The Journal of Neuroscience, July 1988, 8(7): 2381–2393

Oligodendrocytes and CNS Myelin Are Nonpermissive Substrates for Neurite Growth and Fibroblast Spreading *in vitro*

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To study the interaction of neurons with CNS glial cells, dissociated sympathetic or sensory ganglion cells or fetal retinal cells were plated onto cultures of dissociated optic nerve glial cells of young rats. Whereas astrocytes favored neuron adhesion and neurite outgrowth, oligodendrocytes differed markedly in their properties as neuronal substrates. Immature (O_4^+, $A_2B_5^+$, $GalC^-$) oligodendrocytes were frequently contacted by neurons and neurites. In contrast, differentiated oligodendrocytes (O_4^+, $A_2B_5^-$, $GalC^+$) represented a nonpermissive substrate for neuronal adhesion and neurite growth. When neuroblastoma cells or 3T3 fibroblasts were plated into optic nerve glial cultures, the same differences were observed; differentiated oligodendrocytes were nonpermissive for cell adhesion, neurite growth, or fibroblast spreading. These nonpermissive oligodendrocytes were characterized by a radial, highly branched process network, often contained myelin basic protein, and may, therefore, correspond to cells actively involved in the production of myelin-like membranes.

Isolated myelin from adult rat spinal cord was adsorbed to polylysine-coated culture dishes and tested as a substrate for peripheral neurons, neuroblastoma cells, or 3T3 cells. Again, cell attachment, neurite outgrowth, and fibroblast spreading was strongly impaired. General physicochemical properties of myelin were not responsible for this effect, since myelin from rat sciatic nerves favored neuron adhesion and neurite growth as well as spreading of 3T3 cells. These results show that differentiated oligodendrocytes express nonpermissive substrate properties, which may be of importance in CNS development or regeneration.

The reasons why the regeneration of lesioned fiber tracts is almost totally absent in the CNS of higher vertebrates have remained unknown up to now. Transplantation studies of pieces of peripheral nerves into the adult CNS clearly demonstrated the capacity of most types of CNS neurons for regenerative growth and elongation of their processes over long distances (Tello, 1911; Ramon y Cajal, 1928; Benfey and Aguayo, 1982; Richardson et al., 1984; So and Aguayo, 1985). These studies assigned a crucial role to the microenvironment of the growing fibers, whereby peripheral nerve tissue should allow, support, or provoke neurite regeneration. The involvement of neurotrophic and neurotropic factors—produced by Schwann cells but not by CNS glia—was suggested 60 years ago by Ramon y Cajal (1928). In fact, a marked increase in the production of neurotrophic factors and cell adhesion molecules by Schwann cells in response to denervation has recently been observed (Richardson and Ebendal, 1982; Longo et al., 1984; Abrahamson et al., 1986; Daniloff et al., 1986). However, neurotrophic factors are also present in developing and adult CNS, and increased neurotrophic activities were found at sites of CNS lesions (Barde et al., 1982; Korsching et al., 1985; Whittemore et al., 1985, 1986, 1987; Needels et al., 1986; Shelton and Reichardt, 1986).

Recent *in vitro* studies, in which dissociated sensory or sympathetic neurons were confronted with explants of adult rat sciatic (PNS) or optic (CNS) nerves, showed that the differences in regenerative neurite growth within peripheral or central nervous tissue environments persisted in the presence of high amounts of a neurotrophic factor (NGF) (Schwab and Thoenen, 1985). In the same cultures, in which up to several hundred axons could be found in the sciatic nerve explants, neurite ingrowth into optic nerves was strictly absent. The same findings were obtained with previously frozen optic and sciatic nerves that were free of living glial cells. These results strongly argue against the hypothesis that the lack of CNS regeneration is mainly due to an absence, or insufficient production, of neurotrophic factors by denervated glial cells (Ramon y Cajal, 1928). Rather, the differentiated CNS may lack cellular or substrate constituents conducive for neurite growth during development (Liesi, 1985a; Carbonetto et al., 1987), or it may contain components that are nonpermissive or inhibitory for nerve fiber regeneration.

In the present study, dissociated sympathetic, sensory, or retinal neurons were added to cultures of dissociated CNS glial cells grown at low cell density. One cell type with the characteristics of a differentiated oligodendrocyte forming myelin membranes was found to be highly nonpermissive for neurite growth. This cell contact-mediated, nonpermissive substrate effect was observed for primary culture neurons, neuroblastoma cells, and spreading 3T3 fibroblasts. Isolated myelin from the CNS, but not myelin from sciatic nerves, likewise inhibited neurite growth and fibroblast spreading.

Materials and Methods

Glial cell cultures. Optic nerves were dissected from 7- to 12-d-old or young adult (180–220 gm) Wistar rats and collected in plating medium

Received July 7, 1987; revised Sept. 29, 1987; accepted Nov. 6, 1987.

We thank Ch. Müller (Munich), J. Erni, and L. Steinberg for their skillful technical assistance. Initial experiments were done at the Max-Planck-Institute for Psychiatry, Department of Neurochemistry, Martinsried/Munich. We thank Dr. H. Thoenen for his generous support and interest. The gift of antibodies by Drs. D. Dahl (Boston), F. Omlin (Lausanne), M. Schachner (Heidelberg), and M. Willard (St. Louis) is gratefully acknowledged. We are grateful to Mrs. S. Kaufmann for typing and to Dr. D. Kuffler for critically reading the manuscript.

This work was supported by the Swiss National Foundation for Scientific Research (Grant 3.043-0.84) and the Bonizzi-Theler Foundation (Zurich).

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(air-buffered enriched L_{15} with 5% rat serum; Mains and Patterson, 1973). The meninges and blood vessels were carefully removed under a microscope, and the nerves were cut into small pieces. Dissociation of young nerves was done twice for 25 min each in 0.25% trypsin (Sigma) and 0.02% collagenase (Worthington) (Raff et al., 1979) in CMF-PBS (Ca^{2+}/Mg^{2+}-free PBS) at 37°C. Adult optic nerves were dissociated in 0.1% trypsin, 0.1% collagenase for 1 hr at 37°C, followed by 0.5% trypsin for 10 min. After washing and dissociation by trituration with a Pasteur pipet, the cells were plated into the wells of 35 mm tissue culture dishes containing 4 internal wells at a density of 20,000–30,000 cells/well (surface of well, 95 mm^2). For 7- to 10-d-old optic nerves, the yield was about 10,000 cells per nerve. The culture substrate for most of the experiments was polyornithine (PORN, Sigma, 0.5 mg/ml in borate buffer, incubated overnight) or polylysine (PLYS, Sigma, 50 μg/ml in water); in some experiments, a dried collagen film (calf skin collagen, incubation overnight with sterile solution), laminin-coated PORN [purified mouse EHS tumor laminin (gift of Dr. R. Timpl, Munich), 5 μg/ml, incubated for 3 hr on dishes previously coated with PORN], or plain tissue culture plastic was used. The culture medium was an enriched L_{15} medium with 5% rat serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) (Mains and Patterson, 1973). In some experiments, 10% fetal calf serum (FCS) was added instead of the rat serum.

Optic nerves of E13 or E17 chicken embryos were dissociated by brief trypsin/collagenase treatment and cultured for 2–7 d in L_{15} with 5% FCS on PORN-coated culture dishes.

Glia–nerve cell cocultures. Three different types of nerve cells were cocultured with glial cells: sympathetic neurons from the superior cervical ganglion of newborn rats, sensory neurons from dorsal root ganglia of newborn rats, or cells from the retina of E17–E18 embryonic rats. Superior cervical and dorsal root ganglia were dissected and dissociated into single cells as described by Mains and Patterson (1973) and Schwab and Thoenen (1985). Retinas were dissected from the embryos, cleaned from adhering blood vessels, incubated in 0.03% trypsin and 0.03% DNAase for 10 min at 37°C, washed by centrifugation in serum-containing medium, and dissociated by trituration.

The neurons were added to 2- to 10-d-old glial cultures in the same medium, with the addition of NGF (2.5S NGF, 50 or 100 ng/ml) for sensory and sympathetic neurons or brain-derived neurotrophic factor for the retinal cells (Johnson et al., 1986). In order to suppress the Schwann cells added together with the peripheral neurons, pulses of cytosine arabinoside (Ara C; 10^{-5} M) were given twice for 24 hr on the 2nd and 5th d of coculture in some experiments. The cultures were processed for antibody staining after 1–5 d of coculture in the case of retinal cells or after 2 d–3 weeks in the case of peripheral ganglion cells.

Mouse *neuroblastoma cells* (line NB-2A) cultured in DMEM/10% FCS were detached from the culture flasks by a brief treatment with 0.1% trypsin in CMF-Hank's solution terminated by addition of DMEM/FCS. After washing, the cells were added to glial cultures (40,000 or 20,000 cells/well) in DMEM/FCS with either 2 mM dibutyryl-cyclic AMP or glia-derived neurite-promoting factor (GdNPF) (Guenther et al., 1986).

Mouse NIH *3T3 cells,* treated identically to the neuroblastoma cells, were added to 2- to 3-d-old cultures of 7-d-old or newborn rat optic nerves at a concentration of 20,000 or 40,000 cells/well in DMEM containing 10% fetal calf serum or in MEMα supplied with insulin (20 ng/ml) and transferrin (50 ng/ml). Cultures were returned to the incubator for 2–4 hr and then fixed with warm 4% formalin in phosphate buffer and double-stained with the O_1 and O_4 antibodies.

