Studies of Adhesion Molecules Mediating Interactions between Cells of Peripheral Nervous System Indicate a Major Role for L1 in Mediating Sensory Neuron Growth on Schwann Cells in Culture

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Abstract. The involvement of the adhesion molecules L1, N-CAM, and J1 in adhesion and neurite outgrowth in the peripheral nervous system was investigated. We prepared Schwann cells and fibroblasts (from sciatic nerves) and neurons (from dorsal root ganglia) from 1-d mice. These cells were allowed to interact with each other in a short-term adhesion assay. We also measured outgrowth of dorsal root ganglion neurons on Schwann cell and fibroblast monolayers. Schwann cells (which express L1, N-CAM, and J1) adhered most strongly to dorsal root ganglion neurons by an L1-dependent mechanism and less by N-CAM and J1. Schwann cell–Schwann cell adhesion was mediated by L1 and N-CAM, but not J1. Adhesion of fibroblasts (which express N-CAM, but not L1 or J1) to neurons or Schwann cells was mediated by L1 and N-CAM and not J1. However, inhibition by L1 and N-CAM antibodies was found to be less pronounced with fibroblasts than with Schwann cells. N-CAM was also strongly involved in fibroblast–fibroblast adhesion. Neurite outgrowth was most extensive on Schwann cells and less on fibroblasts. A difference in extent of neurite elongation was seen between small- (10–20 μm) and large- (20–35 μm) diameter neurons, with the larger neurons tending to exhibit longer neurites. Fab fragments of polyclonal L1, N-CAM, and J1 antibodies exerted slightly different inhibitory effects on neurite outgrowth, depending on whether the neurites were derived from small or large neurons. L1 antibodies interfered most strikingly with neurite outgrowth on Schwann cells (inhibition of 88% for small and 76% for large neurons), while no inhibition was detectable on fibroblasts. Similarly, although to a smaller extent than L1, N-CAM appeared to be involved in neurite outgrowth on Schwann cells and not on fibroblasts. Antibodies to J1 only showed a very small effect on neurite outgrowth of large neurons on Schwann cells. These observations show for the first time that identified adhesion molecules are potent mediators of glia-dependent neurite formation and attribute to L1 a predominant role in neurite outgrowth on Schwann cells which may be instrumental in regeneration.

NEURITE outgrowth and guidance are important features in development and regeneration of the nervous system, since connectivity between nerve cell processes and their target cells is the decisive factor for functionality. The growing neurite most likely interacts with its cellular or acellular environment to some extent by surface cues which are instrumental not only in supporting growth itself, but also in providing directionality. Glial cells have been repeatedly reported to be an excellent substrate for promoting neurite extension and patterns of neurite outgrowth (Fallon, 1985*a*, *b*; McCafferty et al., 1984; Noble et al., 1984; Mudge, 1984; Denis-Donini et al., 1984; Selak et al., 1985; Banker, 1980; Liesi et al., 1983; Hall et al., 1987). In mammals, glial cells of the central nervous system (CNS)[1] support neurite outgrowth only up to a certain developmental stage, while in lower vertebrates CNS glia retain this capacity into adulthood (for review see Smith et al., 1986). Peripheral nervous system (PNS) glia in mammals, however, are capable of supporting neurite outgrowth not only during development, but also, in the adult animal, after axon degeneration by lesions (for review see Aguayo, 1985). Schwann cells not only support regrowth of PNS, but also of CNS axons (Aguayo, 1985). Thus, Schwann cells are unique in their capacity to function as a permissive substrate

1. *Abbreviations used in this paper*: CMF-HBSS, Ca^{++}- and Mg^{++}-free Hank's balanced salt solution; CNS, central nervous system; DRG, dorsal root ganglion; NGF, nerve growth factor; PNS, peripheral nervous system.

for growth and regrowth of axons at any age. The molecular mechanisms underlying this phenomena, however, have yet to be characterized.

The problem of Schwann cell–neuron interactions has recently been addressed with regard to cell adhesion molecules which are likely candidates for specifying cell contacts. Schwann cells express the cell adhesion molecules L1, N-CAM, the myelin-associated glycoprotein, and the extracellular matrix glycoprotein J1 (Faissner et al., 1984*b*; Daniloff et al., 1986; Martini and Schachner, 1986; Fahrig et al., 1987; Seilheimer and Schachner, 1987). L1 and N-CAM are expressed by non–myelin-forming Schwann cells in the adult animal and by all Schwann cells before the onset of myelination, while the myelin-associated glycoprotein only appears after myelination has been initiated (Martini and Schachner, 1986). Under regenerative conditions (e.g., after transection or crushing of the sciatic nerve), expression of L1 and N-CAM is increased within several days after the trauma (Nieke and Schachner, 1985; Martini and Schachner, 1988).

The present study was undertaken with the aim of characterizing the functional properties of adhesion molecules on Schwann cells with regard to their interactions with neurons. Furthermore, it appeared pertinent to investigate the particular adhesive properties of fibroblasts or fibroblast-like cells in relation to Schwann cells and neurons, since after transection of peripheral nerves the proximal and distal nerve stumps seal in a cap forming a territory through which neurites and Schwann cells have to regrow for successful regeneration (Martini and Schachner, 1988).

Materials and Methods

Animals

NMRI mice were used for all experiments. They were obtained from Zentrale Tierzuchtanlage, Hannover, FRG and maintained at the departmental animal facilities.

Antibodies

Polyclonal antibodies to mouse L1, N-CAM, and J1 have been described (Faissner et al., 1984*b*; Goridis et al., 1983; Kruse et al., 1985). Polyclonal antibodies to mouse liver membranes have also been described (Lindner et al., 1983; Pollerberg et al., 1986). mAbs to the larger molecular mass component of mouse neurofilament were purchased from Boehringer Mannheim GmbH, Mannheim, FRG.

TRITC-, FITC-, or horseradish peroxidase–conjugated antibodies to mouse or rabbit Igs were purchased from Cappel Laboratories, Cochranville, PA or Dakopatts GmbH (Glostrup, Denmark) and generally used at dilutions of 1:100 or 1:200.

Fab fragments of polyclonal antibodies were prepared according to Porter (1959).

Cell Cultures

Schwann cells were prepared from 1-d mouse sciatic nerves as described in detail (Seilheimer and Schachner, 1987).

