Identification of a peripheral nerve neurite growth-promoting activity by development and use of an in vitro bioassay
(axonal regeneration/neurite outgrowth/laminin/growth cones)

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ABSTRACT The effective regeneration of severed neuronal axons in the peripheral nerves of adult mammals may be explained by the presence of molecules in situ that promote the effective elongation of neurites. The absence of such molecules in the central nervous system of these animals may underlie the relative inability of axons to regenerate in this tissue after injury. In an effort to identify neurite growth-promoting molecules in tissues that support effective axonal regeneration, we have developed an in vitro bioassay that is sensitive to substrate-bound factors of peripheral nerve that influence the growth of neurites. In this assay, neonatal rat superior cervical ganglion explants are placed on longitudinal cryostat sections of fresh-frozen sciatic nerve, and the growing axons are visualized by catecholamine histofluorescence. Axons are found to regenerate effectively over sciatic nerve tissue sections. When ganglia are similarly explanted onto cryostat sections of adult rat central nervous system tissue, however, axonal regeneration is virtually absent. We have begun to identify the molecules in peripheral nerve that promote effective axonal regeneration by examining the effect of antibodies that interfere with the activity of previously described neurite growth-promoting factors. Axonal elongation over sciatic nerve tissue was found to be sensitive to the inhibitory effects of INO (for inhibitor of neurite outgrowth), a monoclonal antibody that recognizes and inhibits a neurite growth-promoting activity from PC-12 cell-conditioned medium. The INO antigen appears to be a molecular complex of laminin and heparan sulfate proteoglycan. In contrast, a rabbit antiserum that recognizes laminin purified from mouse Engelbreth–Holm–Swarm (EHS) sarcoma, stains the Schwann cell basal lamina of peripheral nerve, and inhibits neurite growth over purified laminin substrata has no detectable effect on the rate of axonal regeneration in our assay.

In mammals, severed neuronal axons of peripheral nerves regenerate readily, whereas axons of the central nervous system (CNS) fail to regrow effectively (1). Ramon y Cajal (2) was among the first to postulate that the rapid regeneration of peripheral axons was made possible by the presence of "neurotrophic substances" in the environment of elongating growth cones and that the relative inability of central axons to regenerate was due to the lack of these substances in CNS tissue, rather than an inherent defect of these neurons. This hypothesis was based, at least in part, on two observations: (i) regenerating dorsal root axons will elongate until the spinal cord is reached, where most of the fibers are "detained" or deflected to grow back down the root (ref. 2, p. 543), and (ii) sciatic nerve grafts placed into deeply cut adult rabbit cerebral cortex contained numerous axons originating from the surrounding white matter, "behaving in their growth, ramifications, orientation, energetic progress, etc., exactly like the sprouts of the central stump of a cut [peripheral] nerve" (ref. 2, pp. 738–740). These results have been confirmed by more recent experiments (3–5); many CNS neurons possess the ability to elongate axons for considerable distances when provided with a supportive environment—e.g., a peripheral nerve graft (6).

What, then, are the "neurotrophic substances" of peripheral nerves? At least two classes of neurite growth-promoting molecules have been defined in tissue culture studies: soluble and substrate-bound. The best described member of the former type is nerve growth factor (NGF)—a peptide with profound effects on the survival, growth, and differentiation of sympathetic and certain sensory neurons (7)—whose isolation and subsequent purification depended on its ability to produce a vigorous "halo" of neurite outgrowth from sympathetic ganglion explants in vitro (8). Similar neurotrophic molecules have been defined for other neuronal cell types (9). Peripheral nerves may contain soluble neurotrophic activities resembling NGF (10).

