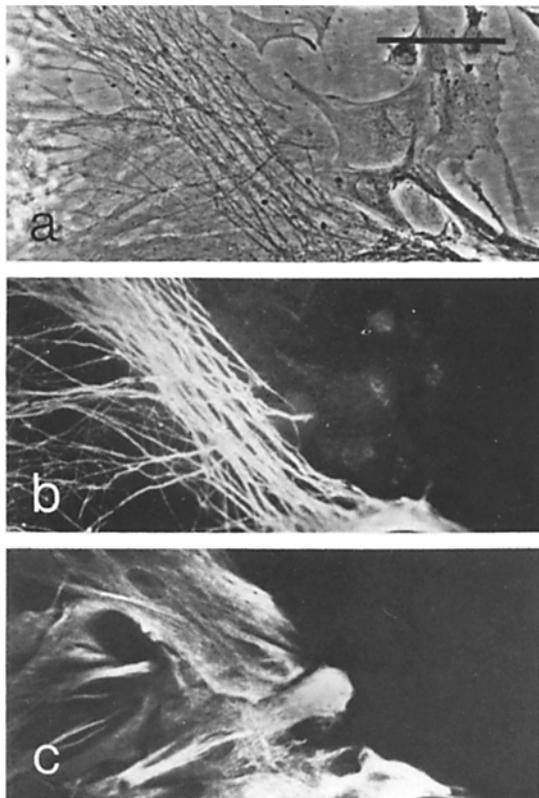


FIGURE 7 Retinal neurites at an astrocyte-fibroblast/polylysine-coated substrate border. This culture, similar to that in Fig. 6, has been double labeled with mouse anti-NF and rabbit anti-GFAP followed by goat anti-Mlg-Rd and goat anti-Rlg-fl. This regimen allows the visualization of the neurites, astrocytes, and fibroblasts in the same field. The field is shown with phase-contrast (a), rhodamine (b), and fluorescein (c) optics. The sharp boundary is seen between the astrocytes on the left (stained with rabbit anti-GFAP [c]) and the fibroblasts (visible by phase-contrast microscopy on the right in a, unstained in b or c). Note the close association of the neurites and the underlying astrocytes; the neurites grow neither onto the fibroblasts nor onto the exposed polylysine-coated substrate. Bar, 100 μ m. \times 170.

TABLE I
Retinal Neurite Outgrowth on Astrocytes and Fibroblasts is Unaffected by Incubation with CM

Source of media	Monolayer cell type	Growth rate μ m/h
Astrocytes	Astrocytes	31 \pm 1
Fibroblasts	Astrocytes	33 \pm 3
Control	Astrocytes	34 \pm 2
Astrocytes	Fibroblasts	<5
Fibroblasts	Fibroblasts	<5
Control	Fibroblasts	<5

Retinal explants were placed on preformed monolayers of 5,000 astrocytes or fibroblasts in 35-mm dishes in the indicated media. The media had been conditioned for 5–7 d by more than 100,000 cells. The distance from the explant border to the outgrowth front was measured 24 h later. The morphology of the neurite outgrowth was not influenced by any of the media tested. Rates are expressed as the mean \pm SE for at least four explants.