Cultures of Dorsal Root Ganglion (DRG) Neurons

Cultures of DRG neurons were established from 1-d mice. Ganglia (~100) were dissected from the animals and incubated for 45 min at 37°C in 0.25% trypsin (Cooper Biomedical, Inc., Frankfurt, FRG) and 0.03% collagenase (Worthington Biomedical Corp., Berlin, FRG) in 2 ml DME containing 10 mM Hepes. After removal of 1.5 ml supernatant, 0.5 ml culture medium containing 0.2% FCS and 0.01% DNase I (Boehringer Mannheim GmbH, Mannheim, FRG) was added, and ganglia were mechanically dissociated in this medium by five cycles of pipetting through a fire-polished Pasteur pipette. Released cells were collected by centrifugation (5 min at 4°C and 80 *g*) and resuspended in 200 μl Eagle's basal medium containing 10 mM Hepes, 10 IU/ml penicillin, 10 IU/ml streptomycin, 10% horse serum, and 100 ng/ml beta-nerve growth factor (β-NGF) from mouse submaxillary glands (culture medium). The cell suspension (~3.5×10^6 cells total) was transferred onto a cushion of 35% Percoll (1 ml, Pharmacia Fine Chemicals, Uppsala, Sweden) and centrifuged for 15 min at 4°C and 200 *g*. The cell pellet (~3×10^5 cells of which ~90–95% are neurons) was washed once with 5 ml culture medium and plated in culture medium on a double layer of rat tail collagen (Bornstein, 1958; Ehrmann and Gey, 1956) at densities of 1 or 3×10^4 cells per 50–100 μl per well in 24-well plates (Nunc, Roskilde, Denmark) or on glass coverslips (15 mm diam). After 12–16 h in vitro, cytosine arabinoside (10^{-5} M, Sigma Chemical GmbH, Munich, FRG) was added to the culture and removed after 3 d by replacement with culture medium. Cultures were maintained for an additional 7 d without change of culture medium.

Preparation of Fibroblasts

Fibronectin-positive fibroblasts or fibroblast-like cells were prepared from 1-d mice by a procedure that followed exactly the preparation of Schwann cells up to the immunocytolysis step (see above, and Seilheimer and Schachner, 1987). Instead of immunocytolysis, the cultures were washed two times with DME and then incubated for 1 min at room temperature in DME containing 1 μg/ml trypsin and 0.02% EDTA. Fibroblasts were then lifted off the layer of Schwann cells by gentle pipetting, collected by centrifugation, resuspended, and seeded onto neurons on coverslips in Eagle's basal medium containing 10% horse serum. Yields were ~1.5×10^6 cells from ~40 mice and purity was between 98.3 and 99% by the criteria of epithelioid morphology and L1 and S-100 negativity (Seilheimer and Schachner, 1987). After 1 h in vitro, the medium was exchanged by culture medium as described for DRG neurons (see above).

Indirect Immunofluorescence and Immunoperoxidase Methods

Indirect immunofluorescence was carried out as described by Schnitzer and Schachner (1981). Indirect immunoperoxidase for neurofilament was carried out also according to Schnitzer and Schachner (1981) except that the first antibody was incubated overnight at 4°C and horseradish peroxidase–conjugated second antibody was used instead of a fluorescein-coupled antibody.

Immunoprecipitation

Immunoprecipitation was carried out according to Seilheimer and Schachner (1987) and Faissner et al. (1985).

Adhesion Test

Adhesion of single cell suspensions of Schwann cells and fibroblasts (probe cells) to monolayer cultures of DRG neurons, Schwann cells, and fibroblasts (target cells) in various combinations was measured by the method of Keilhauer et al. (1985). Target cells were obtained from 1-d mice. Schwann cells and fibroblasts were prepared as described above and plated at 4×10^4 cells per 100 μl per well of a 24-well Nunc tissue culture plate. DRG neurons were first plated at 3×10^4 cells per 100 μl per well with cytosine arabinoside and maintained in vitro for 10 d in culture medium before being used for the adhesion test. Probe cells were obtained from monolayer cultures of pure Schwann cells (Seilheimer and Schachner, 1987) and fibroblasts by treatment with 20 μg/ml trypsin, 0.02% EDTA in Ca^{++}- and Mg^{++}-free Hank's balanced salt solution (CMF-HBSS) for 15 min at room temperature after three washes with CMF-HBSS. To vitally stain the probe cells, fluorescein diacetate (10^{-6} M) was included during the trypsinization step. Trypsinization was stopped by adding 0.5 mg/ml soybean inhibitor and cells were collected by centrifugation (10 min at 4°C, 60 *g*). Treatment with the low trypsin concentration did not visibly reduce the intensities of immunofluorescence staining with L1, N-CAM, J1, and liver membrane antibodies compared to nontrypsinized cells. Cell concentrations were adjusted to 4×10^5 cells/ml CMF-HBSS for Schwann cells and fibroblasts. Probe and target cells were treated with 0.5 ml Fab fragments of antibodies in CMF-HBSS for 20 min on ice before the adhesion test. Target cells were washed three times with CMF-HBSS containing 0.02% EDTA before addition of Fab fragments. Probe cells (4×10^4 Schwann cells or fibroblasts) were added to the target cells and incubated at room temperature in a

reciprocal shaker at 40 cycles/minute or without shaking with identical results. Unbound probe cells were then removed by three gentle washing steps with 1 ml CMF-HBSS/well. Aggregation among probe cells was negligible both in controls and in the presence of Fab fragments. Probe cells always tended to adhere to target cells and not to the free plastic in cases where target cells were not confluent monolayers; i.e., when DRG neurons and Schwann cells were used. Bound cells were scored in an inverted fluorescence microscope (model ICM 405, Zeiss, Oberkochen, FRG) equipped with the appropriate filter combinations. Mean values ± SD were obtained from four independent experiments with each experimental value run in quadruplicate. At least 1,500 cells were counted per well. Without Fab fragments, the binding of the total input cells was 79 ± 4% for Schwann cell–Schwann cell adhesion; 71 ± 3% for Schwann cell–fibroblast adhesion; 81 ± 3% for Schwann cell–neuron adhesion; 69 ± 4% for fibroblast–Schwann cell adhesion; 85 ± 4% for fibroblast–fibroblast adhesion; and 65 ± 3% for fibroblast–neuron adhesion. Inhibition of adhesion in the presence of Fab fragments was calculated as follows: % inhibition = (adhesion [control] − adhesion [+Fab]/adhesion [control]) × 100.

Neurite Outgrowth Test

DRG neurons obtained after the plating step on fetuin (1,000–1,500 cells/30 μl per 1.5 cm coverslip) were plated onto a monolayer of Schwann cells (40,000 cells/coverslip) or fibroblasts (10,000 cells/coverslip) maintained for 2 d in vitro. After allowing the neurons to attach for 4–6 h, Fab fragments of antibodies (0.3 or 1 mg/ml) were added. After another 48 h, cells were fixed and processed for indirect immunoperoxidase staining with mAbs to neurofilament as described above. Coverslips were then embedded in Kayser's gelatine and neurofilament-stained cells scored morphometrically.

Morphometric Measurements

The length of processes of neurofilament-stained DRG neurons was measured with a computer (model M24; Olivetti, AI tektron, Meerbusch, FRG) equipped with a graphic tablet, TV camera, color monitor, and an image analysis program (VIDS III; AI tektron, Meerbusch, FRG). To monitor length of processes and area and circumference of cell bodies, a cursor was used in conjunction with the TV camera.

Results

We first determined the expression of adhesion molecules on Schwann cells, neurons, and sciatic nerve–derived fibroblasts by indirect immunofluorescence and immunoprecipitation.