Immunofluorescence. The following antibodies as markers for oligodendrocytes, astrocytes, neurons, or fibroblasts were used: *oligodendrocytes:* mouse monoclonal antibody (m-AB) O_4 (Sommer and Schachner, 1981); mouse m-AB O_1 (Sommer and Schachner, 1981); specific for galactocerebroside (GalC; Singh and Pfeiffer, 1985); and goat antiserum against myelin basic protein of rabbits (Omlin et al., 1982); *precursor cells:* mouse m-AB A_2B_5 (Sera-Lab, Crawley Down, GB); *astrocytes:* rabbit antiserum against glial fibrillary acid protein (GFAP) (Dahl and Bignami, 1976); *neurons:* mouse m-AB against guinea pig or rabbit neurofilaments (Willard and Simon, 1981); *fibroblasts:* mouse m-AB Ox7 against Thy-1.1 (Sera-Lab); goat antiserum against human fibronectin (LETS protein; Cappel, NC).

The specific antibodies were visualized by the corresponding anti-mouse, anti-rabbit, or anti-goat–FITC or –RITC linked secondary antibodies (Cappel, NC). Prior to staining, the cultures were washed twice with PBS containing 5% sucrose and 0.1% BSA. The antibodies O_1, O_4, and A_2B_5 were directed against surface antigens and were therefore incubated on the living cultures at room temperature for 30 min at a dilution of 1:20 in PBS/sucrose/BSA. Antibodies against Thy-1 were diluted 1:10, anti-fibronectin 1:20. The cultures were then rinsed twice, fixed for 10 min with 4% formalin in PBS, rinsed again, incubated for 1 hr with the labeled secondary antibodies (dilution 1:30–1:100), washed, and mounted in PBS:glycerol (1:1). For double-labeling experiments of A_2B_5 or O_1 antibodies with the O_4 antibody, living cultures were first incubated with antibodies A_2B_5 or O_1, followed by anti-mouse–FITC and then with antibody O_4; after fixation, this was followed by anti-mouse–RITC. In order to detect A_2B_5 or O_1 labeled cells which do not carry the O_4 antigen, the sequence was reversed in some experiments. Staining for GFAP was done on cultures previously fixed in 95% ethanol/5% acetic acid for 30 min at 4°C and rehydrated into PBS. In the case of O_4/GFAP double-labeling experiments, staining with the O_4 antibody was done first on the living cultures, followed by 10 min fixation in 4% formalin and then by ethanol/acetic acid treatment and GFAP staining. For visualization of MBP, the cultures were briefly fixed in 4% formalin, then treated with ethanol/acetic acid, and finally incubated with anti-MBP antiserum (1:500) for 1 hr at room temperature. Ethanol/acetic acid fixation was also used for visualization of neurofilaments.

Evaluation of double-labeled cultures. Cultures were systematically screened in the fluorescence microscope for the presence of one antigen (usually O_4), and every labeled cell was examined for the presence of the other antigen, e.g., A_2B_5, O_1, or GFAP.

Evaluation of cocultures with nerve cells, neuroblastoma cells, or 3T3 cells. Antibody-labeled cultures were systematically screened in the fluorescence microscope, and all O_4-labeled cells were photographed. The same fields were photographed under phase-contrast illumination. The oligodendrocyte surface area occupied by or in contact with neurons, neurites, ganglionic Schwann cells, or 3T3 cells was estimated, and the oligodendrocytes were grouped into 3 categories: cells with <20, 20–80, or >80% of the territory covered by neurons, neurites, or 3T3 cells. (Single thin processes, especially of immature cells, were often excluded from evaluation for reasons of comparability with the dense process network of highly branched oligodendrocytes.) In experiments with retinal cells, total oligodendrocyte territory and areas overlapped by retinal cells were measured with a Hewlett-Packard digitizer. The oligodendrocyte subtypes were identified on the corresponding fluorescence micrographs. The criteria used were cell morphology and antigenic characteristics (O_4/O_1). A_2B_5 staining could not be used as a marker for immature cells, since this antigen was rapidly lost (without a concomitant change in cell morphology—unpublished observations) after coculture with neurons. The distinguishing morphological criteria were shape and size of the cell body, number of primary processes, branching pattern of processes, and the occurrence of anastomoses and membrane sheets within the process network. With these criteria, *highly branched oligodendrocytes and immature oligodendrocytes* could be reproducibly distinguished. Most (but not all) of the highly branched cells were positive for the O_1 antigen; immature cells were consistently negative.

Quantification of the direction of neuroblastoma process outgrowth with respect to highly branched oligodendrocytes was done as illustrated in Figure 6. Highly branched oligodendrocytes were sampled systematically, and neighboring neuroblastoma cells were classified as "adjacent" if the distance between the edge of the oligodendrocyte process network and the NB-2A cell was less than 2 cell body diameters. Further cells were classified as "distant" (see Fig. 5). A circle with 8 sectors (4 classes) was overlaid over the center of each neuroblastoma cell and oriented towards the nearest oligodendrocyte cell body; the neuroblastoma processes were then counted in each sector (see Fig. 6 and Table 2).

Preparation of myelin. Spinal cords were dissected from 200 gm rats, carefully cleaned from adhering dorsal and ventral roots, and homogenized (Polytron, 30 sec at half-maximal speed). Sciatic nerves were dissected, minced, and homogenized. Myelin fractions were isolated by flotation of low-speed supernatants on sucrose density gradients (Colman et al., 1982). In some experiments, to remove possible trapped contaminants, the crude membrane fraction was washed following hypotonic shock. Sedimentation in hypotonic medium was achieved at 10,000 × *g* for 5 min. Membrane fractions in sucrose solutions containing no more than 50 mM ionic species were adsorbed for several hours onto the wells of polylysine-coated tissue culture dishes (about 0.1 mg of protein/cm^2 of tissue culture dish). Unbound membranes were removed by 3 washes with CMF-Hank's solution. Coated dishes

