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Neurite Guidance by Non-neuronal Cells in Culture: Preferential Outgrowth of Peripheral Neurites on Glial as Compared to Nonglial Cell Surfaces[1]

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Abstract

Growing axons in the peripheral nervous system (PNS) encounter a variety of cellular and extracellular substrates. Since it is difficult to sort out the possible contributions of these diverse components of the extracellular environment to axonal guidance *in vivo*, I have developed an *in vitro* system to study neurite outgrowth on two classes of cells which may provide as substrates for growing axons during development or regeneration: glial cells, e.g., astrocytes and Schwann cells, and nonglial cells, e.g., fibroblasts. Although neurites from sympathetic and spinal sensory ganglia explants grew onto preformed monolayers of both glial and nonglial cells, glial cells were a markedly better substrate. On the glial cells the neurites extended at a rate of 25 to 30 μm/hr and traveled singly or in fine fascicles; their growth cones displayed long filopodia and migrated on the upper surface of the monolayer cells. Conditioned media experiments suggested that neurite outgrowth on glial cell monolayers was not mediated by soluble secreted factors. These results indicate that the glial cell surface is an attractive substrate for neurite outgrowth. In contrast, on nonglial cells the rate of outgrowth was only 10 to 15 μm/hr, large neurite fascicles were common, and the growth cones migrated beneath the monolayer cells in contact with the underlying artificial substrate. This location of the growth cone, coupled with the observation that conditioned medium from these cells promoted neurite outgrowth only when bound to artificial substrates, suggests that secreted substrate-associated components may be an important determinant of neurite outgrowth on nonglial cell monolayers. The surface of the nonglial cells thus appears to be either inhibitory or a poor substrate for neurite outgrowth.

The behavior of neurites confronted with a choice of glial and nonglial cells was also tested. When neurites growing on astrocytes were confronted with fibroblasts, most of the neurites turned and stayed on the glial cells. The neurites which did cross to the nonglial portion of the monolayer slowed their growth rate and formed larger fascicles. In contrast, neurites growing on a fibroblast monolayer increased their growth rate and defasciculated when they encountered an adjacent astrocyte monolayer. Taken together, these experiments demonstrate that, although PNS neurites can grow both on glial and nonglial cell monolayers, glial cell surfaces are the preferred substrate. Furthermore, the results suggest that neurites are more adherent to glial as compared to nonglial cell surfaces, thus revealing an adhesive hierarchy between these two major cell classes encountered by growing axons during nerve development and regeneration.

The orderly formation of neural connections depends on the paths chosen by migrating growth cones as they travel from neuronal cell body to target cell. These pathways are often remarkably stereotyped, and factors in the local environment can play an important role in their formation and regeneration (Ramon y Cajal, 1928; Lance-Jones and Landmesser, 1981a, b; Lewis et al., 1981; Bentley and Keshishian, 1982; Tosney and Landmesser, 1984). A major component of this local environment is the substrate encountered by growing axons. This substrate can include other axons or neurons (Keshishian and Bentley, 1983; Bastiani et al., 1984), elements of the extracellular matrix (Roberts, 1976; Bate and Grunewald, 1981; Nordlander and Stinger, 1982; Easter et al., 1984), and non-neuronal cells (Morris et al., 1972; Al-Ghaith and Lewis, 1982; Berlot and Goodman, 1984).

The relative adhesivity between these various substrates and migrating growth cones could be an important factor in guiding axonal growth. In a series of elegant studies, Letourneau (1975a, b) established that growth cones confronted in culture with a choice of artificial substrates of differing adhesivity would grow on the more adherent substrate. He also made the important observation that this choice was not based on the absolute adhesivity of a particular substrate, but rather on its adhesivity relative to another substrate. Thus, a given substrate could be selected or shunned depending on the competition.

However, much less is known about the relative affinities of growth cones and neurites for cellular substrates in culture (see Fallon, 1985, for discussion). For example, are all cells comparable in their ability to serve as substrates, or are some cell types preferred over others? Conversely, do different classes of axons vary in their response to the same cellular substrate? In the past, such questions have been difficult to address because the cell types of interest could not be reliably identified or obtained as highly enriched populations. These obstacles have not been overcome for many of the major cell types of the nervous system (Bignami et al., 1972; Brockes et al., 1979; Raff et al., 1979; McCarthy and de Vellis, 1980), and studies of the interactions between central nervous system (CNS)

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neurons and identified glial cells have recently appeared (Hatten and Liem, 1981; Foucaud et al., 1982; Denis-Donini et al., 1984; Noble et al., 1984; Fallon, 1985). However, there are few reports describing the interactions of peripheral neurites and identified glial cells (Wood and Bunge, 1975; Lindsay, 1979; Bunge et al., 1982).

There is mounting evidence that glial or non-neuronal cells may play a central role in two aspects of the peripheral nervous system (PNS) which distinguish it from the CNS: the structure and composition of its environment during development (Al-Ghaith and Lewis, 1982; Tosney and Landmesser, 1984), and its ability to regenerate (Aguayo et al., 1982). Therefore, in the present study I have used a combination of cell type-specific antibodies and highly enriched cell populations in order to compare neurite outgrowth from PNS explants on glial and nonglial cell monolayers. I have examined the rate, direction, and degree of fasciculation of neurites growing on a single cell type. I have also tested the ability of material secreted by these cell types to influence neurite outgrowth. Finally, in order to establish the *preferred* substrate for neurite outgrowth, I have also studied the behavior of neurites faced with a choice of cell types. Portions of this work have been presented in preliminary form (Fallon, 1983).

Materials and Methods

Cell Cultures. All primary cultures were from Sprague-Dawley rats. Astrocytes from the cerebral cortex were prepared by a modification of the methods of Noble et al. (1984) and McCarthy and de Vellis (1981). Newborn neocortex was dissected free of meninges, gently triturated four or five times through a large-bore pipette (Falcon; 10 ml), and incubated in 0.125% trypsin in HEPES-buffered minimum essential medium (MEM-H) for 20 min at 37°C. Complete medium consisting of Dulbecco's minimum essential medium with 10% fetal calf serum (FCS; Grand Island Biological Co.), 6 gm/liter of glucose, 50 units/ml of penicillin-streptomycin, and 2.5 μg/ml of amphotericin B (Fungizone; Squibb) was then added. The tissue was then gently triturated through a Pasteur pipette, counted in the presence of trypan blue, and plated at a density of 5×10^6 viable cells/25-cm^2 tissue culture flask (Falcon or Nunc). (The plating efficiency varied greatly with different batches of FCS; therefore, when poorer batches of serum were used, the flasks were coated with 5 μg/ml of polylysine (Sigma) and preincubated for 2 hr in complete medium before the addition of the cells. Such modifications did not influence the purity of the final cell populations obtained.) The cultures were fed every 3 days and grown to confluence (usually 8 to 10 days). The flasks were then placed overnight on a rotary shaker at 10 to 20 rpm to remove the neurons, oligodendrocytes, and fibrous astrocytes. If a significant number of these process-bearing cells remained after this treatment, the cultures were rinsed five times in MEM-H without serum, 5 ml of this medium were added, and the flasks were shaken vigorougly by hand for 15 to 60 sec. The remaining adherent cells were then split 1:3 with 0.025% trypsin in 1 mM EDTA and treated with two 48-hr pulses of 10^{-5} M arabinosylcytosine to kill the rapidly dividing leptomeningeal cells. Schwann cells were prepared by the method of Brockes et al. (1979). Skin, muscle, and sciatic nerve fibroblasts were prepared by enzymatic digestion of neonatal tissue and were passaged at least three times before use (Fallon, 1985). The purity of the cell monolayers obtained in each case was judged to be >95% as judged by staining for glial fibrillary acidic protein (GFAP) for astrocytes (Raff et al., 1979), Ran-1 for Schwann cells (Brockes et al., 1979), and fibronectin for fibroblasts (Bartlett et al., 1981).