Detection of Adhesion Molecules on DRG Neurons, Schwann Cells, and Fibroblasts by Indirect Immunofluorescence

We have previously shown that DRG neurons and Schwann cells from sciatic nerve of 1-d mice express the cell adhesion molecules L1 and N-CAM (Seilheimer and Schachner, 1987; Seilheimer, B., E. Persohn, and M. Schachner, manuscript submitted for publication). Schwann cells also express the secreted extracellular adhesion molecule J1 (Seilheimer and Schachner, 1987). Interestingly, fibroblasts from 1-d mouse sciatic nerve expressed N-CAM, but not L1 or J1 (Fig. 1). The polyclonal antibody prepared against liver membranes reacted with all three cell types (Fig. 1, Table I).

Immunoprecipitation of Cell Adhesion Molecules from DRG Neurons, Schwann Cells, and Fibroblasts from Sciatic Nerve of 1-d Mice

To confirm the presence or absence of cell adhesion molecules on the three cell types and to determine their molecular masses, immunoprecipitations were carried out. Expression of L1, N-CAM, and J1 has been shown on Schwann cells to occur in molecular forms slightly different from those expressed by neural cells in the CNS (Seilheimer and Schachner, 1987). L1 is seen as a single component at 210 kD, a molecular mass slightly higher than that observed on CNS neurons. N-CAM is expressed predominantly as the 140-kD form, but with a molecular mass slightly different from those of cerebellar neurons and telencephalic astrocytes (Seilheimer and Schachner, 1987). Also, J1 is expressed in slightly higher molecular mass forms in the cell culture supernatant and in the detergent lysates of Schwann cells than in the CNS (Seilheimer and Schachner, 1987).

Cultured DRG neurons expressed L1 in two molecular forms of 230 and 210 kD (Fig. 2, lane *1*). N-CAM was expressed by these neurons as bands of 180, 140, and 120 kD (Fig. 2, lane *2*). This finding is noteworthy, since in cerebellar granule cell neurons, neuroblastoma, and pheochromocytoma PC12 cells N-CAM was present as 180- and 140-kD components, and the 120-kD component has predominantly been found on glia (Keilhauer et al., 1985; He et al., 1986; Sadoul et al., 1986; Pollerberg et al., 1985). In agreement with the observation that J1 is not detectable by indirect immunofluorescence on DRG neurons, J1 was also not seen by immunoprecipitation (Fig. 2, lane *3*). Only L1 was detectable in culture supernatants with an apparent molecular mass of 210 kD (not shown; see also Sweadner, 1983*a*, *b*; Sadoul et al., 1986).

In cultures of fibroblasts from sciatic nerve, L1 and J1 were not detectable (Fig. 2, lanes *4* and *6*) as expected from the immunofluorescence experiments. N-CAM was detectable on these fibroblasts solely in its 140-kD form (Fig. 2, lane *5*). N-CAM was not detectable in the supernatants of the fibroblast cultures.

Involvement of L1, N-CAM, and J1 in Adhesion between Neurons, Schwann Cells, and Fibroblasts in Several Combinations

To probe whether expression of adhesion molecules on particular cell types is functional in cell adhesion, Schwann cells and fibroblasts were allowed to adhere to neurons and each other in a short-term adhesion assay in the absence and presence of Fab fragments of L1, N-CAM, J1, and liver membrane antibodies (Table II). Antibodies to mouse liver membranes reacting with all three cell types by indirect immunofluorescence (Table I) were used as negative controls in the adhesion assay. Antibodies to L1 interfered most strongly with Schwann cell–neuron adhesion (65% inhibition of adhesion). Schwann cell–Schwann cell adhesion was reduced to 51% in the presence of L1 antibodies. L1 appeared to be less prominently involved in Schwann cell–fibroblast and fibroblast–Schwann cell adhesion, and least prominent in fibroblast–neuron adhesion (29, 29, and 16% inhibition, respectively). Since fibroblasts do not express L1, fibroblast–fibroblast adhesion was not determined in the presence of L1 antibodies.

N-CAM was found to be quite prominently involved in adhesion between all cell types measured. Interestingly, it was most evident in fibroblast–fibroblast adhesion, which was inhibited by N-CAM antibodies by 71%. Inhibition of Schwann cell–neuron adhesion by N-CAM antibodies was 52%, followed by inhibition of Schwann cell–Schwann cell adhesion by 44%, fibroblast–neuron adhesion by 40%, and Schwann cell–fibroblast and fibroblast–Schwann cell adhesion by 30%.