The second type of neurite growth-promoting molecule is one that exerts its effects only after it has been attached to the tissue culture substratum. First described by Collins as a factor in the medium conditioned by heart cell cultures (11), it subsequently has been found in many types of neuronal and nonneuronal cell-conditioned media (12) and has been identified as a molecular complex of several extracellular matrix components—namely, laminin, heparan sulfate proteoglycan, and possibly entactin (13, 14). Laminin, a million-dalton glycoprotein constituent of all basement membranes (15), may account for most, if not all, of the neurite growth-promoting activities present in various conditioned media (14). A monoclonal antibody called "INO" (for inhibitor of neurite outgrowth), which directly inhibits the neurite growth-promoting activity of PC-12 cell-conditioned medium, appears to recognize an epitope consisting of a heparan sulfate proteoglycan bound to a laminin-like molecule (16). Laminin purified from a mouse Engelbreth–Holm–Swarm (EHS) sarcoma cell line is very effective in promoting neurite growth from a variety of peripheral nervous system (PNS) and CNS neurons; this action can be inhibited by a rabbit antiserum raised against the same purified laminin preparation. Curiously, anti-laminin antisera that are effective in removing neurite growth-promoting factors from a variety of conditioned media fail to affect the activity of these factors directly (14). These results suggest either that laminins derived from different cell types exhibit considerable heterogeneity or that the association of laminin with other basal lamina components results in the formation of unique epitopes.

After a peripheral nerve has been severed, axonal and myelin debris are removed from the distal stump by phagocytosis...
cytosis, and Schwann cells proliferate within the confines of their basal laminae to form columns called the “bands of Büngner” (2). Axons regenerating into the distal stump seem to prefer to elongate within the persistent basal laminar tubes and among the Schwann cells (2, 17). Clearly, molecules residing in the basal lamina, as well as molecules of Schwann cell surfaces, could play important roles in promoting the regeneration of peripheral nerve axons. Experiments that have shown that axons are capable of regrowing into segments of sciatic nerve in which all living cells have been destroyed by repeated freezing and thawing (18, 19) suggest an important role for the basal lamina in supporting the regrowth of axons after peripheral nerve injury. Interestingly, even in these experiments, the regenerating axons appear to maintain their preference of growing within the “empty” basal laminar tubes. Since laminin-like immunoreactivity is present in the Schwann cell basal lamina of peripheral nerves (20), it is possible that a laminin-like molecule could play a role in supporting axonal regeneration in this tissue. However, the activity of substrate-bound neurotrophic molecules in peripheral nerve has yet to be studied, and the role of these molecules in axonal regeneration remains poorly defined. We have developed an in vitro bioassay that can be used to identify and characterize neurite growth-promoting activities in rat tissue sections. We will show that axonal regeneration in this assay (i) accurately reflects the differential ability of PNS versus CNS tissue to support effective axonal regrowth, as has been observed in mammals in vivo, and (ii) is inhibited by the monoclonal antibody INO but relatively unperturbed by an anti-laminin antiserum that inhibits the neurite growth-promoting activity of substrates coated with purified laminin. A preliminary report appeared elsewhere (21).

MATERIALS AND METHODS

Preparation of Tissue Sections. Sciatic nerves are removed from adult Sprague-Dawley rats (Charles River Breeding Laboratories) and immediately frozen onto chucks in OCT embedding compound (Miles). Sections (15–20 μm) are cut along the longitudinal axis of the nerve in a cryostat (Bright Instrument, Huntingdon, U.K.) and dried onto 22-mm2 square glass coverslips (Corning) that had been immersed for 12 hr in 0.15 M KCl/0.15 M sodium borate containing poly(D-lysine) (Sigma) at 1 mg/ml. The coverslips are sterilized by a 20-min exposure to the UV lamp in a laminar flow hood, and each is placed into 35 × 10 mm plastic Petri dishes (Falcon) containing 2 ml of L-15-CO2 medium. [L-15-CO2 is a modified L-15 (Leibovitz) medium (North American Biologicals, Miami) and is supplemented with imidazole, stable vitamins, and sodium bicarbonate as described by Mains and Patterson (22).] After two brief rinses in L-15-CO2, the sections are ready for use. If antibody preincubations are to be included in the assay, they are performed at this stage, with hybridoma supernatant added for 2 hr at 37°C followed by washes.