Preparation of Monolayers. Cells to be tested as monolayers were plated onto 31-mm Nunc culture dishes in three 4-mm (inner diameter) cloning rings per dish as described previously (Fallon, 1985). Briefly, cloning rings were prepared by cutting Beckman nitrocellulose Microfuge tubes with a razor blade, and were then placed on dry dishes without sealant. Astrocytes and fibroblasts were plated at 5,000 cells/ring, and Schwann cells were plated at 20,000 cells/ring. The rings were removed after 24 hr, the dishes where washed three times with MEM-H, and the resulting monolayer islands were grown for at least three more days. In order to generate contiguous monolayers of astrocytes and fibroblast for the choice experiments, the cells were treated as above except that one cell population was introduced into a cloning ring and the second was placed as a droplet abutting the outer wall of the ring. After the cells had settled down (12 to 24 hr), the ring was removed and the cells were allowed to migrate into the intervening bare area (~1 mm, representing the thickness of the cloning ring wall). The resulting border between the two cell monolayers was stable for at least 2 or 3 days: staining with either anti-fibronectin (anti-FN) or anti-GFAP showed little intermixing of the cell populations. All monolayers were irradiated with 2000 rad from a ^{60}Co source before use.

Explants. Superior cervical ganglia (SCG) and dorsal root ganglia (DRG) were dissected from embryonic day 20 (E20; plug date is counted as E0) Sprague-Dawley rats and stripped of their connective tissue capsules. The SCG were cut into three or four pieces and the DRG were used whole. The explants were irradiated with 2000 rad from a ^{60}Co source to inhibit division of intrinsic non-neuronal cells and were then plated onto the preformed monolayers using a finely drawn out Pasteur pipette. The irradiation treatment was not found to significantly affect the rate of neurite outgrowth. Partially purified nerve growth factor (NGF) (7 S; Varon et al., 1967) was used at a concentration that gave optimal SCG neurite outgrowth on collagen substrates. Neurite outgrowth was measured at four roughly equidistant points around the explant after 1 day of culture. Twenty-four hours later, the outgrowth along the same axes was again measured and the rate of outgrowth was calculated for this second interval (24 to 48 hr in culture). Neurite outgrowth on Schwann cells was assessed after 24 hr on cultures stained with anti-neurofilament (anti-NF) antibody.

Conditioned media experiments. Conditioned medium (CM) was obtained from confluent cultures of astrocytes or fibroblasts incubated for 7 days and was used the same day it was collected. For studies on soluble factors the CM was diluted 1:1 with fresh medium containing NGF. For testing substrate-bound factors, undiluted CM was incubated on polylysine-coated (5 μg/ml) Nunc dishes overnight at 4°C in a 5% CO_2 atmosphere. The dishes were washed twice in MEM-H and the explants were added in fresh or conditioned media. Outgrowth was measured after 24 hr.

Immunofluorescence and immunoperoxidase labeling. Neurites were labeled by indirect immunofluorescence with a mouse monoclonal anti-NF antibody (1:50 ascites; Wood and Anderton, 1981), and fibroblasts were labeled with anti-FN (1:100; Bartlett et al., 1981) as described previously (Fallon, 1985). Cultures stained for immunofluorescence to permit simultaneous localization of neurites and monolayer cells were viewed in a Zeiss Universal fluorescence microscope equipped with phase contrast and incident ultraviolet illumination. Appropriate dichroic mirrors and barrier filters were used to selectively visualize rhodamine and fluorescein fluorescence. For immunoperoxidase labeling, cultures were fixed in 3.7% formaldehyde (10% formalin in phosphate-buffered saline for 5 to 10 min at room temperature, postfixed with 100% methanol at −20°C, and labeled as described previously (Fallon, 1985).

Electron microscopy. Preparation of tissues for scanning electron microscopy was as described previously (Fallon, 1985). Briefly, cultures were fixed in 2% glutaraldehyde at 37°C, postfixed in 1% osmium tetroxide, dehydrated, critical point dried in liquid CO_2, and sputter coated with gold-palladium.

Results

Neurite outgrowth on astrocytes, Schwann cells, and nonglial cells. The neurite outgrowth from SCG and DRG explants onto preformed monolayers of astrocytes was abundant, radial, and rapid (Table I), the first neurites leaving the explant within 3 to 4 hr of plating. The growth of SCG neurites on astrocyte monolayers is illustrated in Figure 1, *a* and *b*. The neurites formed an anastomosing network of fine fascicles <1 to 3 μm in diameter; fascicles larger than 5 μm were rarely seen. Prominent growth cones were seen at the leading edge of the neurite outgrowth zone (Fig. 2). The growth cones, which migrated on the upper surface of the astrocytes, varied greatly in their morphology: although long, fine filopodia were a

TABLE I

Rate of outgrowth of SCG and DRG neurites on astrocyte and fibroblast monolayers

Irradiated explants were placed on preformed monolayers of astrocytes or skin fibroblasts and grown for 48 hr as described under "Materials and Methods." Growth rates were measured over the second 24 hr in culture. Results are from a representative experiment; in four separate experiments with SCG the mean outgrowth rates were 27.6 ± 0.65 (n = 19) and 13.8 + 0.85 (n = 21) on astrocytes and fibroblasts, respectively. The rates in this and the subsequent tables are expressed as the mean ± the standard error.