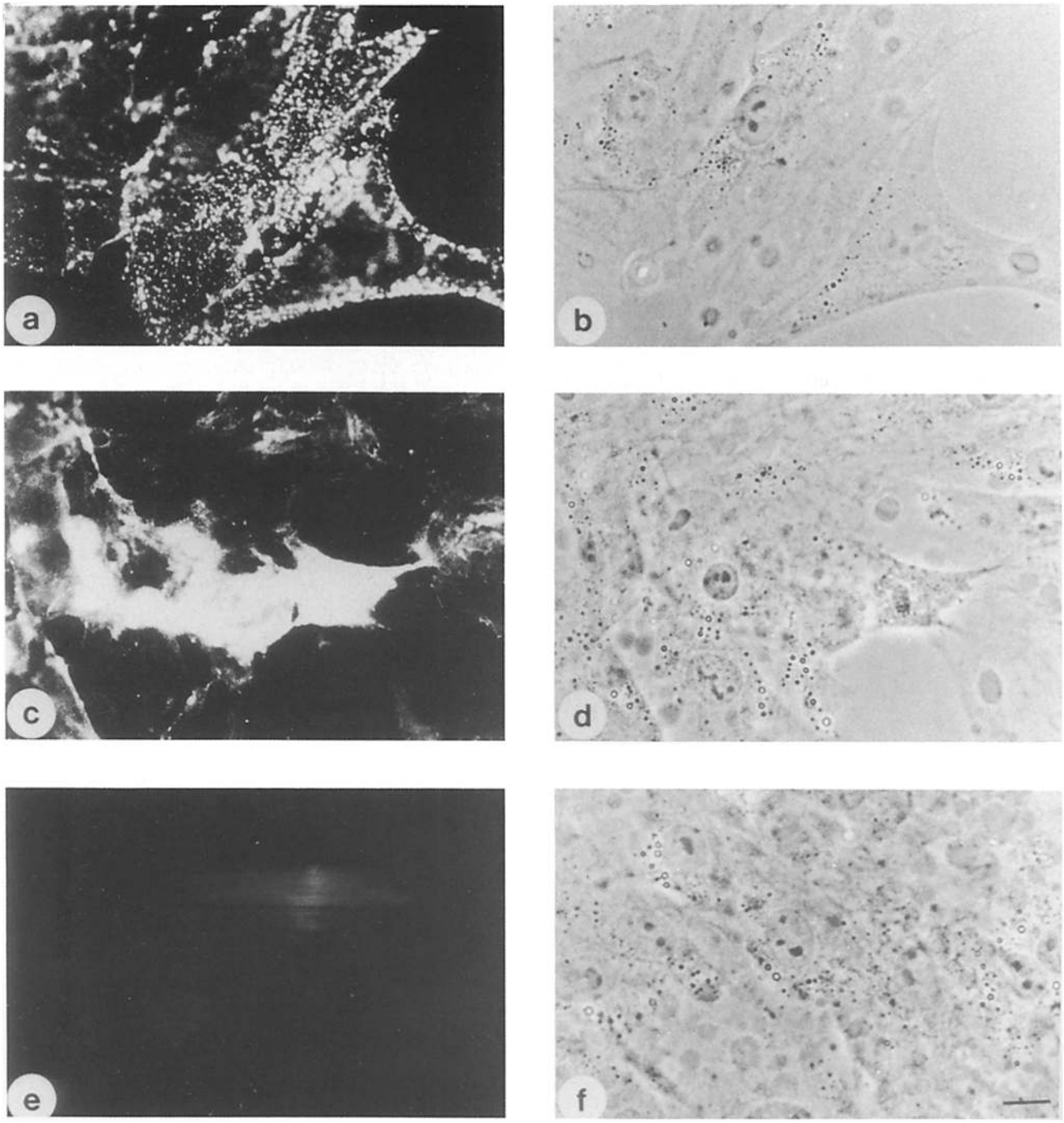


Figure 1. Indirect immunofluorescence on fibroblasts from mouse sciatic nerve using polyclonal N-CAM, liver membrane, and L1 antibodies on live cultured cells maintained in vitro for 7 d. Fluorescence images of (*a*) N-CAM, (*c*) liver membrane, and (*e*) L1. *b*, *d*, and *f* are phase-contrast micrographs corresponding to *a*, *c*, and *e*, respectively. Bar, 4 μm.

J1 was moderately involved in Schwann cell–neuron adhesion (inhibition of adhesion by 36% in the presence of J1 antibodies) but not in Schwann cell–Schwann cell adhesion.

The same results were obtained when the adhesion test was performed in the absence or presence of the divalent cations Ca^{++} and Mg^{++}.

Neurite Outgrowth from DRG Neurons on Monolayers of Schwann Cells and Fibroblasts

To test the influence of adhesion molecules on neurite outgrowth, DRG neurons were plated onto two substrates, Schwann cells and fibroblasts. To measure neurite outgrowth quantitatively, the total length of neurites per neuron was measured and calculated with an image analysis data program. Number of neurites per cell body and branching points per neurite were also determined. Cells were stained by indirect immunoperoxidase for neurofilament, so that neurites and cell bodies could easily be seen. The values of ≥100 cells were pooled in each experiment and compared between cultures maintained in the absence and presence of antibodies.

Table I. Detection of Cell Adhesion Molecules and Surface Membrane Constituents on DRG Neurons, Schwann Cells, and Fibroblasts from Sciatic Nerve by Indirect Immunofluorescence

Cells	L1	N-CAM	J1	Liver membrane
Neurons	+	+	−	+
Schwann cells	+	+	+	+
Fibroblasts	−	+	−	+

Cell adhesion molecules and surface membrane constituents were monitored by indirect immunofluorescence using polyclonal antibodies in cultures of pure neurons, Schwann cells, or fibroblasts after various times in culture (2–21 d) and scored as antigen positive (+) or negative (−) by comparison to negative controls (no first antibody or rabbit antibodies to human hemoglobin).

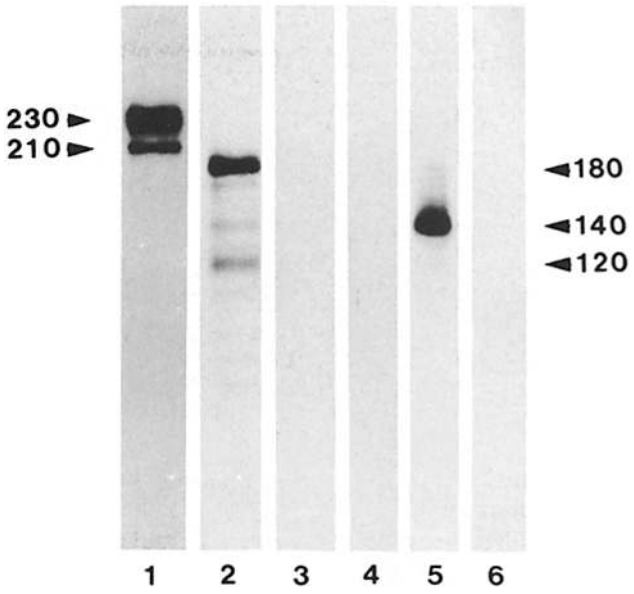


Figure 2. Immunoprecipitation of biosynthetically radiolabeled cultures of DRG neurons (lanes *1–3*) and fibroblasts from sciatic nerve (lanes *4–6*) using polyclonal L1, N-CAM, and J1 antibodies. Pure cultures of DRG neurons were maintained for 14 d in vitro and fibroblasts from sciatic nerve were maintained for 7 d in vitro. Immunoprecipitates were separated by SDS-PAGE (7% gels) and processed for autoradiography. Lanes *1* and *4*, L1; lanes *2* and *5*, N-CAM; and lanes *3* and *6*, J1. Molecular mass markers are indicated at the right and left margins.

It was noted in the first measurements that neurite outgrowth was differentially inhibited by antibodies depending on the size of the cell bodies giving rise to the neurites. Cell body size, as measured by diameter, was therefore included in our quantitative evaluations.

The total length of neurites from single small-diameter neurons was different from large-diameter neurons cultured on Schwann cells and fibroblasts (Table III). Small neurons (~70% of all neurons) were defined as having a diameter of 10–20 μm and large neurons (~30%) a diameter of 20–35 μm. The length of neurites from small-diameter neurons was generally shorter. Schwann cells promoted neurite extension of large neurons significantly better than fibroblasts (1,305 μm vs. 508 μm). Small-diameter neurons extended their neurites slightly but not significantly better on Schwann cells than on fibroblasts (246 vs. 220 μm).

The influence of L1 and mouse liver membrane antibodies on neurite outgrowth on Schwann cells as seen by indirect immunoperoxidase staining for neurofilament and in comparison to controls without antibodies is shown in Fig. 3. L1 antibodies (Fig. 3 *b*) were observed to interfere with neurite extension of a DRG neuron, when compared to the control culture maintained in the absence of antibodies (Fig. 3 *a*) or presence of mouse liver membrane antibodies (Fig. 3 *c*). (It should be noted that the length of neurites in control cultures shown in Fig. 3, *a* and *c*, extended far beyond the microscopic fields shown, while Fig. 3 *b* comprises the extent of neurites almost totally.) Computer tracings of single DRG neurons give an impression of the number of primary neurites and their initial branching patterns on a monolayer of Schwann cells in the absence and presence of antibodies to L1, N-CAM, and J1 (Fig. 4). Whereas in the presence of N-CAM (Fig. 4 *c*) and J1 (Fig. 4 *d*) antibodies the number of primary neurites and branching patterns appeared uninhibited, L1 antibodies reduced the overall length of neurites, number of processes emerging from the cell body, and branching patterns of neurites (Fig. 4 *b*). (Again, the length of neurites cannot be assessed completely from the microscopic fields shown, since they cut through neurites.)