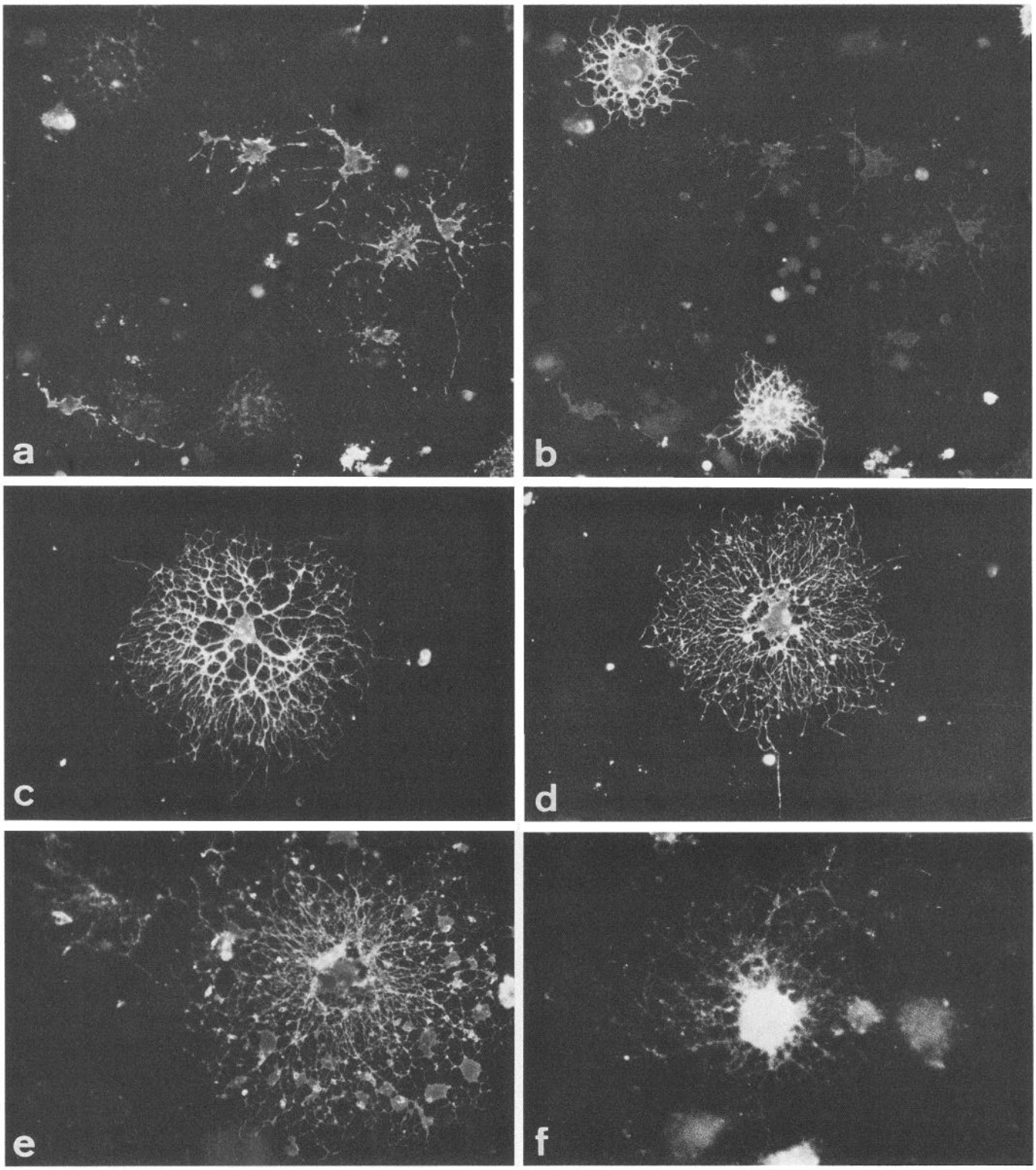

Figure 1. Typical morphologies and antigenic characteristics of immature oligodendrocytes (*a*) and highly branched oligodendrocytes (*b–f*). The antigenic profile ($A_2B_5^-$, O_4^+, O_1^+, often MBP^+) suggests that highly branched oligodendrocytes are actively involved in myelin synthesis. *a* and *b*, Double-stained culture of 7-d-old optic nerve glial cells (2 d *in vitro*): A_2B_5 (*a*) labels immature oligodendrocytes and type II astrocytes; O_1 (*b*) exclusively labels highly branched oligodendrocytes. ×200. *c* and *d*, Highly branched oligodendrocytes from adult rat optic nerves (8 d *in vitro*) stained with antibody O_4 (*c*) or O_1 (*d*). ×300. *e*, Highly branched oligodendrocyte from 10-d-old rat optic nerve (14 d *in vitro*) stained with O_4. Process network contains flat membrane areas. ×300. *f*, MBP-positive oligodendrocyte (8-d-old optic nerve, 2 d *in vitro*). ×700.

Table 1. Oligodendrocyte subpopulations characterized by antibody labeling

Population	Percentage of labeled cells					
	$A_2B_5^+/O_4^-$	$A_2B_5^+/O_4^+$	$A_2B_5^-/O_4^+$	$A_2B_5^+/O_1^-$	$A_2B_5^+/O_1^+$	$A_2B_5^-/O_1^+$
Highly branched oligodendrocytes	0	9 ± 4	91 ± 4	0	7 ± 2	93 ± 2
Cells with irregular or polygonal shapes						
Flat, membranous cells	37 ± 4[a]	51 ± 6	12 ± 6	100	0	0
Process-bearing cells	18 ± 5	74 ± 5	8 ± 2	84 ± 6	14 ± 6	1.5 ± 1.5
Cells with filopodia	0	57 ± 8	43 ± 8	91	1	(8 ± 8)[b]

Dissociated 7- to 10-d-old rat optic nerve cells were cultured on polyornithine for 2 d and labeled by either first antibody A_2B_5 (detected by anti-mouse–FITC) followed by O_4 or O_1 (detected by anti-mouse–RITC) or vice versa. The proportion of double-labeled cells was calculated from the values obtained for $A_2B_5^+/O_{4/1}^-$ and $A_2B_5^-/O_{4/1}^+$ cells. Values represent the means ± SEM of 4–6 cultures (120–200 cells/culture) from 2 separate experiments.

[a] This population of $A_2B_5^+/O_4^-$ cells contains type II astrocytes and precursor cells not expressing any oligodendrocyte marker.

[b] Variable, weak, granular staining.

were then immediately used in substrate testing experiments. In experiments with sympathetic or sensory neurons, small droplets of central or peripheral myelin were deposited in defined patterns over 35 mm culture dishes.

Sympathetic or sensory neurons cultured as described before were examined after 12 hr–4 d, neuroblastoma cells after 5–24 hr, and 3T3 cells after 1–4 hr. For quantification, neuroblastoma cells were classified as round cells, cells with filopodia or short processes, or cells with processes longer than one cell body diameter. 3T3 cells were classified as round cells, cells with filopodia or short processes, or large flat cells. Three to four micrographs per culture were taken at random from 3 cultures for each experimental point.

Results

Cultures of dissociated young or adult rat optic nerves

GFAP-positive astrocytes accounted for about 30% of the cells in dissociated 10-d-old rat optic nerves. About 50% of the cells were positive for the O_4 antigen, a marker for differentiated, (GalC-positive) and immature (A_2B_5-positive) oligodendrocytes. No overlap was seen in the labeling between O_4 and GFAP or O_4 and Thy-1, confirming the specificity of the O_4 antibody as a marker for the oligodendrocyte family (Sommer and Schachner, 1981). Thy-1-positive fibroblasts with large flat morphologies accounted for about 20% of the cells in young rat optic nerves.

Subtypes of oligodendrocytes

In cultures from 7- to 10-d-old rats, about 50% of the O_4-positive cells were A_2B_5-positive. A_2B_5-labeled cells were O_1-negative (Table 1; Fig. 1, *a, b*) and had different morphologies, including cells with irregular processes from polygonal cell bodies, flat cells with peripheral processes, bipolar cells, or cells decorated with filopodia. On the basis of this marker profile ($A_2B_5^+$, O_4^+, O_1^-), and in agreement with Schnitzer and Schachner (1982), we interpret these cells as being precursor and immature oligodendrocytes. They are collectively called "immature oligodendrocytes" in the following. This cell group is probably heterogenous, as suggested also by the different morphologies. Filopodia-carrying cells may be the most advanced (Table 1).

About 50% of the O_4-positive cells were A_2B_5-negative and O_1-positive after 2 d in culture under our culture conditions. Most of these cells showed a typical, highly branched radial process network. Because of this characteristic morphology we called these cells *highly branched oligodendrocytes* (Fig. 1, *b–f*; Table 1). After 2 d in culture, most highly branched oligodendrocytes from optic nerves of 10-d-old rats were stained with an antiserum against myelin basic protein (MBP) (Fig. 1*f*). We therefore interpret these cells as being myelin-forming oligodendrocytes. Their characteristic process network may be the result of an unstable, partially collapsed myelin membrane containing occasional flat membrane areas (Figs. 1*e*, 5*d*).

The total yield of cells from *adult* nerves was very low. Both differentiated O_1-positive highly branched oligodendrocytes (Fig. 1*d*) and immature A_2B_5-positive oligodendrocytes were also present in cultures of adult tissue.

Coculture with sympathetic or sensory neurons

Dissociated cells from newborn rat superior cervical ganglia or dorsal root ganglia were added to glial cells after 2–10 d in culture. In part of the experiments, ganglionic Schwann cells and fibroblasts were eliminated by pulses of Ara C. NGF (50 or 100 ng/ml) was added to the culture medium, leading to a rapid fiber outgrowth and to the formation of dense neurite networks within a few days. NGF alone had no effect on the occurrence and morphology of oligodendrocytes. Glial cell types were identified by antibody staining at the end of the experiments (2 d–2 weeks of coculture).

In cultures with a dense neurite plexus, the most striking observation was the occurrence of "windows" free of neurites, in the center of which cells with radial, highly branched processes could be observed (Fig. 2). Antibody staining identified these cells as highly branched oligodendrocytes. A quantification of the interaction of oligodendrocytes with sympathetic ganglion cells is shown in Figure 3, *A, B*. Astrocytes adjacent to oligodendrocytes were rare in these cultures since the overall glial cell density was low; preferential association with astrocytes, therefore, could not account for this result. Highly branched oligodendrocytes excluded neurons from their territory, irrespective of the culture substrate used. The same "windows" were formed on plain plastic, collagen, polyornithine-, or laminin-coated culture dishes. No difference was seen between sympathetic and sensory neurons; both were excluded from the territory of highly branched oligodendrocytes. Likewise, Schwann cells, when present, did not invade or overgrow the oligodendrocyte process networks (Fig. 2*b*). In contrast, immature oligodendrocytes, characterized by their irregular shapes and the absence of O_1 antigen, did allow neurite growth on their processes and cell bodies (Figs. 2 *e, f*; 3*B*). A_2B_5 could not be used as a marker for immature oligodendrocytes in cocultures with neurons, as this antigen was rapidly lost after addition of the neurons (M. Schwab, unpublished observations). Recent direct observations of the encounter of growth cones with oligodendrocytes showed us that growth cone movement is arrested after filopodial contact is established. Normal growth cone activity