	SCG (μm/hr)	DRG (μm/hr)
Astrocytes	24.9 ± 0.7	27.2 ± 1.4
Fibroblasts	11.0 ± 1.2	9.7 ± 2.7

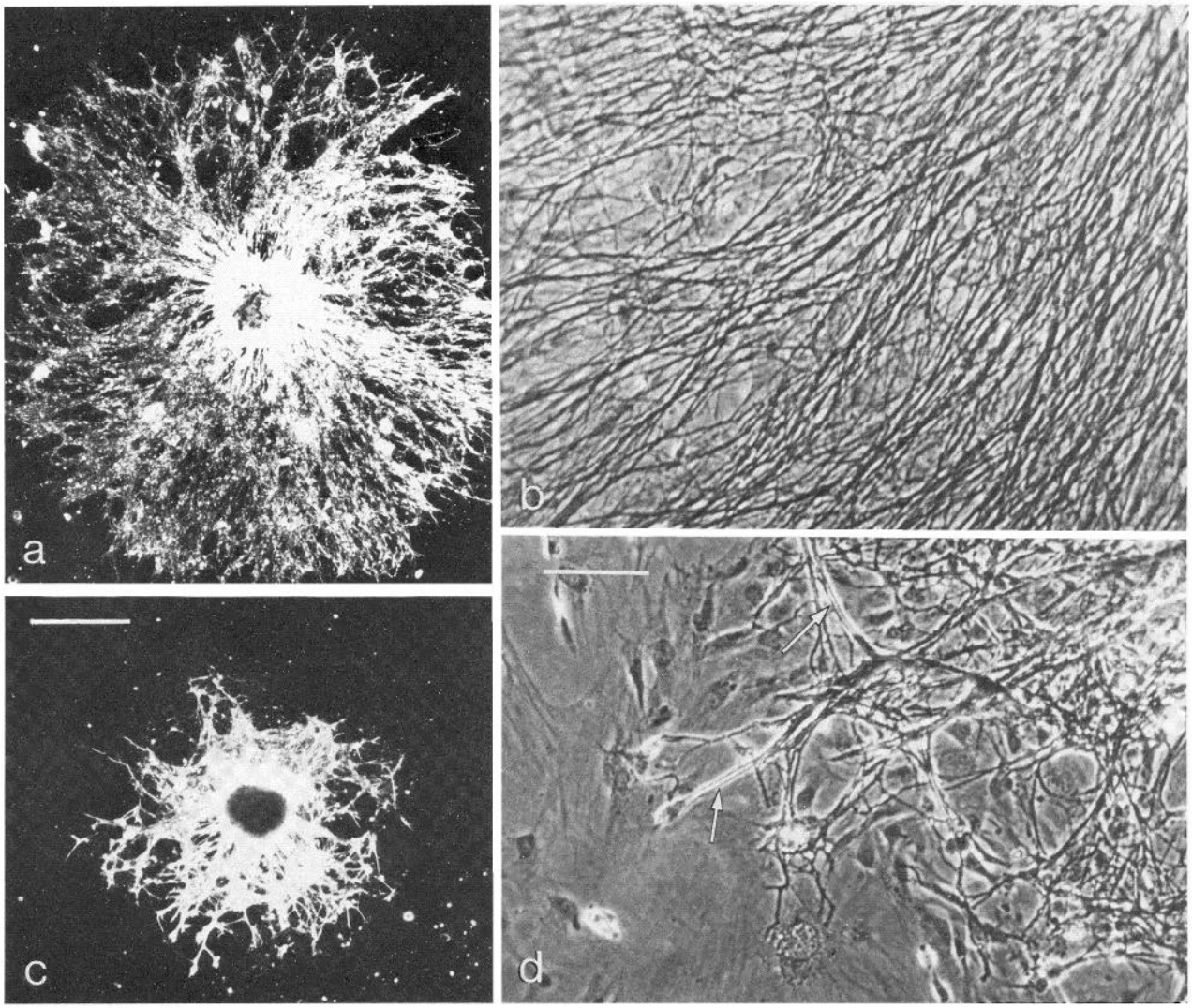

Figure 1. SCG neurite outgrowth on astrocyte monolayers is rapid and finely fasciculated as compared to growth on fibroblast monolayers. Confluent monolayers of astrocytes or fibroblasts were prepared as described under "Materials and Methods." X-irradiated E20 SCG explants were plated onto the monolayers and grown for 48 hr. The cultures were then fixed and the neurites were visualized with an anti-NF antibody followed by a horseradish peroxidase-conjugated second layer. *a* is an overview and *b* shows detail of explants on astrocyte monolayers. The neurite outgrowth on these glial cells is abundant, rapid, and in fine fascicles. *c* is an overview and *d* shows detail of SCG explants grown on fibroblast monolayers. Note that the neurite outgrowth on these nonglial cells is slower and prominent phase-bright fascicles are seen (*arrows*). *a* and *c*, Darkfield optics. Magnification × 27; *bar* = 0.5 mm. *b* and *d*, Phase contrast. Magnification × 140; *bar* = 100 μm.

regular feature, the lamellopodia ranged from broad and flat to more elongated and rounded configurations.

Both the sympathetic (SCG) and the sensory (DRG) ganglia were comparable in their neurite outgrowth patterns on glial as compared to nonglial cells (Table I); however, as the SCG contains a more homogeneous population of neurons and does not project to the CNS, this ganglion was chosen for the remainder of the studies described here. It is important to note that, in either case, few non-neuronal cells were observed migrating from the irradiated explants onto the monolayers. In addition, staining with anti-FN (for example, see Fig. 6*c*), which marks fibroblasts, or anti-Ran-1 (not shown), which labels Schwann cells (Brockes et al., 1979), failed to demonstrate significant contamination of the monolayers with these explant-derived cells.

Sciatic nerve Schwann cells also offered an attractive substrate for SCG neurite outgrowth. The initial outgrowth was similar to that seen on astrocytes, being rapid and in small fascicles (Fig. 3). In addition, through-focusing of cultures stained with anti-NF antibody showed that the distal neurites and growth cones were on the upper surface of the Schwann cells (Fig. 4). However, whereas the neurites usually extended more than 500 μm in the first 24 hr, the Schwann cells underwent extensive movement when contacted by the neurites; therefore, the outgrowth pattern was far less even and more variable than that seen on astrocytes or artificial substrates. This variability made it difficult to accurately assess growth rates on these glial cells.

SCG neurites also grew onto monolayers of skin fibroblasts (Fig. 1, *c* and *d*), but in a manner that differed from that on glial cells in several important respects. First, the overall rate of outgrowth was 30 to 50% slower (Table I). Second, neurites growing on fibroblast monolayers tended to form larger fascicles than those on glial cells: 5- to 10-μm-thick phase-bright bundles of neurites were prominent in the outgrowth zone (Fig. 1*d*). Third, the growth cone and distal portion of the neurites were found under the fibroblasts. This mode of outgrowth is illustrated in Figure 5. When the leading edge of the neurite outgrowth zone was examined by phase microscopy in living cultures, the fascicles often appeared to end bluntly and characteristic growth cones were not readily observed. However, closer