The quantitative evaluation of total neurite length in the presence of L1, N-CAM, J1, and liver membrane antibodies on Schwann cells and fibroblasts is summarized in Table IV. Outgrowth of neurites on a monolayer of fibroblasts was not interfered with by L1 and N-CAM antibodies and both small

Table II. Influence of Fab Fragments of Polyclonal L1, N-CAM, J1, and Liver Membrane Antibodies on Adhesion between Neurons, Schwann Cells, and Fibroblasts in Several Combinations of Probe to Target Cells

Antibody	Fab concentration	Schwann cell* -neuron‡ adhesion	Fibroblast* -neuron‡ adhesion	Schwann cell* -Schwann cell‡ adhesion	Schwann cell* -fibroblast‡ adhesion	Fibroblast* -Schwann cell‡ adhesion	Fibroblast* -fibroblast‡ adhesion
	mg/ml						
None	0	0 ± 4 (4)	0 ± 1 (3)	0 ± 3 (4)	0 ± 3 (4)	0 ± 3 (3)	0 ± 4 (6)
L1	1.0	65 ± 3 (4)	16 ± 3 (3)	51 ± 3 (4)	29 ± 1 (4)	29 ± 1 (3)	ND
N-CAM	1.0	52 ± 1 (4)	40 ± 2 (3)	44 ± 2 (4)	30 ± 2 (4)	30 ± 3 (3)	71 ± 4 (6)
J1	1.0	36 ± 4 (2)	ND	9 ± 4 (2)	ND	ND	1 ± 0.3 (2)
Liver membrane	1.0	1 ± 3 (4)	−3 ± 1 (3)	3 ± 3 (4)	3 ± 3 (4)	3 ± 3 (3)	1 ± 4 (6)

Percent inhibition of adhesion is given as mean value ± SD. Numbers in parentheses represent numbers of experiments run each in quadruplicate.
* Probe cells.
‡ Target cells.

Table III. Total Length of Neurites from One DRG Neuron with Small or Large Cell Body Maintained on Schwann Cells and Fibroblasts

Diameter of neurons	Schwann cells	Fibroblasts
	μm	μm
Small (10–20 μm)	246 ± 31	220 ± 20
Large (20–35 μm)	1,305 ± 66	508 ± 39

Neurons were maintained for 2 d in vitro. Diameters of cell bodies were taken at their largest value. Mean values ± SD are from ≥100 neurons in each of three independent experiments.

and large DRG neurons were equally refractory to inhibition. The most drastic inhibitory effect on neurite outgrowth was seen with L1 antibodies on Schwann cells. Neurite outgrowth of small neurons was inhibited by 88% and of large neurons by 76%. N-CAM antibodies interfered with neurite outgrowth of large neurons by 34% and of small neurons by only 16%. Conversely, J1 antibodies interfered slightly with neurite outgrowth of large neurons (17% inhibition), but not of small neurons.

On Schwann cells, L1 antibodies also reduced the number of neurites extending from the neuronal cell body (from a maximum of 4 to 2) (Fig. 5 *A*) and the number of branch points per neurite (from a maximum of 11 to 6) (Fig. 6 *A*), whereas the other antibodies did not affect these parameters (Figs. 5 and 6). On fibroblasts the number of neurites per cell body and branch points per neurite were not affected by any of the antibodies (Figs. 5 *B* and 6 *B*).

To verify that alteration in cell shape and neurite outgrowth is not due to decreased viability of cells in the presence of antibodies, antibodies were removed after 2 d and neurite outgrowth was observed to resume its normal pattern within several days. Furthermore, neurons were never seen to disintegrate and be reduced in number in the presence of antibodies even up to 14 d.

Discussion

The present study was instigated by the question of the individual contributions of cell adhesion molecules to adhesion between and neurite outgrowth on the accessory cells of the PNS, the Schwann cells, and fibroblasts or fibroblast-like cells. As contributors to cell interactions in the developing and regenerating nervous system not only Schwann cells but also fibroblasts have to be considered. Endoneurial fibroblasts are not only partners of Schwann cells at the basement membrane side of Schwann cells, opposite to the side where Schwann cells meet axons, but fibroblasts also exist as a prominent cell type in the cap sealing the proximal and distal nerve stumps after transection or nerve crushing (Nieke and Schachner, 1985; Daniloff et al., 1986; Martini and Schachner, 1988). Fibroblasts in this cap are strikingly N-CAM positive, in contrast to the endo-, peri-, and epineurial fibroblasts which do not express N-CAM at any postnatal developmental stage studied (Martini and Schachner, 1988; Martini, R., personal communication). Since regenerating axons have to grow through this N-CAM-positive territory of fibroblasts, the contribution of neuron adhesion and neurite outgrowth with regard to these cells appeared pertinent to investigate. Since cultured fibroblasts from sciatic nerve do indeed express N-CAM in our culture system in contrast to the N-CAM-negative fibroblasts from skin and brain (our unpublished observations), the contribution of these cells to adhesion and neurite outgrowth could be tested.

Expression of adhesion molecules in the PNS occurs in a cell type-specific fashion. As reported previously, Schwann cells express the three adhesion molecules L1, N-CAM, and J1 (Seilheimer and Schachner, 1987). Neurons only express L1 and N-CAM, whereas fibroblasts only express N-CAM. Thus, adhesion between these three cell types is expected to occur also in a cell type-specific manner. N-CAM is involved in the interactions between all three cell types: Schwann cell–neuron, fibroblast–neuron, Schwann cell–Schwann cell, Schwann cell–fibroblast, and most strongly in fibroblast–fibroblast adhesion. L1 shows a more graded contribution to cell adhesion in that it most strongly contributes to Schwann cell–neuron adhesion but also contributes to Schwann cell–Schwann cell adhesion and, to a lower degree, to fibroblast–neuron and Schwann cell–fibroblast adhesion. Since fibroblasts do not express L1, these cells must carry an L1 binding molecule different from L1 itself. J1 shows the most cell type-restricted involvement in cell adhesion in that it only mediates Schwann cell–neuron adhesion, not Schwann cell–Schwann cell adhesion, confirming that J1 is a neuron–glia adhesion molecule (Kruse et al., 1985) mediating inter-

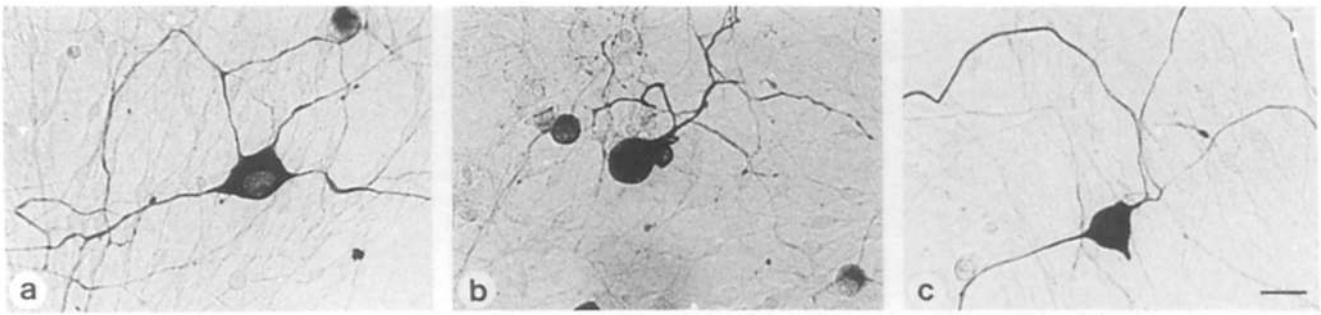


Figure 3. Neurite outgrowth of DRG neurons maintained on a monolayer of Schwann cells for 3 d in vitro in the absence (*a*) and presence of Fab of polyclonal L1 (*b*) and liver membrane (*c*) antibodies. Neurites of DRG neurons were visualized by indirect immunoperoxidase staining using a mAb to neurofilament. Total lengths of neurites cannot be assessed from the microscopic fields shown, since these cut through most neurites in *a*, one neurite in *b*, and all neurites in *c*. Bar, 8 μm.