Preparation of Ganglionic Explants. Superior cervical ganglia are removed from 1- to 7-day-old Sprague–Dawley rats and placed into L-15 medium supplemented with 0.6% glucose. After blood vessels and other adhering tissue are dissected and removed, we will show that ganglia are transferred to 2 mg of collagenase (Type CLS, Cooper Biomedical, Malvern, PA) per ml of Hank’s basal salt solution (GIBCO) supplemented with 0.6% wt/vol glucose and are incubated at 37°C for 30 min; this treatment removes the connective tissue sheath encapsulating the ganglion. The ganglia subsequently are rinsed free of collagenase with several rinses of L-15-CO2 and transferred to L-15-CO2 medium supplemented with 0.6% wt/vol glucose, 2 mM glutamine, 100 units of penicillin per ml, 100 μg of streptomycin per ml, 1% vol/vol fresh vitamin mix (23), 5% (vol/vol) rat serum, 1 μg of 7S NGF per ml, and 10 μM cytosine arabinoside; the ganglia were maintained in a humidified 5% CO2/95% air 37°C incubator for 8–20 hr. [7S NGF had been partially purified from male mouse salivary glands by gel filtration through Sephadex G-100 (24), followed by DEAE-cellulose chromatography (25).] Just prior to plating the ganglia, the medium in the Petri dishes containing the tissue sections is replaced with 1 ml of L-15-CO2 growth medium containing 0.6% glucose, 2 mM glutamine, 100 units of penicillin per ml, 100 μg of streptomycin per ml, 1% fresh vitamin mix, and 5% rat serum. The ganglia are then removed from the incubator, cut in half, and placed (with the cut surface down) directly onto the tissue sections. The level of medium in each Petri dish is lowered until the ganglionic explants are somewhat flattened against the tissue section by surface tension, and the cultures are gently returned to the incubator.

Visualization of Regrowing Axons. After 36–48 hr in culture, the sections containing ganglionic explants are examined for the presence of regenerating axons. The regenerating axons are routinely visualized by catecholamine histofluorescence using the modified glyoxylic acid technique of de la Torre and Surgeon (26). Prior to treating with glyoxylic acid (Sigma), fluorescence intensity in living axons is optimized by incubating the cultures for 30 min at 37°C in the presence of 10 μM α-methyl norepinephrine (Regis, Morton Grove, IL) and 100 μM pargyline (Sigma). Following the addition of 0.01% bromphenol blue in Dulbecco’s phosphate-buffered saline (GIBCO), the coverslips are examined with a Zeiss microscope equipped with epifluorescence and a catecholamine filter set (excitation, 400–440 nm; barrier, LP 470 nm).

Quantification of Neurite Growth. Low power (×200) fluorescence photomicrographs of regions along the tissue sections directly adjacent to ganglionic explants (similar to Fig. 1) are developed and printed identically and optically scanned (Electronic Information Technology, personal scanner); the digitized images are stored in a computer (International Business Machines personal computer). These images are organized in memory as scan lines (200 scan lines per inch; 1 inch = 2.54 cm) of data (240 bits per inch), in which the location of every white (fluorescent) dot on a given line is assigned a bit value of 1 and every black (background) dot on the same line is assigned a bit value of 0. Therefore, the sum of bit values on any given scan line is equivalent to the amount of fluorescence present in that line. Since only viable sympathetic axons take up catecholamine and exhibit histo-fluorescence after treatment with glyoxylic acid, the sum of the bit values per scan line is a measure of the amount of axonal material present in that line. We insert the fluorescence photomicrographs into the optical scanner so that the scan lines are oriented perpendicular to the axons. By programming the computer to sum up the bit values of each scan line and to assign the total bit values of 100 scan lines (equal to one-half inch along the photomicrographs, or 64 microns along the nerve) into bins numbered 1 to 12, we were able to plot histograms. The amount of fluorescence in each half-inch strip along the photomicrographs (in bits per bin) is plotted against the distance from the edge of the ganglionic explant (in bins).