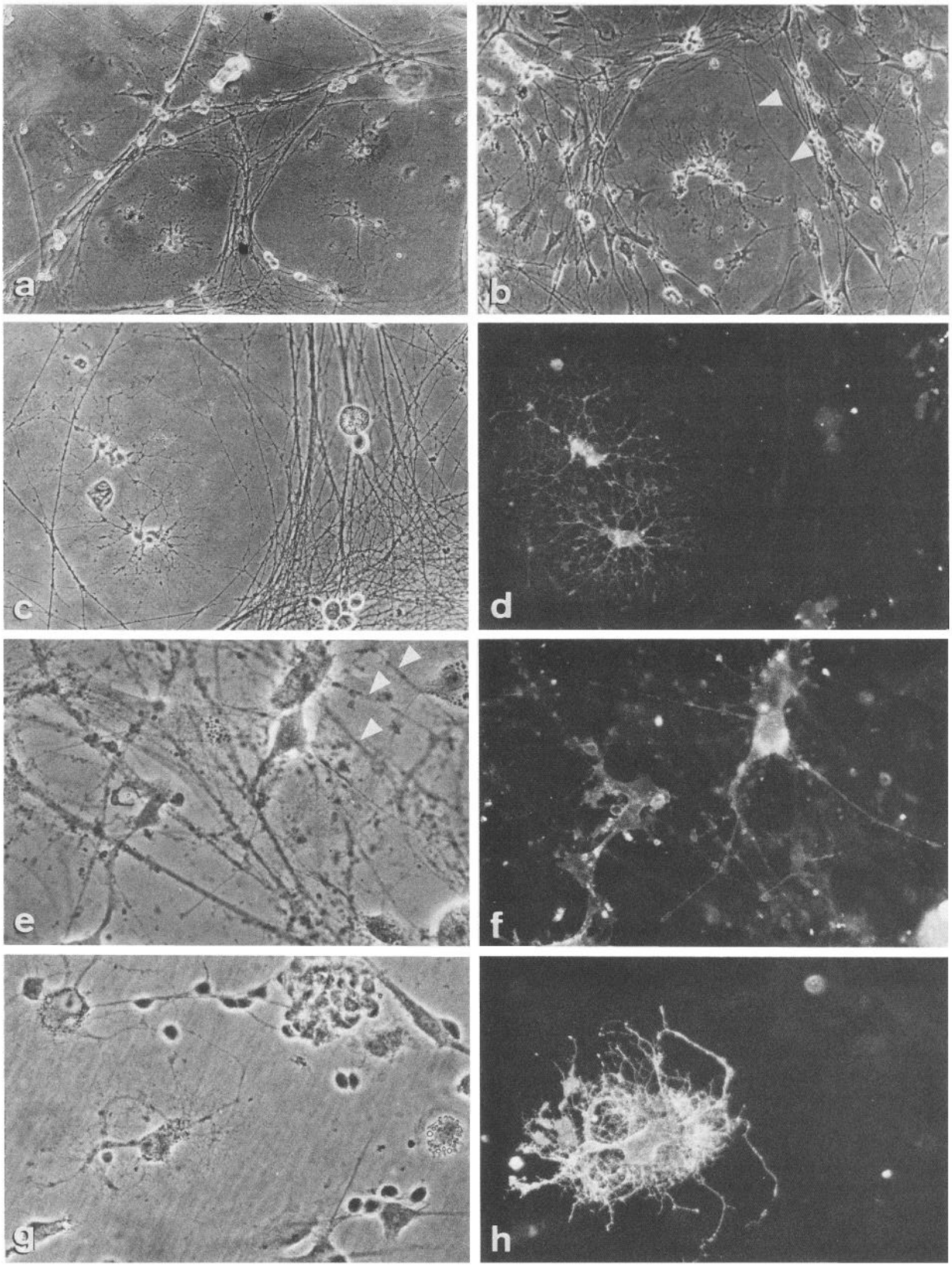

Figure 2. Sympathetic (*a–f*) or retinal (*g, h*) neurons plated into cultures of optic nerve glial cells show nonpermissive substrate effect of highly branched oligodendrocytes and its absence in immature oligodendrocytes. *a* and *b,* "Windows" formed by highly branched oligodendrocytes (10-d-old optic nerves, 18 d *in vitro*) in the neurite plexus of sympathetic neurons (*a,* 8 d *in vitro*; *b,* 4 d *in vitro*). ×120. In *b,* a neurite changing its direction is seen (*arrowhead*). Schwann cells also avoid the oligodendrocyte. *c* and *d,* O_4-positive oligodendrocytes (from 10-d-old optic nerves, 23 d *in vitro*) surrounded by plexus of sympathetic neurites (13 d *in vitro*). ×220. Neurites characteristically "loop around" the oligodendrocytes. The occasional spanning of neurite bundles over nonpermissive oligodendrocytes occurs as a secondary event. *a* and *c,* no astrocytes are present in the field. *e* and *f,* O_4-positive cells with typical morphology of immature oligodendrocytes are permissive for sympathetic neurites (*arrowheads*) (5 d *in vitro*). ×380. *g* and *h,* E20 rat retinal cells (2 d *in vitro*) do not adhere or grow neurites onto highly branched, O_1-positive (*h*) oligodendrocyte. ×200.

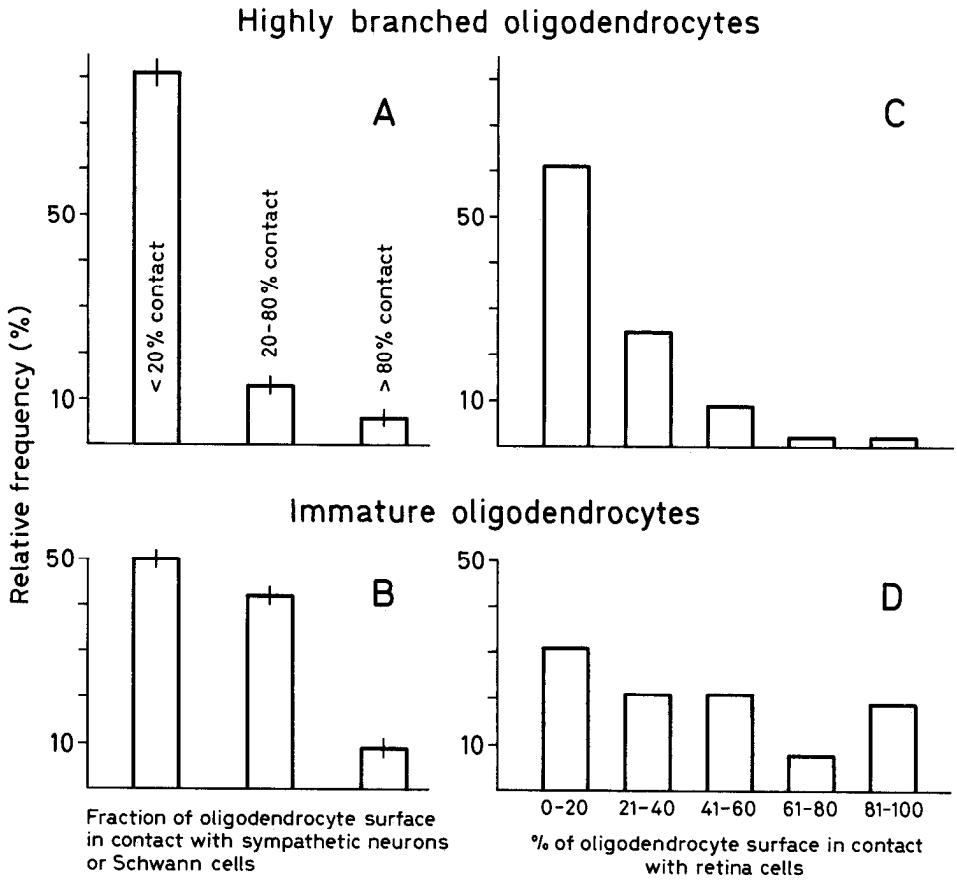

Figure 3. *A* and *B*, Histograms showing the frequency of interactions/overlap of sympathetic neurites and Schwann cells with highly branched (*A*) or immature (*B*) oligodendrocytes. Glial cells from 8- to 10-d-old optic nerves (2 d *in vitro*) were cocultured for additional 2 d with dissociated neurons from superior cervical ganglia and then stained with O_4. Oligodendrocytes were classified by morphology on coded fluorescence pictures. On phase-contrast pictures, the fractional area of contact with neurites and Schwann cells was determined and classified into 3 categories: <20, 20–80, or >80% of oligodendrocyte territory covered by neurites or Schwann cells. Values represent mean frequencies of cells in the 3 categories ± SEM (4 cultures; 70–130 systematically sampled cells per culture). *C* and *D*, Histograms showing the interaction on retinal cells with highly branched (*C*) or immature (*D*) oligodendrocytes. Glial cultures from adult rat optic nerves (6–11 d *in vitro*) were cocultured for 1–5 d with embryonic rat retinal cells. O_4-stained oligodendrocytes were classified morphologically, and the total area occupied by each oligodendrocyte, as well as the fraction occupied by retinal cells, was determined by measuring with a graphic tablet. n = 109.

was seen during contact and crossing of immature cells (T. Zachleder and M. Schwab, unpublished observations). These observations also exclude that the "windows" were formed secondarily in the neurite plexus.

Astrocytes in the same cultures were often overgrown by single neurites or neurite bundles (Fig. 4, *a, b*). This was true for both morphological types, flat and stellate cells.

Cocultures with fetal rat retinal cells

After plating retinal cells at monolayer density on top of 5-d-old cultures of optic nerve non-neuronal cells, a typical rearrangement of the retinal cells was observed: whereas oligodendrocyte precursor cells were often contacted by retinal cells, the highly branched oligodendrocytes were mostly free of them (Figs. 2, *g, h*; 3, *C, D*). Again, astrocytes were preferred as a substrate over polyornithine (Fig. 4, *c, d*).