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REFERENCES

- Aguayo, A. J., S. David, P. Richardson, and G. M. Bray. 1982. *Adv. Cell. Neurobiol.* 3:215–234.
- Aliot, F., and B. Pessac. 1984. Astrocytic cell clones derived established cultures of 8-day postnatal mouse cerebella. *Brain Res.* 306:283–291.
- Bartlett, P. B., M. D. Noble, R. M. Pruss, M. C. Raff, S. Rattray, and C. Williams. 1981. Rat neural antigen-2: a cell surface antigen on astrocytes, ependymal cells, Muller cells and leptomeningeal cells defined by a monoclonal antibody. *Brain Res.* 204:339–351.
- Bohn, R. C., P. J. Reier, and E. B. Sourbeer. 1982. Axonal interactions with connective tissue and glial substrata during optic nerve regeneration in *Xenopus* larvae and adults. *Am. J. Anat.* 165:397–419.
- Bjorklund, A., U. Stenevi, S. B. Dunnett, and F. H. Gage. 1982. Cross species neural graft in a rat model of Parkinson's disease. *Nature (Lond.)* 298:652–654.
- Bottenstein, J. E., and G. H. Sato. 1979. Growth of a rat neuroblastoma cell line in serum free supplemented media. *Proc. Natl. Acad. Sci. USA.* 76:514–517.
- Brookes, J. P., K. L. 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.
- Denis-Donini, S., J. Glowinski, and A. Prochiantz. 1984. Glial heterogeneity may define the three-dimensional shape of mouse mesencephalic dopaminergic neurons. *Nature (Lond.)* 307:641–643.
- Dribbin, L. B., and J. N. Barrett. 1980. Conditioned medium enhances neurite outgrowth from rat spinal cord explants. *Dev. Biol.* 74:184–195.
- Fallon, J. R. 1983. Preferential outgrowth of CNS and PNS neurites on astrocytes and Schwann cells as compared to non-glial cells *in vitro*. *Soc. Neurosci. Abstr.* 9:207a. (Abstr.)
- Fallon, J. R., and M. C. Raff. 1982. Specificity and character of neurite outgrowth on defined cell types *in vitro*. *Soc. Neurosci. Abstr.* 8:302a. (Abstr.)
- Foucoud, R., M. Reeb, M. Sensenbrenner, and G. Gombos. 1982. Kinetic and morphological analysis of the preferential adhesion of chick embryo neuronal cells to astroglial cells in culture. *Exp. Cell Res.* 137:285–294.
- Grumet, M., and G. M. Edelman. 1984. Heterotypic binding between neuronal membrane vesicles and glial cells is mediated by a specific cell adhesion molecule. *J. Cell Biol.* 98:1746–1756.
- Guillery, R. W., H. M. Sobkowicz, and G. L. Scott. 1970. Relationships between glial and neuronal elements in the development of long-term cultures of the spinal cord of the fetal mouse. *J. Comp. Neurol.* 140:1–34.
- Hatten, M. E., and R. H. K. Liem. 1981. Astroglial cells provide a template for the positioning of developing cerebellar neurons *in vitro*. *J. Cell Biol.* 90:622–630.
- Hinds, J. E., and P. L. Hinds. 1974. Early ganglionic cell differentiation in the mouse retina: an electron microscopic analysis utilizing serial sections. *Dev. Biol.* 37:381–416.
- Johnston, R. N., and N. K. Wessels. 1980. Regulation of the elongating nerve fiber. *Curr. Top. Dev. Biol.* 16:165–206.
- Krayanek, S., and S. Goldberg. 1981. Oriented extracellular channels and axonal guidance in embryonic chick retina. *Dev. Biol.* 84:41–50.
- Kuwabara, T. 1975. Development of the optic nerve of the rat. *Invest. Ophthalmol.* 14:732–745.
- Lake, P., E. A. Clark, M. Khorshidi, and G. H. Sunshine. 1980. Production and characterization of cytotoxic Thy-1 antibody-secreting hybrid cell lines. Detection of T cell subsets. *Eur. J. Immunol.* 9:875–886.
- Lander, A. D., D. Fujii, D. Gospodarowicz, and L. Reichardt. 1982. Characterization of a factor that promotes neurite outgrowth: evidence linking activity to a heparan sulfate proteoglycan. *J. Cell Biol.* 94:574–586.
- Letourneau, P. C. 1975. Cell-to-substratum adhesion and guidance of axonal elongation. *Dev. Biol.* 44:92–101.