Antibodies. The rabbit antiserum to laminin was purchased from Bethesda Research Laboratories and dialyzed against 104 volumes of phosphate-buffered saline (to remove the sodium azide in which the antiserum was shipped); aliquots were taken and stored at –20°C. The production of the monoclonal antibody INO has been described (27). The hybridomas were grown in L-15-CO2 supplemented with 0.6% glucose, 2 mM glutamine, 100 units of penicillin per ml, and 100 μg of streptomycin per ml; hybridoma supernatants were obtained by centrifugation (500 × g for 3 min) of the medium in which hybridomas had grown for 1 week at a density of 104–105 cells per ml.
RESULTS

Characteristics of Axons Regenerating over Sciatic Nerve Sections. Two-day-old rat superior cervical ganglia were explanted onto longitudinal sections of adult rat sciatic nerve as described and incubated for 36 hr. Fig. 1a shows a region immediately adjacent to a ganglionic explant along one of these tissue sections, as viewed by phase-contrast microscopy. The sciatic nerve section retained its structural integrity, and, although the explant itself is visible, it is impossible to determine whether neuronal axons extended out of the ganglion. Fig. 1b shows a similar field, viewed by fluorescence microscopy, after the same culture was incubated in α-methylnorepinephrine and processed for catecholamine histofluorescence as described. It is now evident that numerous axons regrew vigorously over sciatic nerve tissue, the longest axons extending about 1 mm from the edge of the ganglion by this time. Axons from sympathetic ganglia always regenerated in roughly parallel arrays along the long axis of peripheral nerve sections as seen in Fig. 1b. Furthermore, the regenerating axons preferred to grow within the boundaries of the tissue section—they rarely extended onto the polylysine-coated glass coverslip. The growth of neurites over sections of the distal stump of previously severed sciatic nerves was qualitatively similar (data not shown).

Since whole ganglia were the source of regenerating axons in these experiments, we were concerned that, despite prolonged treatment with cytosine arabinoside (which selectively destroys proliferating cells), the migration of the remaining viable nonneuronal cells out of the ganglia may have preceded the growth of neurites over the tissue sections and thereby provided the actual substrate for neurite growth. In experiments in which both neuronal axons as well as nonneuronal cells could be visualized by the use of a compound that is incorporated and metabolized into a fluorescent compound by all living cells (fluorescein diacetate; see ref. 28), we determined that the migration of nonneuronal cells trailed behind the leading edge of elongating neurites (data not shown). Therefore, the outgrowth of axons over sciatic nerve sections appears to be rapid and independent of the relatively sluggish migration of nonneuronal cells.

Axonal Regeneration over Sections of Adult CNS. We wondered whether CNS tissue sections also possessed the ability to promote the rapid regeneration of neuronal axons in this bioassay. An adult rat optic nerve was removed and embedded directly adjacent to a sciatic nerve from the same rat in such a way that each cryostat section would contain longitudinal sections of both nerves in direct apposition. A single newborn rat superior cervical ganglion was explanted so that it rested across tissue sections of both nerve types. After a 36-hr incubation period, the cultures were processed for catecholamine histofluorescence. Axons regenerated almost exclusively along the sciatic nerve section (Fig. 2); there was no detectable regrowth over the optic nerve section, except for a tuft of processes that had grown along the edge over what is presumably the connective tissue sheath that invests the nerve. These results are typical of repeated attempts at growing axons over a variety of adult rat CNS tissue sections, including cerebellum and spinal cord: neurons seem to be unable to extend neurites over this environment.