Response of other cell types to highly branched oligodendrocytes

Neuroblastoma cells (line NB-2A) were plated at high cell density into dissociated optic nerve cultures and stimulated for fiber production by 2 mM dibutyryl-cyclic AMP or by GdNPF. At 7, 24, or 48 hr, the cultures were fixed and oligodendrocytes were identified by antibodies O_4 and O_1. Again, the territories of highly branched oligodendrocytes were clearly spared by neuroblastoma cells (Fig. 5, *a, b*). Processes produced by neuroblastoma cells situated close to oligodendrocytes were pointing away from the oligodendrocytes (Figs. 5, *a, b*; 6 and Table 2).

Primary culture *fibroblasts* and astrocytes in the optic nerve preparations and mouse *3T3 cells* showed a drastic "avoidance behavior" towards highly branched oligodendrocytes. 3T3 cells plated at high cell density into optic nerve glial cultures attached and flattened out between 30 min and 3 hr on the polyornithine substrate. In these forming monolayers, characteristic "windows" appeared corresponding to the territories of highly branched oligodendrocytes (Fig. 5*c–f*). At the sites of contact 3T3 cells formed a crescent-shaped bulge of cytoplasm. Lamellipodia were absent in this region. Significantly, fibroblasts that landed directly on highly branched oligodendrocytes completely failed to spread. As for neurons, immature oligodendrocytes were not visibly avoided by 3T3 cells (Figs. 5, *e, f*; 7).

Absence of species specificity

In addition to O_4-positive/A_2B_5-positive precursor cells, dissociated non-neuronal cells from E13 and E17 chick optic nerve also contained the characteristic O_4-positive/A_2B_5-negative/O_1-positive highly branched oligodendrocytes. 3T3 cells plated on top of chicken non-neuronal cells formed characteristic "windows" around these chick oligodendrocytes. These findings show that neither the specific morphology nor the unfavorable substrate property of oligodendrocytes is species specific (data not shown).

Myelin as a substrate

Since myelin consists of spirally wrapped oligodendrocyte membranes, we were interested in testing the properties of myelin as a substrate for neurons or fibroblasts. Crude myelin fractions from adult rat spinal cord or sciatic nerve were prepared by flotation on a sucrose gradient. The myelin was adsorbed to polylysine-coated tissue culture dishes and tested for its sub-

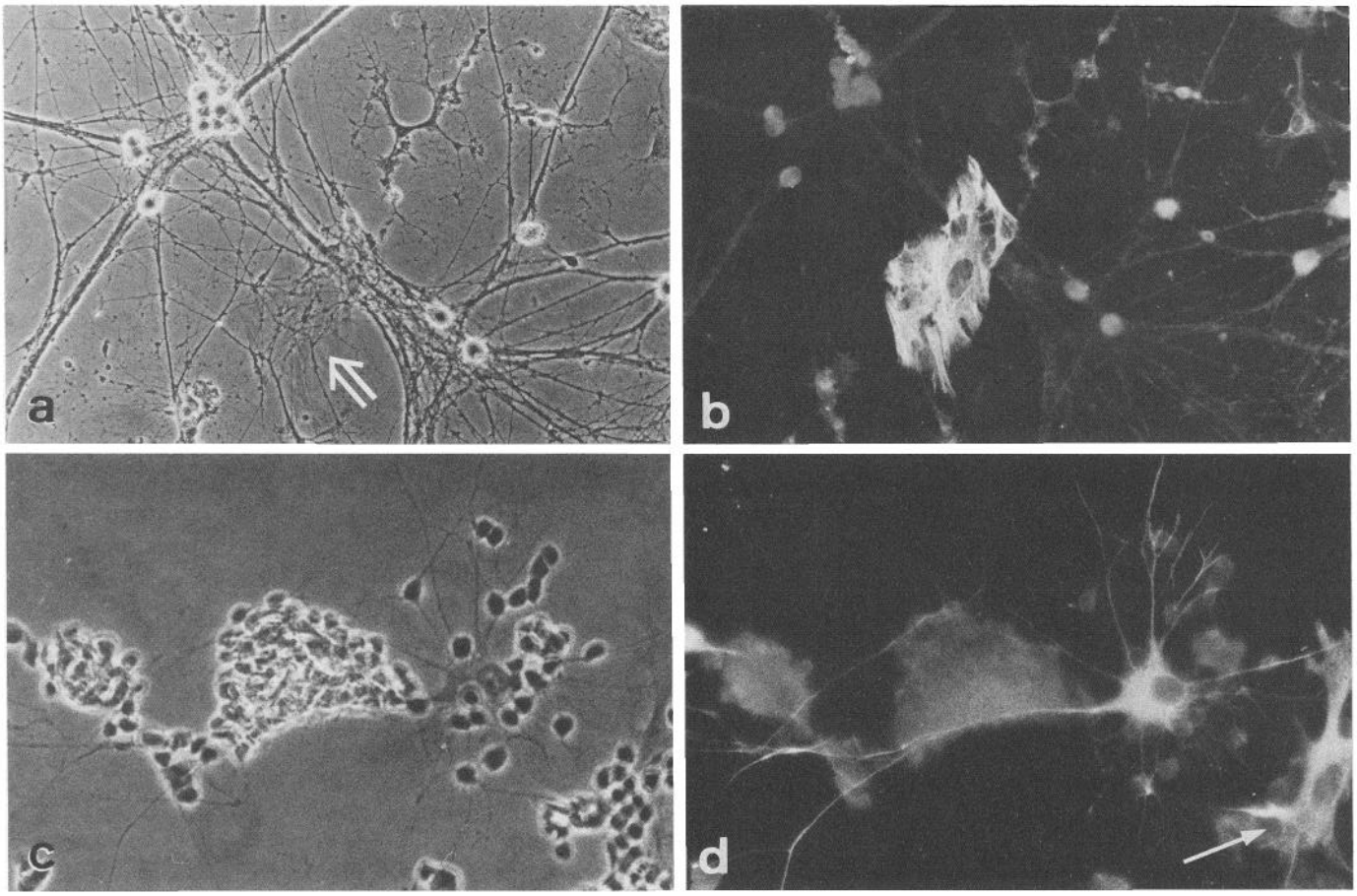

Figure 4. Astrocytes represent an adhesive substrate for neurons and neurites. *a* and *b,* Sympathetic neurites (13 d *in vitro*) growing on reactive, GFAP-positive (*b*) protoplasmic astrocyte (*arrow* in *a*) (from 10-d-old rat optic nerve, 23 d *in vitro*). ×220. *c* and *d,* Retinal cells (from E17 retina, 2 d *in vitro*) adhering to astrocytes (GFAP-positive; from 10-d-old optic nerve, 9 d *in vitro*) with long and with short (*arrow*) processes. ×400.

strate properties for superior cervical ganglion cells, dorsal root ganglion cells, neuroblastoma cells, and 3T3 cells. All 4 cell types were attaching poorly to CNS myelin and showed marked difficulty in their process outgrowth. Sympathetic and sensory neurons on CNS myelin remained round or produced short, abortive fibers despite the presence of NGF (50 or 100 ng/ml; Fig. 8, *a, c*). In contrast, long fibers were produced on islets of sciatic nerve myelin in the same culture dishes (Fig. 8, *b, d*). Small CNS myelin islets on polylysine appeared as "windows" outlined by excluded neurites, whereas PNS myelin–polylysine boundaries were apparently not detected by growing neurites (Fig. 8, *c, d*).

Process outgrowth from *neuroblastoma cells* (line NB-2A) in the presence of dibutyryl-cyclic AMP was significantly reduced by CNS myelin (Fig. 9*A*).

Spreading of *3T3 fibroblasts* was strongly inhibited by CNS myelin (Figs. 9*B,* 10). 3T3 cells remained round or produced spindle-shaped or polygonal morphologies with a minimal cell substrate interaction. In contrast, large flat membranes were produced within 20–30 min on polylysine and, with a somewhat slower time course, also on myelin from the PNS (Figs. 9*B,* 10). Nonpermissiveness was associated, at least in large part, with myelin membranes since sedimentation at 10,000 *g* for 5 min under hypotonic conditions (see Materials and Methods) was sufficient to pellet most nonpermissive membranes. Under these conditions, most surface membrane components floating to densities smaller than the one of 0.85 M sucrose would not be expected to sediment.

These experiments show that, in parallel to the effects of living, highly branched oligodendrocytes, myelin from the CNS is also a strongly nonpermissive substrate for primary culture neurons, neuroblastoma cells, and 3T3 fibroblasts. Myelin from the PNS does not show a comparable nonpermissive substrate effect. Our experiments do not exclude the possibility that PNS myelin permissiveness is due to contaminating basement membrane components, which would adhere tightly to the myelin membranes. CNS myelin nonpermissiveness, however, is not due to astrocyte membranes, since a cell membrane preparation from CNS tissue containing minimal amounts of white matter (superficial cortical layers) was a permissive substrate for fibroblast spreading (P. Caroni and M. E. Schwab, unpublished observations).