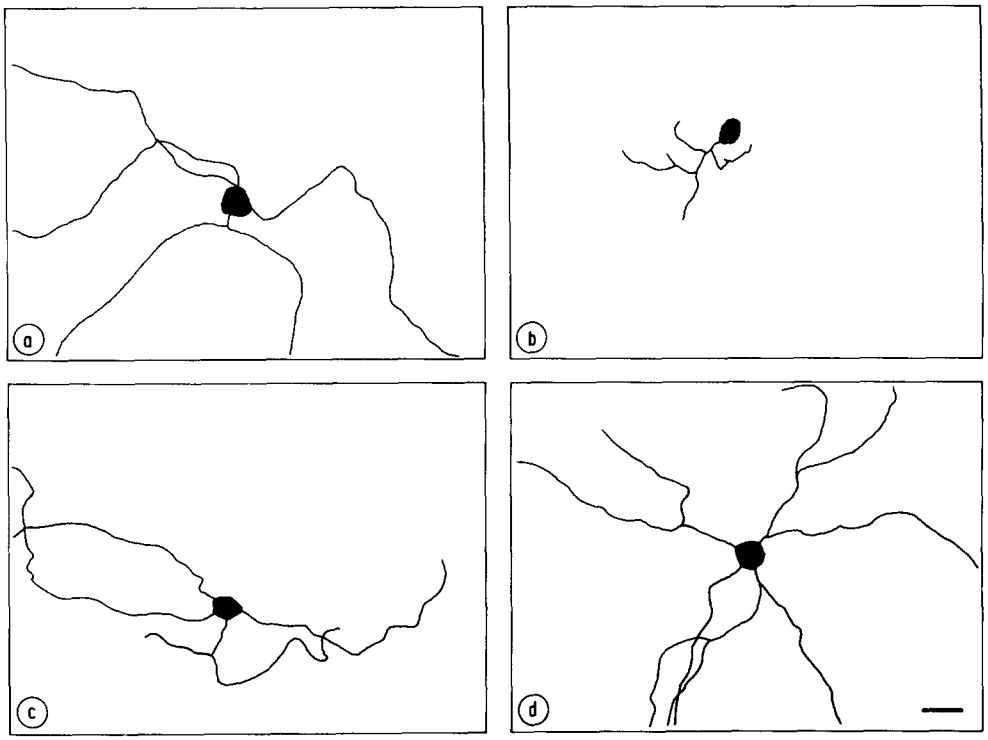


Figure 4. Computer tracing of neurites of DRG neurons maintained on a monolayer of Schwann cells for 2 d in the absence (*a*) and presence of Fab fragments of polyclonal L1 (*b*), N-CAM (*c*), and J1 (*d*) antibodies. Total lengths of neurites cannot be assessed from the fields shown, since these cut through all neurites limited by the frame in *a*, *c*, and *d*. *b* shows an entire cell. Bar, 45 μm.

actions between J1-positive glial cells and J1-negative neurons, but not among J1-positive glial cells. The prominent involvement of N-CAM in fibroblast–fibroblast adhesion could suggest that N-CAM expression on fibroblasts is the predominant mechanism in adhesion of fibroblasts with each other. This type of adhesion may be important in the formation of the fibroblastic cap sealing the proximal and distal nerve stumps after nerve injury. It is interesting in this context that during postnatal development of the sciatic nerve, endoneurial fibroblasts are observed rarely in contact with each other, but usually adjacent to Schwann cells, and have never been seen to express N-CAM (Martini and Schachner, 1986). On the other hand, denervated muscle fibroblasts were observed to express N-CAM (Covault and Sanes, 1985) and regenerating limb buds in newt were N-CAM positive in mesenchymal structures (Maier et al., 1986).

The contribution of the individual cell adhesion molecules to neurite outgrowth is somewhat distinct from the pattern of their involvement in cell adhesion as far as neuron–fibroblast interactions are concerned. Neither L1 nor N-CAM appear to be involved in neurite outgrowth on fibroblasts, although at least N-CAM quite distinctly mediates adhesion between fibroblasts and neurons. On the other hand, with regard to neuron–Schwann cell interactions, the degree of involvement of each adhesion molecule in adhesion is paralleled by that in neurite outgrowth, implicating neurite length,

Table IV. Influence of Fab Fragments of Polyclonal L1, N-CAM, and J1 Antibodies on Neurite Length of DRG Neurons on Schwann Cells, and Fibroblasts

Substrate	Antibody	Fab concentration	Small neurons*	Large neurons‡
		mg/ml		
Schwann cells	None	0	0 ± 3	0 ± 3
	L1	0.3	88 ± 6	76 ± 6
	N-CAM	0.3	16 ± 3	34 ± 4
	J1	0.3	6 ± 3	17 ± 4
	Liver membrane	0.3	1 ± 2	2 ± 2
Fibroblasts	None	0	0 ± 2§	
	L1	0.3	−1 ± 1	
	N-CAM	0.3	2 ± 1	
	Liver membrane	0.3	1 ± 2	

Percent inhibition of neurite outgrowth of DRG neurons after 2 d in vitro is given as mean value ± SD calculated from three independent experiments. At least 100 neurons were measured in each experiment.

* Neurons with a cell body diameter of 10–20 μm.

‡ Neurons with a cell body diameter of 20–35 μm.

§ No distinction was made between small and large diameter neurons.