Axonal Regeneration over Sciatic Nerve Sections Treated with Anti-Laminin Antiserum. We investigated whether the presence of laminin in the Schwann cell basal lamina of peripheral nerves explains the ability of this tissue to support axonal regeneration by testing the effect of a rabbit antiserum to purified mouse EHS sarcoma laminin on the growth of neurites over adult rat sciatic nerve sections. The antiserum

![Fig. 1](image1.png)

**Fig. 1.** Axons regenerating over sciatic nerve sections can be visualized by catecholamine histofluorescence. Superior cervical ganglia from 2-day-old rats were explanted onto longitudinal sections of adult rat sciatic nerve and incubated for 36 hr as described. The cultures were photographed under phase-contrast microscopy (a), incubated in α-methylnorepinephrine, processed for catecholamine histofluorescence as described, and rephotographed under fluorescence microscopy (b). (Bar = 200 μm.)

![Fig. 2](image2.png)

**Fig. 2.** Comparison of neurite growth from the same ganglion over optic nerve vs. sciatic nerve sections. A single ganglion was placed over longitudinal sections of a sciatic nerve and an optic nerve that were aligned directly next to one another, and the bioassay was carried out in the usual manner. (a) Region directly adjacent to the ganglion, as viewed by phase-contrast microscopy. The ganglion is along the left edge of the photomicrograph. (b) Identical field as viewed through a fluorescence microscope equipped with a catecholamine filter. Numerous axons have grown out over the sciatic nerve section. In contrast, the only axons to have regenerated over the optic nerve section are along the edge of the section, which is presumably the outer connective tissue sheath covering the nerve. (Bar = 200 μm.)
was used to pretreat the tissue sections, and it was included in the growth medium of the bioassay as well. A 1:100 dilution of the antiserum had little measurable effect on the extent of axonal regeneration after 48 hr as compared with sister cultures grown for the same length of time in a 1:100 dilution of normal rabbit serum (Fig. 3; see Fig. 5a). To verify that the anti-laminin antiserum used in this experiment inhibits the neurite-promoting activity of EHS sarcoma laminin, ganglia were plated onto polylysine-treated glass coverslips that had been incubated in purified laminin (Bethesda Research Laboratories) at 50 μg/ml for 12 hr at room temperature. These cultures were maintained in growth medium containing either a 1:100 dilution of normal rabbit serum or a 1:100 dilution of the antiserum used for the bioassay of Fig. 3. Treatment of laminin-coated coverslips with anti-laminin antiserum resulted in a dramatic inhibition of neurite growth (data not shown). In repeated attempts, we have been unable to detect any significant effect of the anti-laminin antiserum on the regeneration of axons on cryostat sections—even at dilutions as low as 1:20—despite the fact that, by radioimmunoassay on tissue sections of sciatic nerve identical to those used in the bioassay, binding of this antiserum to sciatic nerve saturates at a dilution of 1:200 (data not shown). To ascertain the stability of laminin-like immunoreactivity under the culture conditions required by the bioassay, sciatic nerve sections that had been in culture for 48 hr were stained with the same rabbit antiserum to laminin used in the bioassay. Laminin immunoreactivity remained in these sections despite the prolonged incubation at 37°C (data not shown). Thus, even though the sciatic nerve sections in the bioassay contain laminin-like immunoreactivity, saturation of these sections with an antiserum that blocks the activity of a purified laminin preparation has no detectable effect on the growth of neurites over this tissue.

Axonal Regeneration over Sciatic Nerve Sections Treated with the INO Antibody. The INO antibody appears to stain the extracellular matrix material between the myelinated and unmyelinated axons in peripheral nerve (16); therefore, the INO antigen, like laminin, may be present in the Schwann cell basal lamina. Since the INO antibody is capable of directly inhibiting the neurite growth-promoting activity of PC-12 cell-conditioned medium, we wondered whether it also would interfere with the ability of sciatic nerve sections to promote vigorous axonal regeneration. To test this possibility, we carried out the bioassay in the presence of saturating concentrations of the INO antibody, both by preincubating the sections with undiluted INO hybridoma supernatant and by including an equal volume of INO hybridoma supernatant in the usual growth medium of the bioassay. [From RIA experiments, the concentration of antibody in the INO hybridoma supernatant was found to be sufficiently high so as to saturate the sciatic nerve sections at a dilution of 1:4 (data not shown).] For the control, we substituted the INO hybridoma supernatant with supernatant from another hybridoma that secretes a monoclonal antibody (called RN1A2) of the same isotype (IgM) that also binds to the Schwann cell basal lamina of peripheral nerves. Fig. 4 shows the extent of neurite growth after 48 hr over sciatic nerve sections in the presence of the control antibody (Fig. 4a) and in the presence of the INO antibody (Fig. 4b). The length of neurites appears to be substantially reduced when the INO antibody is present in the growth medium; this impression is verified by the histogram of Fig. 5b. Similar results were obtained when sciatic nerve sections that have been preincubated with INO supernatant and rinsed were assayed for neurite growth-promoting activity in the absence of soluble antibody (data not shown). Thus, the INO antigen appears to be at least partially responsible for promoting the vigorous regeneration of axons over sciatic nerve sections.