Discussion

Cell attachment, spreading, and motility, and, in particular, neurite outgrowth, are strongly dependent on cell–substrate interactions (Sanes, 1983; Carbonetto, 1984). An increasing number of substrate molecules favoring neuroblast migration or neurite outgrowth are currently being found and characterized in central and peripheral nervous tissue (Cornbrooks et al., 1983; Edelman, 1984; Liesi, 1985b; Stallcup et al., 1985; Chiu et al.,

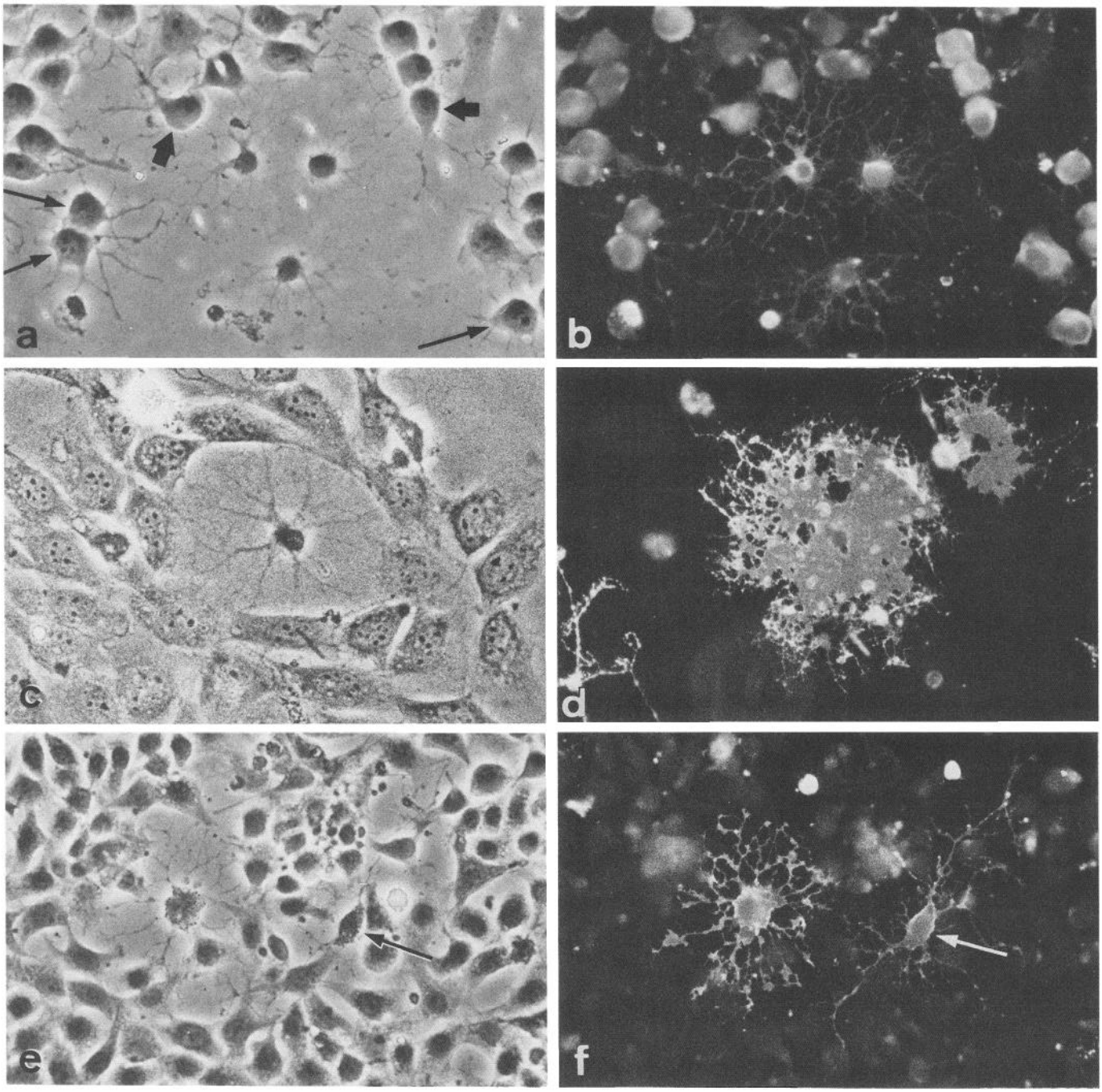

Figure 5. *a* and *b*, Highly branched oligodendrocytes (O_4-positive) are nonpermissive for attachment and fiber outgrowth of NB-2A neuroblastoma cells. NB-2A cells were cultured for 24 hr on optic nerve glial cells (6-d-old rat optic nerves, 3 d in culture) and stimulated for neurite outgrowth by GdNPF (Guenther et al., 1986). NB-2A cells *adjacent* to oligodendrocytes (*short arrows*) show asymmetric outgrowth, *distant* cells (*long arrows*) show random orientation of outgrowth (see Fig. 6). ×260. *c–f*, 3T3 fibroblasts plated at high cell densities into optic nerve glial cultures show nonpermissive substrate effect of highly branched oligodendrocytes (*c* and *e*). The oligodendrocyte in *c* and *d* has large membrane areas connecting its process network. An immature oligodendrocyte (*e* and *f*: *arrow*; O_4-positive, irregular morphology) is overgrown by spreading fibroblasts. Ten- (*c*, *d*) and 12- (*e*, *f*) d-old optic nerves, 2 d *in vitro*; 3T3 added for 3 hr. *d* and *f*, O_4 staining; *c* and *d*; ×300; *e* and *f*, ×250.

1986; Fischer et al., 1986; Lindner et al., 1986; Mirsky et al., 1986; Carbonetto et al., 1987). The appearance of some of these constituents can be correlated with specific developmental stages and, in the PNS, also with denervation (Edelman, 1984; Liesi, 1985b; Stallcup et al., 1985; Daniloff et al., 1986; Carbonetto et al., 1987). The absence of one such component, laminin, in the differentiated mammalian CNS, in contrast to the PNS or lower vertebrate CNS, suggested the hypothesis that the absence of this favorable substrate could be crucial for the absence of neurite regeneration in the CNS of higher vertebrates (Hopkins et al., 1985; Liesi, 1985a; Carbonetto et al., 1987). In the present study, we observed that myelin-forming oligodendrocytes and isolated CNS myelin exert a nonpermissive substrate effect on outgrowing neurites of sympathetic and sensory neurons and neuroblastoma cells, as well as for the attachment of retinal cells and the spreading of fibroblasts. In cerebellar cultures, a similar

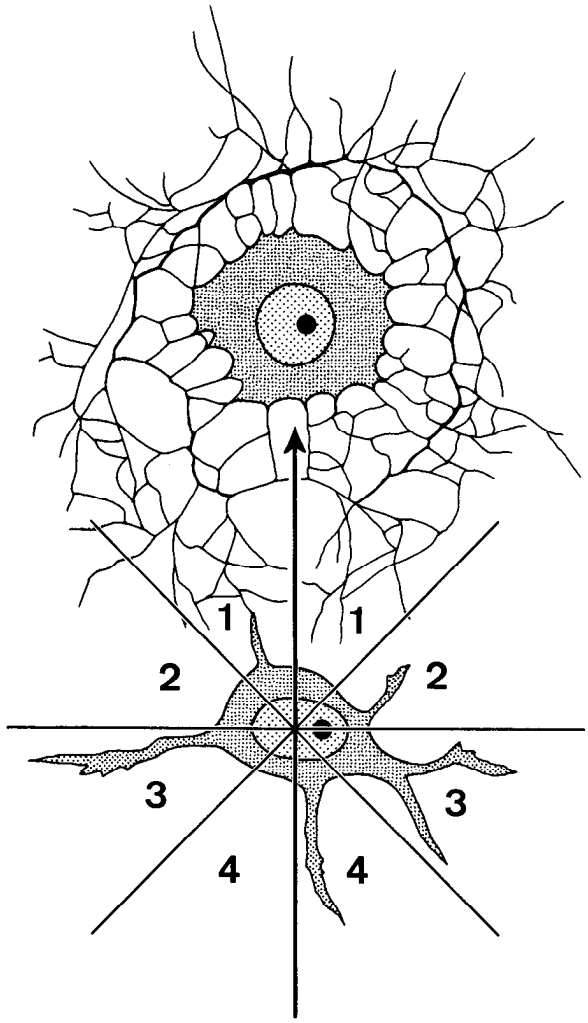

Figure 6. Orientation of neuroblastoma process outgrowth in relation to highly branched oligodendrocytes. Optic nerve glial cells (2 or 6 d *in vitro*) were cocultured with NB-2A cells for 24 hr in the presence of GdNPF or dibutyryl cAMP. O_4-positive, highly branched oligodendrocytes were systematically sampled and neighboring neuroblastoma cells were classified as *adjacent* when the distance between the edge of oligodendrocyte process network and neuroblastoma cell body was less than 2 cell body diameters (Fig. 5, *a, b*). Neuroblastoma cells at greater distances were classified as *distant*. Neuroblastoma processes were assigned to 4 sectors (1–4) according to their direction with regard to the closest oligodendrocyte as illustrated. Values in Table 2 represent means ± SEM of 3 experiments (60–100 neurites from 3 cultures per experiment). * $p < 0.05$; *** $p < 0.001$.

Table 2. Orientation of neuroblastoma processes with regard to highly branched oligodendrocytes

Percentage of processes in each sector

Sector	Adjacent neuroblastoma cells	Distant neuroblastoma cells
1	7 ± 1.4	25 ± 2.4[a]
2	34 ± 1.2	26 ± 1.2[a]
3	33 ± 2.7	25 ± 2.3[b]
4	26 ± 2.3	24 ± 2.7

[a] $p < 0.001$.
[b] $p < 0.05$.