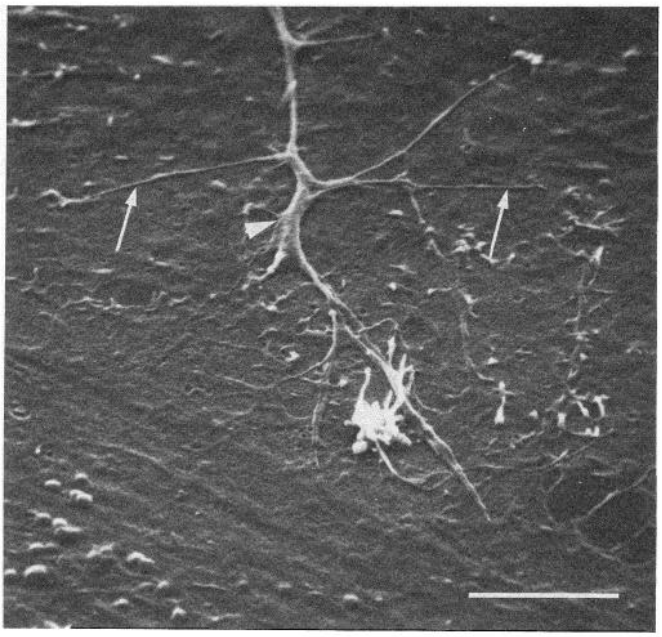

Figure 2. Growth cones migrate on the dorsal surface of astrocytes and display prominent filopodia, as shown in this scanning electron micrograph of DRG growth cone at a neurite outgrowth front on an astrocyte monolayer. Note the long filopodia (*arrows*). The lamellopodium is rounded (*arrowhead*); broad, flattened lamellopodia were also routinely observed. Magnification × 4000; *bar*, 5 μm.

examination of this region revealed a faint ghost of the fascicles extending from the blunt end. Through-focusing of these low contrast structures showed them to be under the fibroblasts; staining with anti-NF antibodies established that they consisted of neurites (Fig. 5, *a* and *b*). Scanning electron microscopy confirmed this interpretation (Fig. 5*c*). The distal segment of the neurite fascicle, including a growth cone, is seen under the fibroblasts. Thus, the growth cones migrate directly on the culture substrate and/or on the ventral aspect of the fibroblasts rather than on the upper surface of the fibroblasts. It should be noted that, whereas the growth cones and distal ends of the neurites were usually under the fibroblasts, the remainder of the neurite shaft was found on top of the cell monolayer. Possible mechanisms which might underlie this arrangement are considered under "Discussion."

A variety of other non-neural cell monolayers were tested for their ability to support neurite outgrowth from SCG explants including leptomeningeal cells, fibroblasts from sciatic nerve, lung and skeletal muscle, the epithelial cell line PTK-1, and the fibroblastic cell lines 3T3 and RAT-1. All of these gave results similar to those seen with the skin fibroblasts. The results were also similar if the monolayers were grown on polylysine- or collagen-coated tissue culture plastic or glass coverslips. The outgrowth of SCG neurites on astrocytes as well as all other cell types tested required the presence of added NGF.

SCG and DRG explants from animals ranging from E14 to postnatal day 2 (P2) also showed similar patterns of growth on glial and nonglial cells. However, the rate of outgrowth varied with explant age: neurites from E14 explants often grew more than 1 mm on astrocytes in the first 24 hr, whereas neurites from postnatal explants grew more slowly than those from the E20 explants.

Substrate-bound but not soluble conditioned media factors stimulate SCG neurite outgrowth. When CM from astrocytes or fibroblasts was added to SCG explants growing on either fibroblast or astrocyte monolayers, no significant change in neurite outgrowth rate was observed (Table II). The degree of neurite fasciculation was also unaffected (not shown). However, enhanced SCG neurite outgrowth was observed when SCG (or DRG) explants were plated onto bare polylysine-coated substrates that had been preincubated with such CM from astrocytes or fibroblasts (Table III). Similar effects were seen with CM from PTK-1, leptomenigeal, C6 glioma, and RN22 Schwannoma cells (not shown). The neurite outgrowth on CM-treated substrates was similar in some respects to that observed on astrocytes: neurites were in radially oriented, fine fascicles, and their growth required the presence of added NGF. However, although the stimulation of neurite outgrowth by glial cell monolayers was independent of the kind of artificial substrate used, these substrate-bound CM effects were not observed when collagen-coated or untreated culture dishes were used.

SCG neurites grow preferentially on astrocyte as compared to fibroblast monolayers. The experiments presented above indicate that SCG neurites grow on glial and nonglial cells, but in markedly different fashions. I next tested whether SCG neurites showed a preference for growth on glial cells as compared with the other substrates. In these experiments I observed the behavior of growing neurites as they encountered a well defined glial cell/nonglial cell border between adjacent astrocyte and fibroblast monolayers.

When SCG explants were placed on the astrocyte side of an astrocyte/fibroblast border, the neurites initially grew in the radial, finely fasciculated pattern characteristic of neurite outgrowth on glial cells. However, as shown in Figure 6, when neurites encountered the fibroblast border only a small number crossed onto the fibroblasts: most turned abruptly and stayed on the astrocytes. The neurites which crossed over to the fibroblasts tended to form larger

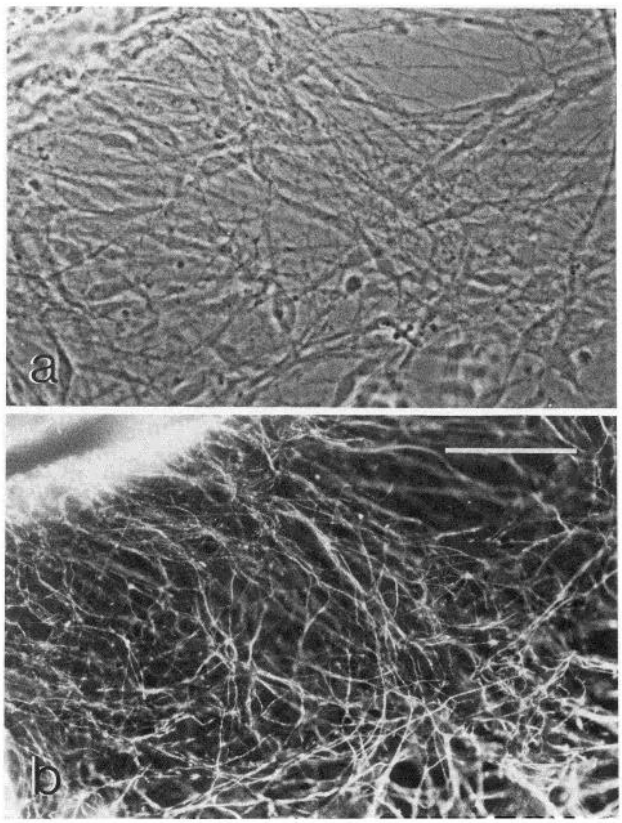

Figure 3. Neurite outgrowth on Schwann cells is abundant and in fine fascicles. SCG explants were plated onto preformed monolayers of sciatic nerve Schwann cells and grown for 48 hr. The cultures were then stained with a mouse anti-NF antibody followed by a rhodamine-conjugated anti-mouse Ig. The same field is seen *a* viewed with phase contrast and in *b* with fluorescence optics. The explant is at *upper left*. Note the finely fasciculated neurite outgrowth. The growth pattern is less coherent than that seen on astrocytes (cf. Fig. 1*b*), apparently reflecting the elongated, bipolar shape of the underlying Schwann cells (seen more clearly in Fig. 4). Magnification × 170; *bar*, 100 μm.