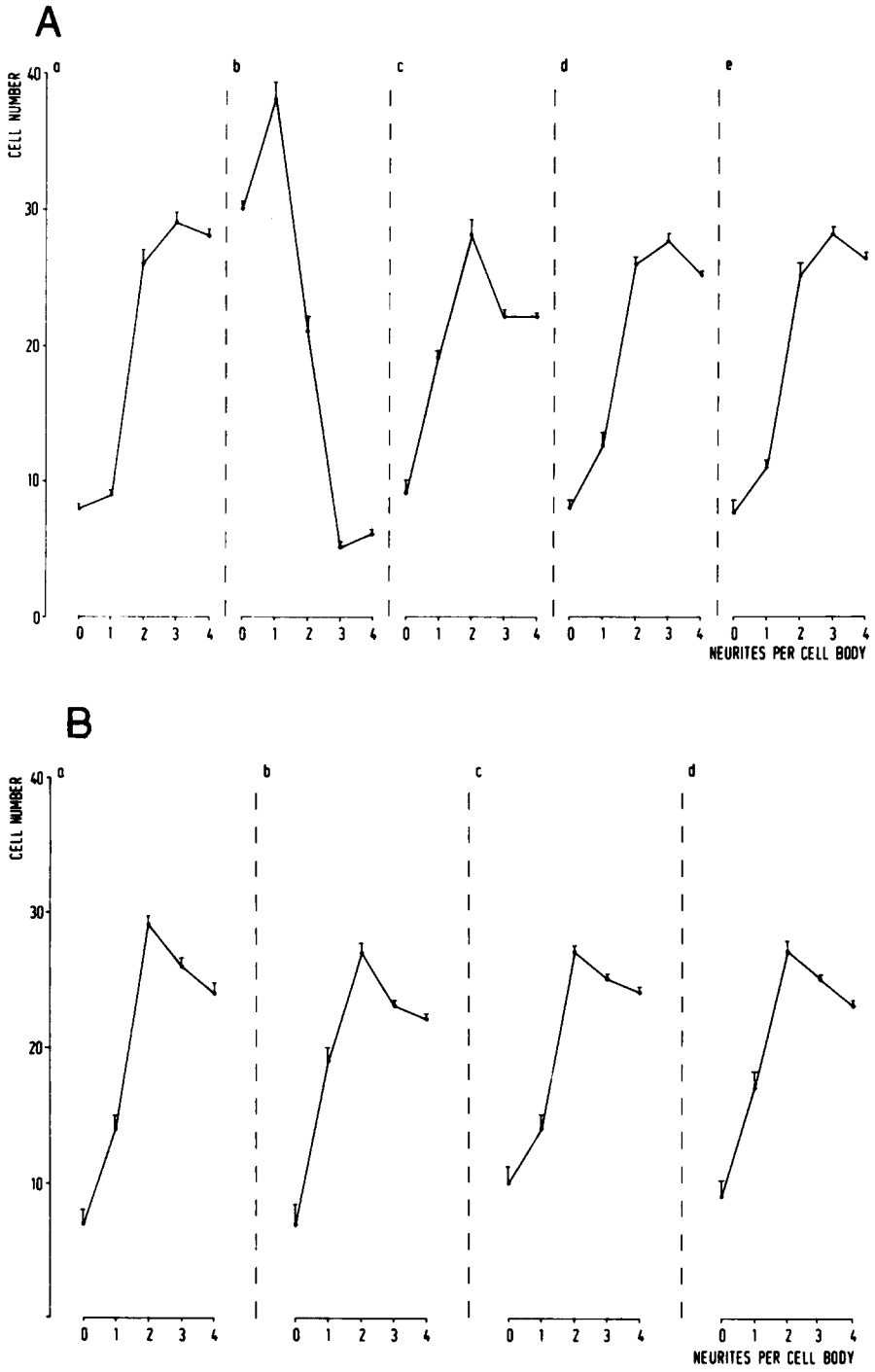
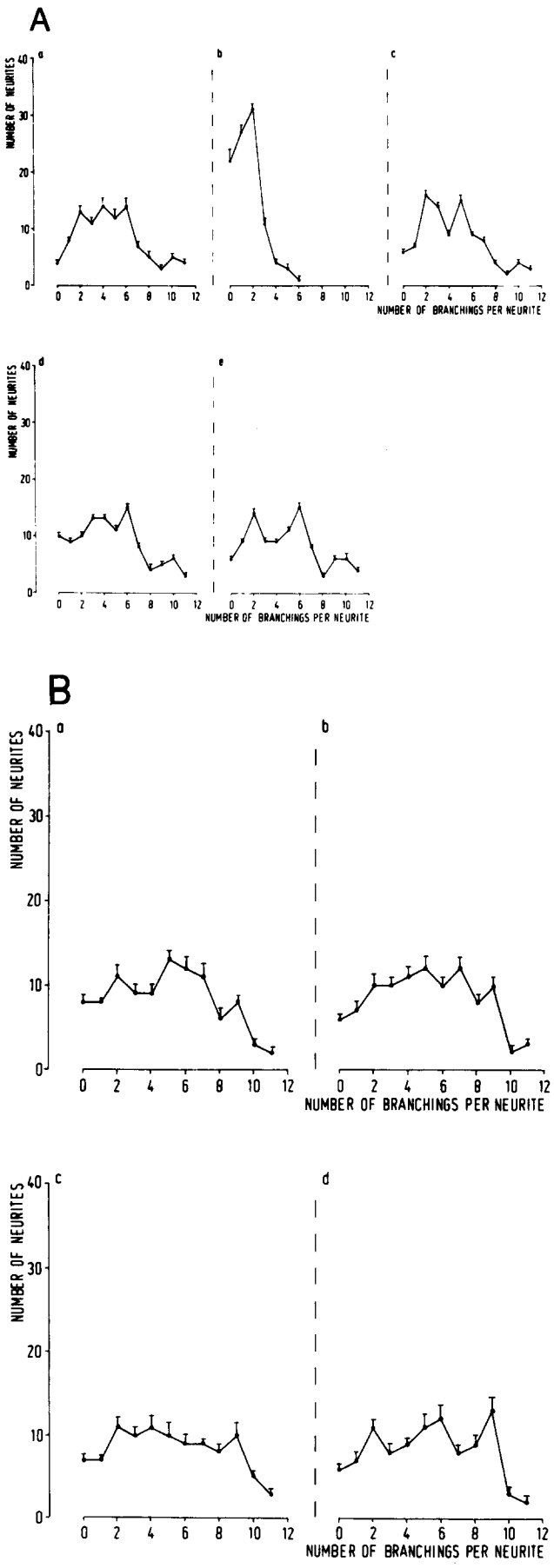


Figure 5. Number of neurites per cell body of DRG neurons cultured on Schwann cells (*A*) and fibroblasts (*B*) in the absence (*A*, *a*; *B*, *a*) and presence of Fab fragments of polyclonal L1 (*A*, *b*; *B*, *b*), N-CAM (*A*, *c*; *B*, *c*), J1 (*A*, *d*), and liver membrane (*A*, *e*; *B*, *d*) antibodies. Values from three independent experiments with a total of ≥100 cells counted ± SD are given.

number of neurites per cell, and number of branching points per neurite. Thus, N-CAM and J1 appear to be involved not only in Schwann cell–neuron but also in promoting neurite outgrowth on Schwann cells. Also, L1 is not only the most potent mediator of Schwann cell–neuron adhesion but also appears to be the predominant adhesion molecule involved in neurite outgrowth on Schwann cells. Thus, all three adhesion molecules tend towards parallel effects in the two assay systems, neuron–Schwann cell adhesion and neurite outgrowth, suggesting some dependence in molecular mechanisms in the more static adherence and the more dynamic process of neurite outgrowth.