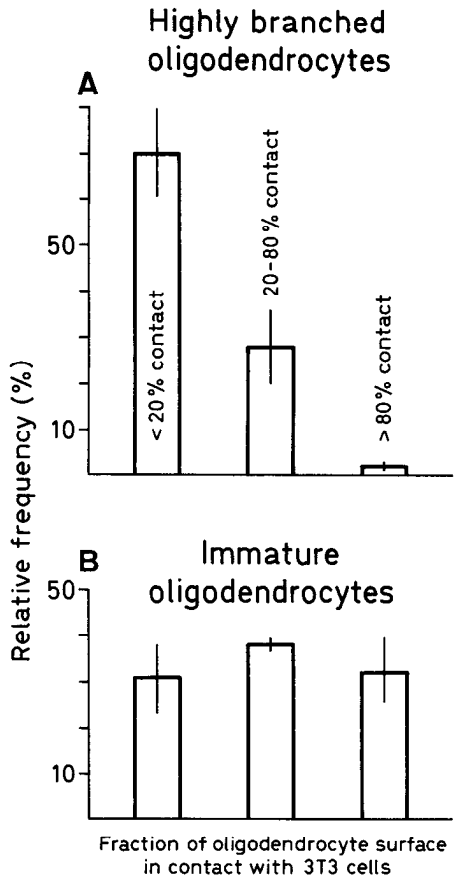

Figure 7. Histograms showing the overlap of 3T3 cells with highly branched (*A*) or immature (*B*) oligodendrocytes. 3T3 cells were cocultured for 3–4 hr on optic nerve glial cells at high cell density, and cultures were fixed and stained with O_4. Oligodendrocytes were sampled systematically, classified as highly branched or immature oligodendrocytes and their overlap with 3T3 cells determined in the 3 categories indicated. Values represent means ± SEM of 4 experiments (70–170 cells).

lack of association of neurons with GalC-positive oligodendrocytes, in contrast to astrocytes, was described by Hatten et al. (1984).

Several classes of cells were present in short-term cultures of dissociated rat optic nerves: oligodendrocytes, astrocytes (GFAP-positive), fibroblasts (Thy-1-, fibronectin-positive), and several types of precursor cells. Within the oligodendrocyte family (O_4-positive; Sommer and Schachner, 1981), one main cell subtype was characterized by the absence of the O_1 antigen (GalC) and of MBP, 2 components highly characteristic of myelin (Mirsky et al., 1980), and the presence of binding sites for the antibody A_2B_5. A_2B_5 was shown to be a marker for oligodendrocyte/type II astrocyte precursors, type II astrocytes, and neurons (Abney et al., 1981; Schnitzer and Schachner, 1982; Raff et al., 1983). Therefore, we considered this cell class to represent immature oligodendrocytes, probably including precursors as those described by Dubois-Dalcq (1986) and Sommer and Noble (1986). The presence of O_4 distinguishes these cells from the O2A precursors (Raff et al., 1983). These immature cells showed irregular and variable morphologies with bipolar shapes or polygonal cell bodies and irregular processes, often decorated with filopodia. The cell class is probably heterogenous; cell division could be observed (F. Dutly and M. E. Schwab, unpublished observations). The second main oligodendrocyte subclass consisted

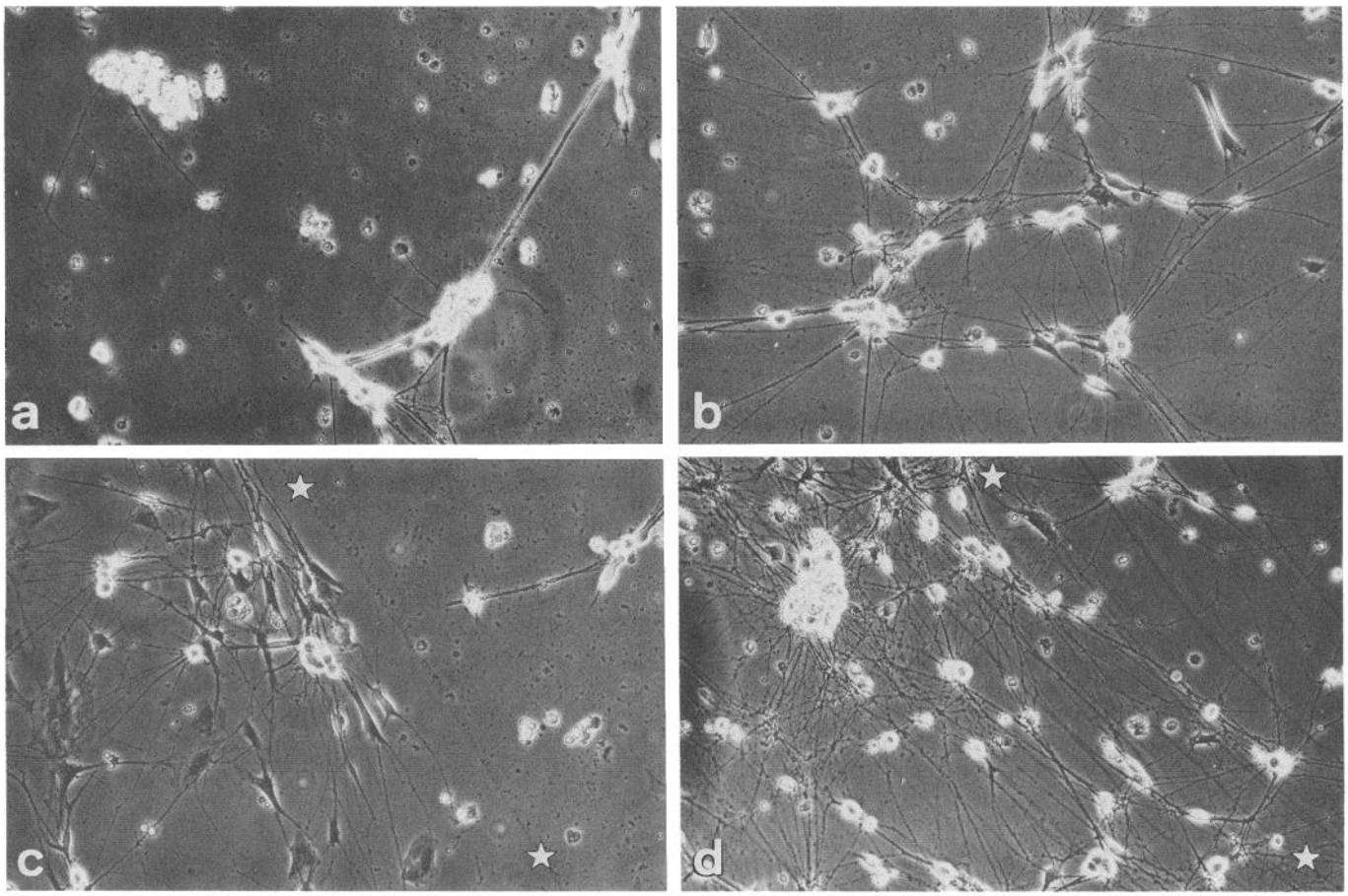

Figure 8. Inhibition of neurite outgrowth by CNS myelin as a substrate. Sympathetic neurons (from 1-d-old rat superior cervical ganglia) cultured in presence of 100 ng/ml NGF for 26 hr on polylysine-coated culture dish containing focal spots of CNS or PNS myelin. CNS myelin (*a, c*) strongly inhibits neurite outgrowth; PNS myelin (*b, d*) is a permissive substrate. In *c* and *d,* the border of a myelin islet on the polylysine is shown (*asterisk*). ×75.

of A_2B_5-negative, GalC-positive cells possessing a radial, highly branched, and anastomosing process network. Most of these highly branched oligodendrocytes in 2-d-old cultures of 10-d-old rat optic nerves were positive for MBP under our culture conditions. We thus interpret this frequent cell type as representing oligodendrocytes actively involved in the synthesis of myelin membranes. In the absence of axons, these membranes are deposited flat on the culture substrate; as such they are unstable and collapse to form the characteristic, anastomosing process network. This cell type has been described as "hairy eyeball cell" (Sommer and Schachner, 1981), and formation of whorls of compact myelin by such cells has been observed after prolonged times in culture (Rome et al., 1986; Yim et al., 1986).

Both immature and myelin-forming oligodendrocytes were seen in cultures of 7- to 10-d-old or adult rat optic nerves and also in cultures of 1-d-old rat optic nerves, newborn rat spinal cord, and adult rat corpus callosum (unpublished observations), as well as in cultures of spinal cord and optic nerves of E13 or E17 chick embryos. Immature cells were clearly predominant in dissociates from younger stages, but the large drop in cell yield upon dissociation with increasing age precluded any quantitative population analysis. However, immature oligodendrocytes could also be obtained consistently from adult rat white matter tissues, confirming earlier observations by ffrench-Constant and Raff (1986).