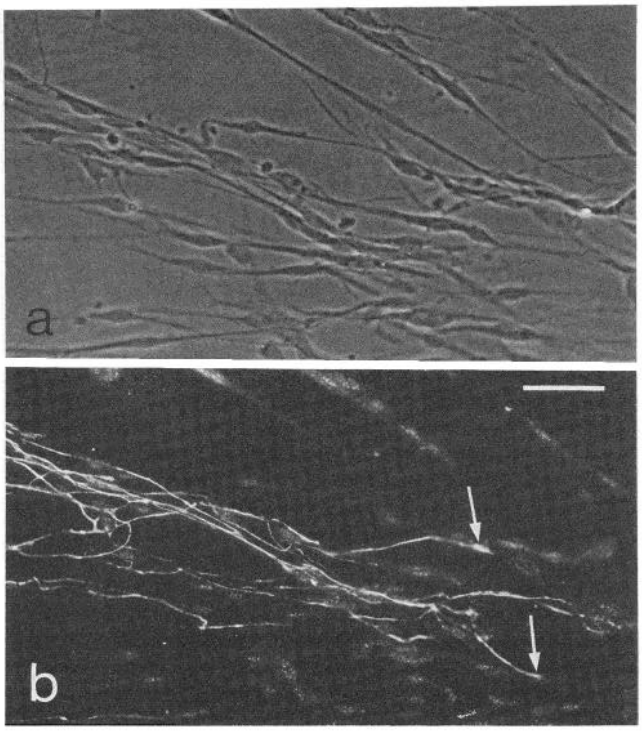

Figure 4. Growth cones and neurites on Schwann cell monolayers. Neurite outgrowth front in SCG-Schwann cell culture prepared as in Figure 3. The same field is shown in *a* with phase contrast optics and in *b* with fluorescence optics to selectively visualize the neurites. Note that the neurites are single or in fine fascicles and closely follow the Schwann cells. Through-focusing showed that the growth cones (*arrows*) were on the dorsal surface of the Schwann cells. Unlike the astrocyte monolayers (Figs. 1*b* and 2), the originally confluent Schwann cells underwent significant movement when contacted by neurites, the cells clearing out from some areas and piling up in others. Magnification × 210; *bar*, 25 μm.

fascicles and grew more slowly than neurites from the same explant which remained on the astrocytes. Conversely, when explants were plated on the fibroblast side, the neurites readily crossed the border, defasciculated, and increased their rate of growth (Fig. 7). In both cases, then, neurites showed a preference for growth on glial as compared to nonglial cell monolayers. This experiment also illustrates the highly localized effect of the astrocytes: only the neurites in direct association with the glial cells defasciculated and increased their rate of growth.

Discussion

In this study I have used cell type-specific markers combined with highly enriched cell populations to examine the growth characteristics of PNS neurites on identified cells *in vitro*. The experiments demonstrate that PNS neurites grow on both glial and nonglial cell monolayers, but that glial cells are the preferred substrate. Several lines of evidence indicate that factors associated with the glial cell surface are responsible for their superior neurite outgrowth-promoting effects: (*1*) the rapid and finely fasciculated neurite outgrowth on the upper surface of the cells (Table 1, Fig. 1), (*2*) the distinctive morphology of the growth cones characteristic of growth on a highly adhesive substrate (Fig. 2; Letourneau, 1979; Bray 1982), (*3*) the lack of soluble CM effects (Table II), and (*4*) the highly localized influence of the glial cells demonstrated in the choice experiments (Figs. 6 and 7; see also Fallon, 1985, for further discussion). These results confirm and extend those of others who have reported scientific interactions between peripheral neurites and morphologically defined "glial" cells (Letourneau, 1975a; Wessells et al., 1980). In contrast, the slow and fasciculated outgrowth of PNS neurites on fibroblasts and other nonglial cell monolayers tested was markedly different from that seen on glial cells. The character of the neurite outgrowth on these cells appeared to be influenced by two types of interactions: the growth cones with the artificial substrate and/or the underside of the fibroblasts, and the neurites with one another. No evidence was found for soluble factors which might mediate these differences (Table II). In addition, it is not likely that the distinctive behavior of neurites on glial versus nonglial cells reflects the increased availability of the artificial substrate to growth cones migrating on fibroblast monolayers: neurites grow rapidly and in fine fascicles both on confluent (Figs. 1 and 2) and subconfluent glial cell monolayers.

In order to accurately interpret the results of these experiments, it is important to establish that the neurites are growing on the cells of the test monolayer, and not on ganglion-derived non-neuronal cells. Indeed, there is evidence that such cells can be effective substrates for neurite growth *in vitro* (Adler and Varon, 1981b; Roufa et al., 1983). Several lines of evidence indicate that the neurites studied here are in fact growing on the glial and nonglial cells of the test monolayers: (*1*) explants were routinely x-irradiated with 2000 rad, a treatment which effectively kills all dividing cells; (*2*) cultures labeled with anti-Ran-1 or anti-FN, markers for Schwann cells and fibroblasts, respectively, revealed that few of these cells had migrated onto the neurite outgrowth zone; and (*3*) the stimulation of neurite outgrowth was seen on monolayers plated on a wide range of substrates; in contrast, outgrowth promoted by cells of ganglionic origin is substrate dependent (Adler and Varon, 1981b; Roufa et al., 1983).

The identity of the glial cell surface molecules which may mediate the interaction with neurites is not known. One candidate is laminin, a component of the extracellular matrix that is known to be a highly effective substrate for neurite outgrowth *in vitro* (Baron-Van Evercooren et al., 1982; Manthorpe et al., 1983; Rogers et al., 1983; Lander et al., 1984) and is found on the surface of cultured Schwann cells (Cornbrooks et al., 1983). However, although immunofluorescent staining revealed the presence of laminin in association with the fibroblast monolayers, it was not seen on the secondary astrocytes used here (unpublished observations; see also Liesi et al., 1983). Interestingly, although fibronectin has been reported to stimulate neurite outgrowth when bound to artificial surfaces *in vitro* (Akers et al., 1981; Baron-Van Evercooren et al., 1982; Rogers et al., 1983), it does not appear to be an attractive substrate when bound to the cell surfaces (Fig. 6*c*). It is also possible that the neural cell adhesion molecule N-CAM (Edelman, 1983; Rutishauser, 1984), which has been reported to be a surface component of some astrocytes (Aliot and Pessac, 1984), or Ng-CAM (Grumet and Edelman, 1984) may be involved in these interactions.

The relationship between the neurite outgrowth-promoting factors present on the surfaces of astrocytes and Schwann cells is also not known. However, the fact that both glial cell types promote retinal (Fallon, 1985) as well as SCG neurite outgrowth, whereas neither combination normally occurs *in vivo*, raises the possibility that the molecules mediating these interactions might be common to central and peripheral glial cells and, perhaps, to other non-neuronal cells that interact with axons during development.