**DISCUSSION**

We report here the development of a method useful for assaying the activity of molecules in tissues that support the regeneration of axons. A major advantage of this bioassay is that it measures the activity of neurite growth-promoting factors in their native state—i.e., in normal tissues—while allowing access to function-blocking antibodies, which can be used to probe their biochemical identity. In addition, we have shown that the bioassay reflects the phenomenology of axonal regeneration in adult mammals in that tissue sections

![Fig. 3. The effect of a rabbit antiserum to laminin on axonal regeneration over sciatic nerve sections. The bioassay was carried out in the usual manner, except that a 1:100 dilution of normal rabbit serum (a) or rabbit anti-laminin antiserum (b) was included in the growth medium; the cultures were incubated at 37°C for 48 hr. (Bar = 100 μm.)](image)

![Fig. 4. The effect of the monoclonal antibody INO on axonal regeneration over sciatic nerve sections. The bioassay was carried out in the usual manner, except that a 1:1 dilution of control hybridoma supernatant (a) or INO hybridoma supernatant (b) was included in the growth medium. (Bar = 100 μm.)](image)
of peripheral nerve promote vigorous axonal regeneration, whereas tissue sections of optic nerve do not; this result suggests that molecular, rather than mechanical (e.g., glial scarring), differences between PNS and CNS tissues may underlie the differential ability of these two tissues to support the effective regrowth of axons.

The inability of CNS tissues to support effective axonal regrowth may be explained by the absence of neurite growth-promoting factors or by the presence of inhibitory factors (see ref. 29) in the brain and spinal cord. We have begun to seek evidence in favor of the former hypothesis by identifying the molecules in peripheral nerve that underlie the ability of this tissue to promote axonal regrowth so effectively. Our experiments indicate that the monoclonal antibody INO binds directly to a factor in peripheral nerve in such a way as to interfere with its ability to promote the rapid regeneration of axons; the INO antigen is not present in CNS fiber tracts (16). In contrast, an anti-laminin antisera— which inhibits the neurite growth-promoting activity of purified laminin— failed to measurably perturb axonal regeneration over peripheral nerve tissue sections, despite the fact that the tissue sections retain high concentrations of laminin-like immunoreactivity during the course of the bioassay. In PC12 cell-conditioned medium, the INO antigen is thought to consist of a molecular complex of a heparan sulfate proteoglycan and a laminin-like molecule (16). Does the INO antibody recognize a form of laminin that more closely resembles the molecule in its native form than that obtained from the mouse sarcoma cell line? It is possible that the combination of laminin with proteoglycan in situ results in the sequestration of a neurite growth-promoting site blocked by anti-laminin antisera and leads to the formation of a new neurite growth-promoting site blocked by the INO antibody. In this regard, it is interesting that one heparin binding site appears to be located in the same region of the laminin molecule as the neurite growth-promoting site (30). Purification of the INO antigen from peripheral nerve may help to resolve this issue.

Since the rapid regeneration of axons over sciatic nerve sections is only partially inhibited by the INO antibody, it is likely that other neurite growth-promoting factors are also present in this tissue. The bioassay should be useful in searching for additional molecules that promote the growth of regenerating axons in peripheral nerves.

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