A striking observation in our experiments is that neurons with small cell bodies (10–20 μm) are influenced differently by antibodies than large neurons (20–35 μm) in their neurite outgrowth patterns on Schwann cell monolayers. N-CAM antibodies do not interfere much with neurite outgrowth of small neurons but significantly reduce neurite outgrowth of large neurons. L1 antibodies reduce neurite outgrowth of small neurons only slightly more than that of large neurons.



On the other hand, J1 antibodies interfere more with the outgrowth of neurites from large neurons than from small neurons. Since both large and small neurons express L1 and N-CAM, an obvious interpretation of these observations is at present difficult. It is possible that large neurons express higher levels of N-CAM than small ones, leading to a higher inhibition of neurite outgrowth in the presence of N-CAM antibodies. Similarly, it could be argued that small neurons express fewer receptors for J1 than large neurons, resulting in a higher inhibition of neurite outgrowth of large neurons in the presence of J1 antibodies. However, these interpretations are highly speculative and remain to be reassessed in view of our knowledge of the functional properties of neurons falling into different classes by their size, modalities they convey, and expression of carbohydrate structures (Dodd and Jessell, 1985).

The most striking observation of our study is the high degree of dependence of neurite outgrowth on Schwann cells on the cell adhesion molecule L1. This observation is interesting from several points of view. First, since Schwann cells cultured in the absence of neurites do not assemble morphologically distinguishable basement membrane nor synthesize the particular components associated with it (Bunge et al., 1986), our experiments show that Schwann cell surfaces themselves are a good substrate for neurite outgrowth and that basement membrane is not the unique required feature for neurite outgrowth (see also Tomaselli et al., 1986; Kleitman et al., 1988). This feature is important in the situation where axon outgrowth in vivo depends on the presence of Schwann cells not yet contacted by neurons as it has been postulated for the more distal parts of axon ingrowth in the PNS during development (for review see Keynes, 1987) and regeneration (Martini and Schachner, 1988).

Second, L1 and to a lesser degree also N-CAM and J1 can be individually defined as mediators of neurite outgrowth. The involvement of N-cadherin and integrin in addition to L1 has recently also been demonstrated (Bixby et al., 1988) indicating that neurite outgrowth on Schwann cells is regulated individually by several distinct adhesion molecules. In contrast, neurite outgrowth on muscle appears to be mediated by a combination of the functions of several cell adhesion molecules; i.e., N-CAM, N-cadherin, and integrin (CSAT) (Tomaselli et al., 1986). Thus, the demonstration that several adhesion molecules can be individually recognized as mediators of neurite outgrowth on Schwann cell substrate is noteworthy and shows that the Schwann cell–secreted laminin and fibronectin and their respective receptors on the neuronal cell surface (Bozycko and Horwitz, 1986; Hall et al., 1987; Pesheva et al., 1987) are not the overriding determinants in neurite outgrowth. In view of the predominant involvement of L1 in adhesion between neurons and Schwann

Figure 6. Number of branching points per neurite of DRG neurons cultured on Schwann cells (*A*) and fibroblasts (*B*) in the absence (*A*, *a*; *B*, *a*) and presence of Fab fragments of polyclonal L1 (*A*, *b*; *B*, *b*), N-CAM (*A*, *c*; *B*, *c*), J1 (*A*, *d*), and liver membrane (*A*, *e*; *B*, *d*) antibodies. Values from three independent experiments with a total of ≥100 cells counted ± SD are given.

cells and neurite outgrowth on Schwann cells and the difficulty in interpreting less than additive or more than additive effects in blocking (Faissner et al., 1984*a*; Keilhauer et al., 1985), we did not add various mixtures of antibodies to the assays.

Third, antibodies to L1, N-CAM, or J1 have not been observed to interfere with neurite outgrowth on astrocytes. It should be emphasized here that glial cells in the CNS do not express L1 but, like Schwann cells, express N-CAM and J1. We would like to propose, therefore, that expression of L1 on Schwann cells is the decisive factor for neurite outgrowth and that its presence on the neuronal cell surface is a necessary, but by itself possibly not sufficient, parameter for neurite outgrowth (see also Martini and Schachner, 1988). It is worth mentioning in this context that L1 but not N-CAM has been shown to mediate extension of L1-positive neurites on each other (Chang et al., 1987). Furthermore, purified L1 is an excellent substrate for neurite outgrowth in vitro (Lagenaur and Lemmon, 1987; Pohlmann, M., and M. Schachner, unpublished observations). These observations beg the questions as to the molecular nature of the L1 receptor on both neurons and Schwann cells. Our experiments further substantiate the notion that the presence of L1 on both interactive surfaces would assure a particular quality of neurite outgrowth that is distinct from neurite outgrowth on L1-negative substrates. It is worth mentioning in this context that in the cerebellum stellate and basket cell axons, which are oriented at a 90° angle to the fasciculating L1-positive granule cell axons, are L1 negative (Persohn and Schachner, 1987). Furthermore, we observed a long time ago that commisural axons in the spinal cord only start to express L1 in addition to N-CAM, when they turn at a 90° angle from a nonfasciculating to a fasciculating state (Holley, J., and M. Schachner, unpublished observations as cited in Holley and Silver, 1987; Persohn and Schachner, 1987; Schachner et al., 1985, 1987). It remains to be seen whether L1 acts by itself as an adhesive ligand or in interdependence with N-CAM (Faissner et al., 1984*a*; Keilhauer et al., 1985; Thor et al., 1986) which so far has always been found to be coexpressed with L1. It is worth mentioning that L1/Ng-CAM has recently been implicated in a homophilic binding mechanism among neurons and a heterophilic interaction between neurons and astrocytes (Grumet et al., 1988).

Finally, the function of adhesion molecules on Schwann cells deserves particular attention in the context of regeneration in the PNS. It has been reported that L1 and N-CAM are expressed on more Schwann cells after cutting or crushing the adult sciatic nerve than in the nonsevered state (Nieke and Schachner, 1985; Daniloff et al., 1986; Martini and Schachner, 1988). This increase in expression was more extensive in the denervated distal part of the nerve stump than in the lesioned part at the distal end of the proximal stump. Thus, it appeared that denervation was a decisive factor in increasing reexpression of the two adhesion molecules. First hints as to the molecular mechanisms involved in the regulation of increased L1 expression stem from the observation that β-NGF increases the expression of L1 in cultures of pure Schwann cells (Seilheimer and Schachner, 1987). Since expression of β-NGF and β-NGF receptor is up-regulated in Schwann cells under regenerative conditions (Heumann et al., 1987; Taniuchi et al., 1986), a dual role for the effect of β-NGF on successful regeneration can be delineated. On one hand, β-NGF increases L1/NILE expression on neurites (Lee et al., 1981; Bock et al., 1985; Friedlander et al., 1986); on the other hand, L1 expression on Schwann cells is increased by β-NGF in an autocrine mechanism (Seilheimer and Schachner, 1987). The involvement of L1 in mediating neurite outgrowth on Schwann cells is thus extremely plausible in terms of biological significance and merits further investigation with regard to the molecular mechanisms of regulation of L1 expression and mode of action.

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