The migration of growth cones under the cells of the fibroblast monolayers, in contrast to their course on the upper surface of the glial cells, indicates that they prefer to grow directly on the culture substratum and/or ventral surface of the nonglial cells. However, unlike the growth cones and the distal part of the neurites, the proximal portion of the neurites was found on top of the fibroblasts and tended to form larger fascicles (Figs. 1 and 5). Two observations suggest that the location of the growth cones on the top of the astrocytes is not due to their having been physically excluded from the underside of these glial cells: (*1*) cell processes from monolayer astrocytes readily underlap those of their neighbors (Fig. 2 and unpublished observations; see also McCarthy and de Vellis, 1980), and (*2*) neurites are found on the top of glial cells and exhibit characteristic outgrowth rates even when the monolayers are cul-

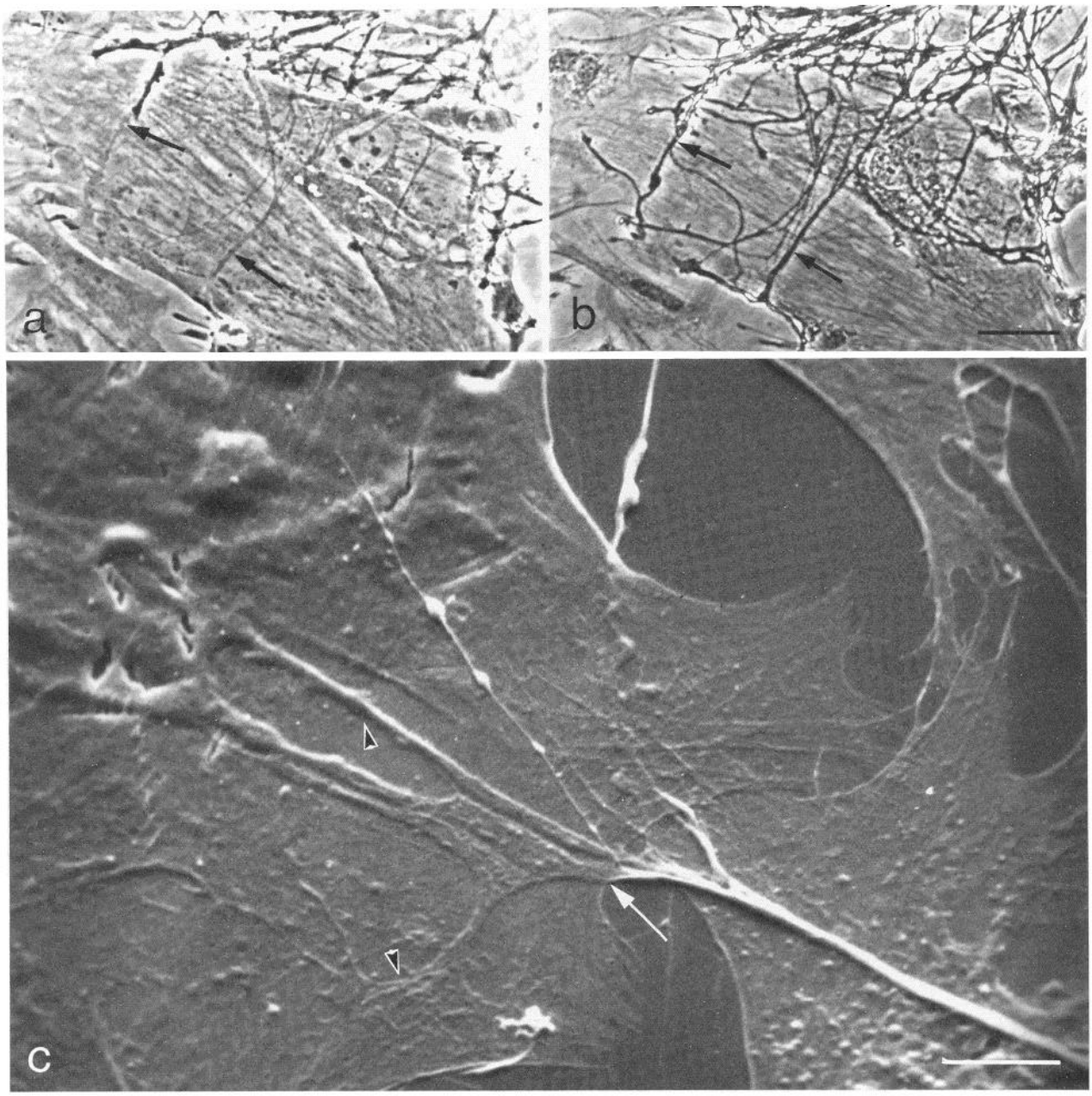

Figure 5. The growth cones and distal segments of PNS neurites migrate under fibroblast monolayers. *a*, Phase micrograph of the leading edge of the neurite outgrowth front of a living SCG-fibroblast monolayer culture. The direction of neurite outgrowth is from *top* to *bottom*. Note that the phase-dark fascicles appear to end abruptly, but that faint extensions are seen to continue onward (*arrow*). *b*, The same field after the culture had been fixed and stained with anti-NF antibody (as in Fig. 1, *b* and *d*). The ends of the neurite fasicles are now clearly seen. Through-focusing of both the living and stained cultures revealed that the distal portion of the neurites were under the fibroblasts. *Arrows* indicate the same neurite fascicles in living and fixed cultures. Magnification (*a* and *b*) × 250; *bar*, 50 μm. *c*, Scanning electron micrograph of an area similar to that illustrated in *a* and *b*. The direction of neurite outgrowth is from *right* to *left*. A neurite fascicle is seen first dorsal to one monolayer cell and then dipping under a neighboring fibroblast at *left* (*arrow*). The distal neurite and a growth cone-like structure are seen outlined under the cell (*arrowheads*). Magnification × 3600; *bar*, 5 μm.

tured on less adherent artificial substrates such as collagen or untreated tissue culture plastic. A more likely explanation for the divergent behavior of the different segments of the neurite assumes that the fibroblasts as well as the growth cones are more adherent to the artificial substrate than is the proximal portion of the neurite. Such differences in adhesivity between growth cones and their proximal neurite have been well documented in studies of neurite outgrowth on artificial substrates (Letourneau, 1979; Bray, 1982). Thus, as the growth cone grew under and past a given area of fibroblasts, the fibroblasts would in turn fill in under the trailing, less adherent, proximal neurite. Fasciculation could then occur by either one of two mechanisms: (*1*) as the result of lateral associations between neurites which had grown out independently (Nakai, 1960) or (*2*) by the growth of new neurites along the pre-existing ones. At present, it is not possible to distinguish between these mechanisms as the techniques used here could only resolve the growth cones at the leading edge of the neurite outgrowth front. Further work will be needed to determine the relative contribution of these two postulated mechanisms to the fasciculation observed here.