The addition of neurons to established glial cultures showed dramatic differences in substrate properties for neuronal attachment and fiber outgrowth among the various types of nonneuronal cells. Astrocytes, in particular the flat reactive protoplasmic astrocytes were adhesive and favorable for neuronal attachment and outgrowth, in agreement with earlier observations (Foucaud et al., 1982; Hatten et al., 1984; Noble et al., 1984; Fallon, 1985). Immature oligodendrocytes were also frequently contacted by neurites or nerve cell bodies. This behavior could have significant physiological relevance. During development, oligodendrocyte precursors migrate into the already formed axonal bundles and extend processes to contact a certain number of axons. These processes then start to enwrap and spiral around the axons, thus forming the structure called myelin (Wood and Bunge, 1984).

In sharp contrast to astrocytes and oligodendrocyte precursors, we found that myelin-forming oligodendrocytes display strongly nonpermissive substrate properties for neuronal attachment and fiber outgrowth, as well as for fibroblast attachment and spreading. This effect was strong and pronounced even on laminin-coated culture dishes, which otherwise represent an excellent substrate for neurite growth (Manthorpe et al., 1983; Rogers et al., 1983). This effect could not be overcome by high doses of NGF in cultures of sympathetic and sensory neurons or GdNPF or dibutyryl-cyclic AMP in cultures of neuroblas-

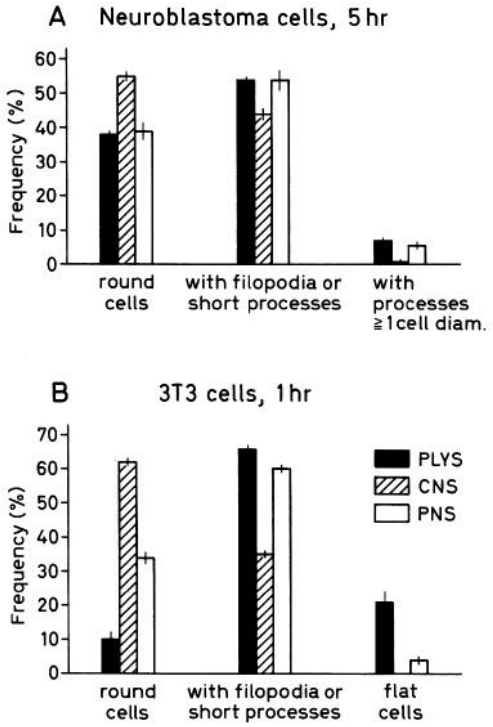

Figure 9. Nonpermissive substrate effects of CNS myelin but *not* PNS myelin for neurite outgrowth from neuroblastoma cells (*A*) and for 3T3 cell spreading (*B*). *A,* Neuroblastoma cells cultured for 5 hr in presence of 2 mM dibutyryl cAMP on polylysine (*solid bars*), CNS myelin-coated polylysine (*hatched bars*), or PNS myelin-coated polylysine (*open bars*). Cells were classified as round cells, filopodia or short process carrying cells or cells with processes longer than 1 cell diameter. Values represent means ± SEM of 3 cultures (250–450 cells per culture). *B,* 3T3 cells cultured for 1 hr on polylysine, CNS myelin-coated polylysine, or PNS myelin-coated polylysine. Cells were classified as round cells, cells with filopodia or short processes, or large flat cells. Values represent means ± SEM of 3 cultures (300–400 cells per culture).

toma cells. A similar or identical nonpermissive substrate property was associated with rat CNS myelin but not with myelin from peripheral nerves. The effect was strictly contact-dependent, since nerve cells or fibroblasts grew well and were free to move in the immediate surrounding of these oligodendrocytes or of CNS myelin islets. Mouse 3T3 cells were also inhibited by chicken oligodendrocytes, showing that this effect is not species specific. Recent biochemical studies have shown that the activity is due to specific myelin and oligodendrocyte membrane proteins (Caroni and Schwab, 1988a, b). As expected from the difference from peripheral myelin, the general physicochemical properties of myelin and the myelin lipids are unrelated to this effect. The fact that mobility of neuronal growth cones, as well as of fibroblast lamellipodia, is inhibited suggests a common underlying cell biological mechanism, which is currently under investigation.

In the rat optic nerve, the peak number of axons is reached at embryonic day 20, followed by a dramatic loss of axons (Crespo et al., 1985). Oligodendrocyte precursors appear from E17 on (Raff et al., 1985) and express GalC around birth (Miller et al., 1985). The first myelin detectable by electron microscopy appears at postnatal day 6 (Hildebrand and Waxman, 1984). This clear-cut temporal dissociation between axonal growth and myelin formation is also present in chicken optic nerves (Rager, 1980) and, although less well studied, in many white matter

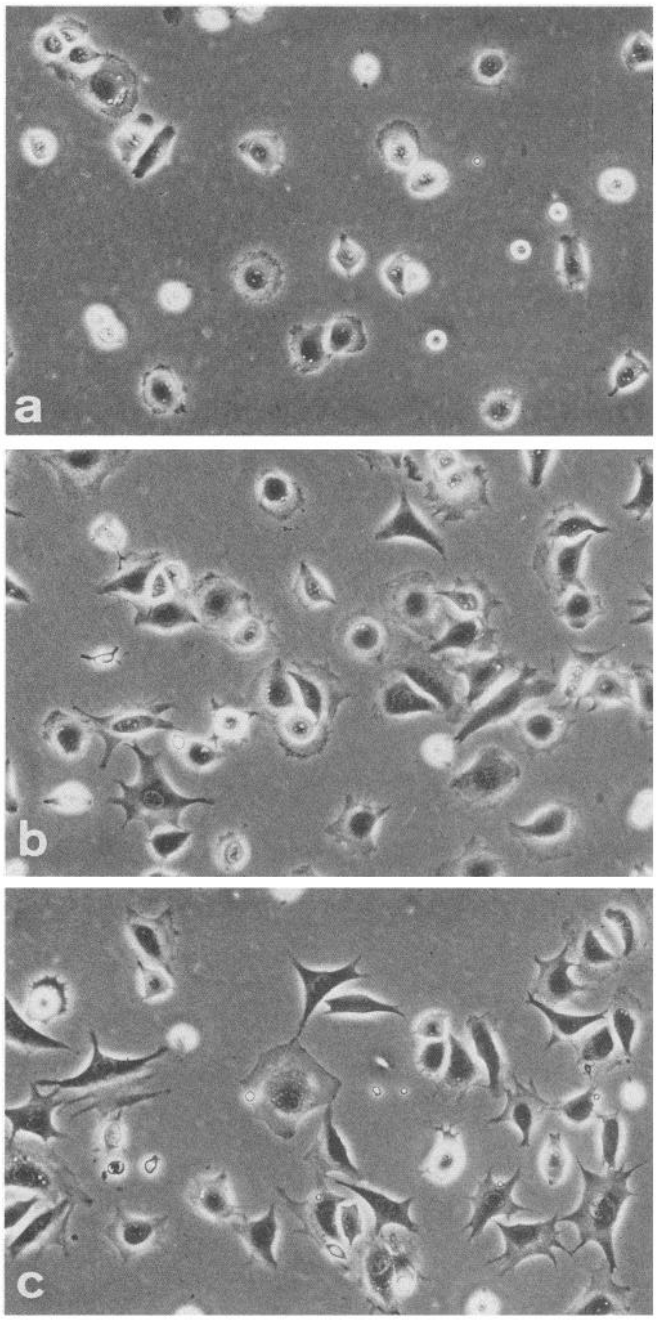

Figure 10. Spreading of 3T3 cells (1 hr) on polylysine coated with CNS myelin (*a*), PNS myelin (*b*), or on polylysine alone (*c*). Round cells or cells with short processes are predominant on the CNS myelin. ×170.

tracts of the CNS (Matthews and Duncan, 1971; Looney and Elberger, 1986). During normal development, growing axons therefore probably never encounter myelin or myelinating oligodendrocytes within their fascicles but, rather, interact with precursors and immature oligodendrocytes. The extremely slow time course observed for *in vitro* myelination (Wood et al., 1980; Wood and Williams, 1984) could be consistent with a situation in which undifferentiated oligodendrocytes first interact with axons and are then induced to differentiate and form myelin.

The physiological significance of the potent nonpermissive substrate property of oligodendrocytes and myelin remains to be investigated. In contrast to development, axonal growth cones

or sprouts do encounter mature oligodendrocytes and myelin during CNS regeneration. Substrate properties of CNS tissue, in particular the absence of potent neurite promoting substrates like laminin in the differentiated CNS of higher vertebrates, are important aspects in the context of CNS regeneration (Liesi, 1985a; Carbonetto et al., 1987). However, since myelin and oligodendrocytes persist for a long time in denervated CNS tracts (Fulcrand and Privat, 1977; Bignami et al., 1981), the absence of any fiber regeneration in white matter areas, in contrast to peripheral nerves and PNS/CNS transplants, could be related to these nonpermissive substrate factors. Implantations of fetal, well-regenerating adrenergic or cholinergic neurons into spinal cords or hippocampus have shown that some long-distance fiber growth can be observed in adult brain tissue, which is, however, strictly confined to gray matter areas (Kromer et al., 1981; Nornes et al., 1983; Björklund and Stenevi, 1984; Commissiong, 1984).

Under normal conditions, blocking certain territories for later-growing axonal populations during development, antagonism between favorable and nonpermissive substrate molecules during development of CNS projection patterns, or spatial limitation of sprouting in the differentiated CNS are possible functions for this oligodendrocyte-associated nonpermissive substrate property.

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