- Letourneau, P. C. 1975. Possible roles for cell-to-substratum adhesion in neuronal morphogenesis. *Dev. Biol.* 44:77–91.
- Levitt, P., and P. Rakic. 1980. Immunoperoxidase localization of GFAP in radial glial cells and astrocytes of the developing Rhesus monkey brain. *J. Comp. Neurol.* 193:815–840.
- Liesi, P., D. Dahl, and A. Vaheri. 1983. Laminin is produced by early rat astrocytes in primary cultures. *J. Cell Biol.* 96:920–924.
- Lindsay, R. M. 1979. Adult rat brain astrocytes support survival of both NGF-dependent and NGF-insensitive neurones. *Nature (Lond.)* 282:80–82.
- Manthorpe, M., S. Varon, and R. Adler. 1981. Neurite promoting factor (NPF) in conditioned media from RN22 Schwann cultures: bioassay, fractionation and other properties. *J. Neurochem.* 57:789–69.
- McCarthy, K. D., and J. DeVellis. 1980. Preparation of separate astroglial and oligodendroglial cultures from rat cerebral tissue. *J. Cell Biol.* 85:890–902.
- Mudge, A. W. 1984. Schwann cells induce morphological transformation of sensory neurons *in vitro*. *Nature (Lond.)* 309:367–369.
- Noble, M. N., J. Fok-Seang, and J. Cohen. 1984. Glia are a unique substrate for the *in vitro* growth of central nervous system neurons. *J. Neurosci.* 4:1892–1903.
- Pruss, R. 1979. Thy 1.1 antigen on astrocytes in long term cultures of rat central nervous system. *Nature (Lond.)* 280:688–690.
- Raff, M. C., K. L. Fields, S. Hakomori, R. Mirsky, R. Pruss, and J. Winter. 1980. Cell-type specific markers for distinguishing and studying neurons and the major classes of glial cells in culture. *Brain Res.* 174:283–308.
- Raff, M. C., R. H. Miller, and M. Noble. 1983. A glial progenitor cell that develops *in vitro* into an astrocyte or an oligodendrocyte depending on culture medium. *Nature (Lond.)* 303:390–396.
- Rakic, P. 1971. Neuron-glia relationships during cell migration in developing cerebellar cortex. A Golgi electron microscopic study in *Macaca rhesus*. *J. Comp. Neurol.* 141:283–312.
- Ranscht, B., P. A. Claphaw, J. Price, M. Noble, and W. Seifert. 1982. Development of oligodendrocytes and Schwann cells studied with a monoclonal antibody against galactocerebroside. *Proc. Natl. Acad. Sci. USA.* 79:2709–2713.
- Rutishauser, U. 1984. Developmental biology of a neural cell adhesion molecule. *Nature (Lond.)* 310:549–554.
- Rutishauser, U., M. Grumet, and G. M. Edelman. 1983. Neural cell adhesion molecule mediates initial interactions between spinal cord neurons and muscle cells in culture. *J. Cell Biol.* 98:145–52.
- Silver, J., S. E. Lorenz, D. Wahlsten, and J. Coughlin. 1982. Axonal guidance during development of the great cerebral commissures: descriptive and experimental studies *in vivo*, on the role of preformed glial pathways. *J. Comp. Neurol.* 210:10–29.
- Silver, J., and J. Sapiro. 1981. Axonal guidance during development of the optic nerve:

- the role of pigmented epithelia and other extrinsic factors. *J. Comp. Neurol.* 202:521-538.
40. Silver, J., and R. L. Sidman. 1980. A mechanism for the guidance and topographic patterning of retinal ganglion cell axons. *J. Comp. Neurol.* 189:101-111.
41. Singer, M., R. H. Nordlander, and M. Egar. 1979. Axonal guidance during embryogenesis and regeneration in the spinal cord of the newt: the blueprint hypothesis of neuronal pathway patterning. *J. Comp. Neurol.* 185:1-22.
42. Trenkner E., and R. L. Sidman. 1977. Histogenesis of mouse cerebellum in microwell cultures. Cell reaggregation and migration, fiber and synapse formation. *J. Cell Biol.* 75:915-940.
43. Wood, J. N., and B. Anderton. 1981. Monoclonal antibodies to mammalian neurofilaments. *Biosci. Rep.* 1:263-268.