An unexpected finding of this study was that, of the cells tested, the surfaces of only the glial cells were capable of supporting vigorous neurite outgrowth, yet all cell types examined secreted components which will promote neurite outgrowth when bound to polylysine-coated substrates (Table III). What is the relationship

TABLE II

Soluble conditioned media factors do not affect SCG neurite outgrowth on astrocytes and fibroblasts

SCG explants were plated in the specified medium onto 5000-cell islands of the indicated monolayer. There were three explants per island in each 35-mm dish. CM from 7-day confluent cultures of astrocytes or fibroblasts (~5 × 10^6 cells/25-cm^2 flask) was collected on the day of use and diluted 1:1 with fresh medium containing NGF. Neurite outgrowth was measured 24 hr later. The degree of fasciculation was unaffected by the type of medium used. Rates are from at least four explants.

Source of Media	Monolayer Cell Type	Growth (μm/hr)
Astrocytes	Astrocytes	24.1 ± 1.4
Fibroblasts	Astrocytes	22.6 ± 0.5
Fresh medium	Astrocytes	23.8 ± 0.6
Astrocytes	Fibroblasts	7.5 ± 0.6
Fibroblasts	Fibroblasts	8.1 ± 1.4
Fresh medium	Fibroblasts	8.9 ± 2.6

between the molecules which mediate these two types of interactions? One possibility is that many cell types secrete an outgrowth-promoting factor, but only glial cells are capable of binding it to their surface. In such a case one molecule could mediate both interactions. Conversely, the secreted and the surface-bound molecules may be different. Two lines of evidence suggest that in fact the

TABLE III

Substrate-bound conditioned media factors stimulate SCG neurite outgrowth

Poly-L-lysine-coated dishes were incubated overnight with CM harvested from cultures grown for 7 days after reaching confluence. The dishes were rinsed and the explants were added in fresh medium containing NGF. Outgrowth was measured after 24 hr.

Source of Medium	Outgrowth (μm/hr)
Astrocytes	20.2 ± 0.3
Fibroblasts	24.1 ± 1.2
Fresh medium	15.2 ± 2.5

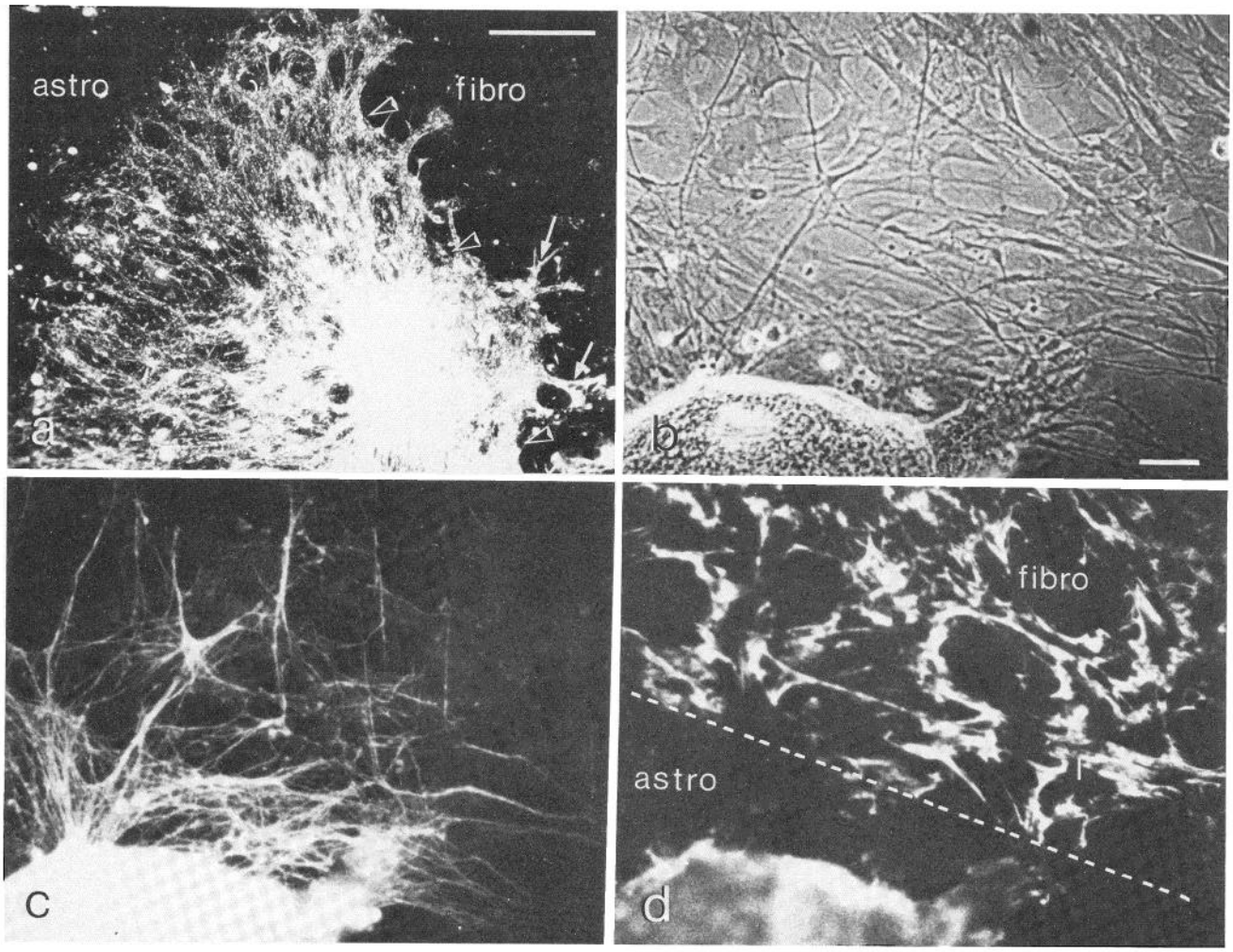

Figure 6. SCG neurites will cross from astrocyte to fibroblast monolayers but slow their rate of growth and increase their degree of fasciculation as they do so. Adjacent monolayers of astrocytes and fibroblasts were constructed as described under "Materials and Methods." Explants were placed on the astrocyte side and grown for 48 hr. *a*, overview of culture stained with anti-NF as in Figure 1*a*. Astrocytes (*astro*) are on the *left* and fibroblasts (*fibro*) are on the *right* of the line indicated by the row of *arrowheads*. Note that the bulk of the neurites do not encroach onto the fibroblasts. Magnification × 30; *bar*, 0.5 mm. *b* to *d*, Detail of neurite behavior of the astrocyte/fibroblast border. The culture had been double-labeled with anti-NF and anti-FN (see "Materials and Methods"). The field is shown in *b* with phase contrast, in *c* with rhodamine optics to visualize the anti-NF-stained neurites, and in *d* with fluorescein optics to visualize the FN-stained fibroblasts and ganglion capsule. The border between the astrocytes (*bottom left*) and the fibroblasts (*top right*) is indicated by the *dashed line* in *d*. Note that most of the neurites turn when they reach the border and do not proceed onto the fibroblast monolayer. Magnification × 175; *bar*, 50 μm.

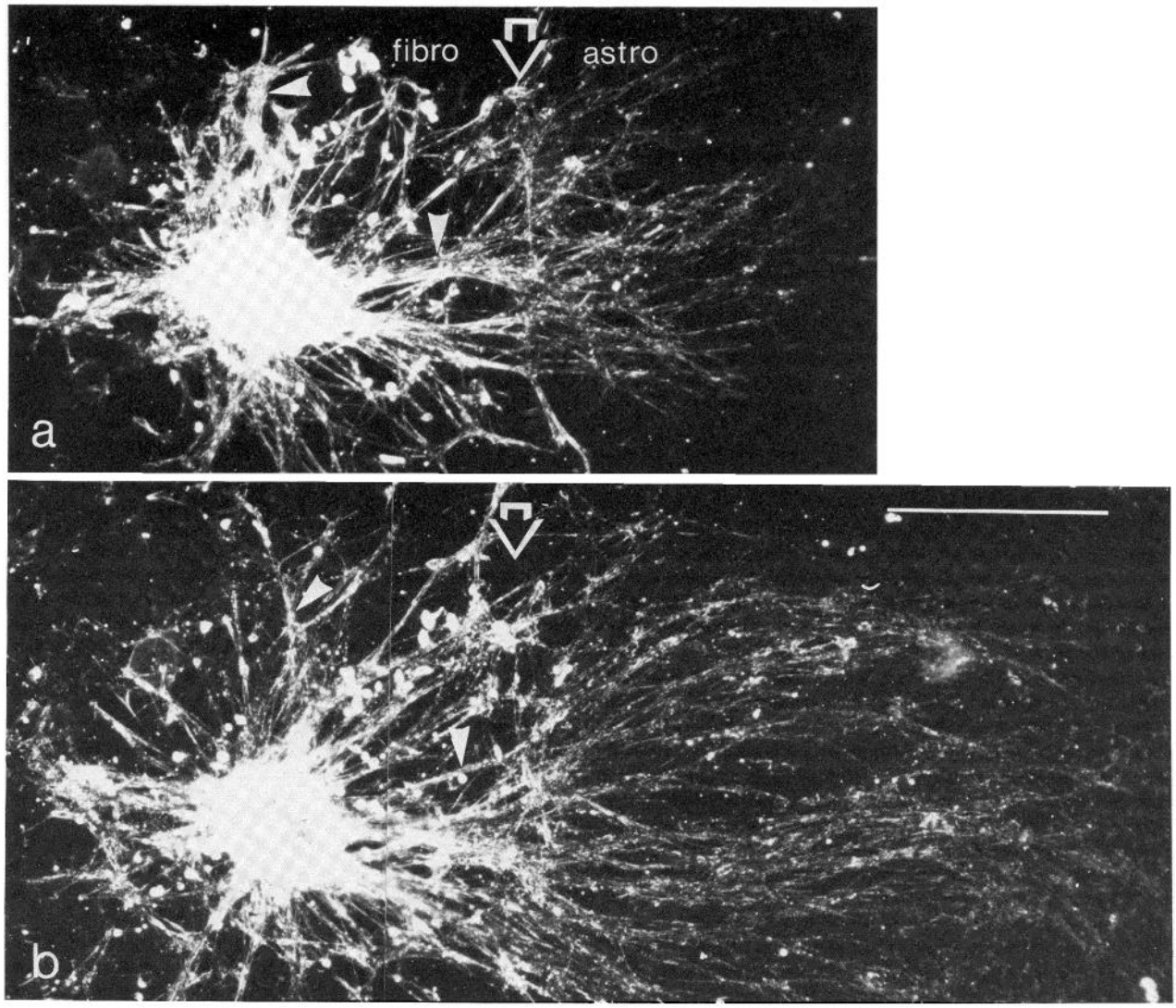

Figure 7. SCG neurites crossing from fibroblast to astrocyte monolayers defasciculate and increase their rate of growth. Adjacent monolayers of fibroblasts (*left*) and astrocytes (*right*) were constructed. The *broad arrow* indicates the borderline between the monolayers. The same explant is shown 48 (*a*) and 72 (*b*) hr after plating onto the fibroblast side. In *a*, some neurites have crossed onto the astrocytes and have defasciculated. Twenty-four hours later these neurites have undergone extensive, finely fasciculated growth (*b*). In contrast, the neurites which remained on the fibroblast monolayer retained their slow, fasciculated growth pattern (*arrowheads*). Magnification × 63; *bar*, 0.5 mm.

molecules are distinct: (*1*) CNS neurites grow vigorously on glial cell surfaces but poorly on nonglial cell monolayers or CM-coated artificial substrates (Fallon, 1985), and (*2*) there is increasing evidence that laminin may be a key component of the neurite outgrowth-promoting factors found in various conditioned media (Manthorpe et al., 1983; Lander et al., 1984). As noted above, laminin was not found in association with the astrocytes used in this study.

It seems likely, then, that the PNS growth cones can respond to at least one of two types of molecules depending on the nature of monolayer used: one tightly bound to the glial cell surface, and a second secreted and bound to the substrate. The second class, which could be the predominant influence on neurite outgrowth on nonglial monolayers, may be analogous to CM factors described by others (Dribbin and Barrett, 1980; Adler and Varon, 1981a; Lander et al., 1982; Collins and Lee, 1984). Indeed, growth cones in the periphery have been observed to grow both on formed elements of the extracellular matrix, such as basement membrane (Roberts, 1976), and in close association with non-neuronal cell surfaces (Al-Ghaith and Lewis, 1982; Berlot and Goodman, 1984). It will be of interest to determine the relative affinity of neurites for these cellular and acellular substrates.

The choice experiments, where growing neurites were confronted with astrocytes and fibroblasts in the same dish, demonstrated that astrocytes are a preferred substrate for neurite outgrowth. Most neurites growing on astrocytes did not continue on to the fibroblast border but turned and stayed on the glial cells. In contrast, neurites growing on the fibroblast side defasciculated and increased their rate of growth when they encountered the astrocytes. The ease with which the neurites cross in the fibroblast-to-astrocyte direction indicates that the border did not present a physical barrier to neurite outgrowth. These experiments also underlined the marked difference in behavior of the neurites on the astrocytes and fibroblasts: the same neurites either fasciculated and slowed their rate of growth or defasciculated and increased their rate of growth depending on the underlying cell type. Furthermore, these changes in behavior at the border occurred within a single cell diameter, providing further evidence that the neurite-glial interactions are mediated by factors tightly associated with the cell surface.

Taken together, the results presented here suggest that an adhesion hierarchy exists among the surfaces of glial and nonglial cells whereby the interaction between neurites and glial cells is stronger than that between neurites and nonglial cells. Although one must be cautious in extrapolating from results in culture to phenomena *in vivo*, my findings suggest that, *in vivo*, such a hierarchy could

influence the rate, direction, and degree of fasciculation of growing axons during development and regeneration.

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