Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 1319–1322, February 1990
Neurobiology

A dual laminin/collagen receptor acts in peripheral nerve regeneration

(extracellular matrix/integrin)

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Communicated by Richard L. Sidman, November 28, 1989 (received for review July 5, 1989)

ABSTRACT **A regeneration chamber was created *in vivo* by suturing a synthetic tube sealed at its distal end onto the proximal stump of a severed rat sciatic nerve. Nerves regenerated into tubes coated with laminin at a rate of 0.33 mm/day after a lag of about 2 days. At 25 days, regenerating nerves had extended 23% farther into laminin-coated tubes as compared with uncoated ones. Monoclonal antibody 3A3, which functionally interferes with a dual laminin/collagen receptor, inhibited nerve regeneration into laminin-coated tubes by 32%. In contrast, monoclonal antibody JG22, which inhibits chicken matrix receptors, had no significant effect on regeneration. Immunohistochemical studies of teased adult rat sciatic nerves indicate that 3A3 bound to Schwann cells and possibly to axons. In other studies, the heterodimeric, laminin/collagen receptor recognized by 3A3 has been shown to be a member of the integrin superfamily of adhesive receptors. These data provide evidence that an integrin receptor functions in nerve regeneration *in vivo*.**

In adult mammals, damaged axons can regenerate for many centimeters within the peripheral nervous system. These regenerating axons are typically found within conduits of basement membranes, often in contact with Schwann cells (1, 2). Like many other basement membranes, those in the peripheral nervous system are comprised of laminin, collagen, fibronectin, and other extracellular matrix (ECM) components (3–7). In culture, purified fibronectin, collagen, and especially laminin stimulate outgrowth as well as guide nerve processes (8–12). This growth requires binding of cell-surface receptors to ECM adhesive proteins. Several ECM receptors and binding proteins have been identified, most notably a superfamily of heterodimeric proteins called integrins. Integrins are found in a wide variety of cell types where they mediate both cell–cell and cell–matrix adhesion (13–15). Of particular note are observations that integrins mediate neurite outgrowth in culture (16–19).

Prompted by recent studies implicating laminin in nerve regeneration *in vivo* (20, 21), we have sought to identify ECM receptors responsible for matrix-mediated nerve regeneration. To accomplish this, we have employed a method for isolating *in vivo* regenerating peripheral nerves within synthetic tubes (21–24). Peripheral nerves are severed, and their proximal stumps are capped with a tube sealed at its distal end (24) to form a chamber. The tubes are then filled with a solution containing monoclonal antibody (mAb) 3A3 (IgG1), which inhibits neurite outgrowth by PC12 cells (a rat pheochromocytoma cell line) on laminin and collagen culture substrata (25). It has been shown elsewhere (25) that mAb 3A3 binds to a heterodimeric receptor complex (185 kDa, 125 kDa) of the integrin superfamily. mAb 3A3 appears to recognize the α subunit of this laminin/collagen receptor, which can be immunoprecipitated from detergent extracts not only of PC12 cells but also of embryonic rat neural tissues (26). We show here that (*i*) laminin coated onto the nerve tubes enhances sciatic nerve regeneration and (*ii*) mAb 3A3 inhibits regeneration. These data suggest that the laminin/collagen integrin recognized by this antibody functions in regeneration *in vivo*.

MATERIALS AND METHODS

Cell Culture. Cultures of dissociated chicken dorsal root ganglia were prepared as described (27). Briefly, ganglia were removed from 9-day-old chicken embryos treated with trypsin (0.025%) for 20 min at 37°C. The ganglia were washed three times with culture medium (Dulbecco's modified Eagle's medium, 10% fetal calf serum, 7S nerve growth factor at 100 ng/ml) and then triturated to yield a suspension of dissociated cells. These cells were preplated on serum-coated dishes to remove fibroblasts, and the unattached cells which were enriched for neurons were harvested and seeded onto culture substrata. After 24 hr, cultures were fixed with 4% (wt/vol) paraformaldehyde in 0.1 M phosphate buffer at room temperature for 15 min and stained with 0.1% Coomassie blue for 10 min.

Nerve Regeneration Assay. Two sets of experiments were carried out. The first set of experiments were done to estimate the rate of nerve regeneration through laminin-coated nerve tubes. Nerve tubes synthesized from interstitial collagens and cross-linked with formaldehyde [obtained from American Biomaterials (Plainsboro, NJ); 2 cm in length and 1-mm inside diameter; 3- to 4-nm pore size, impermeable to myoglobin; cross-linked for 60 min] were washed extensively in Hanks' balanced salt solution and incubated overnight at 4°C in laminin [400 μg/ml in phosphate-buffered saline (PBS)]. Laminin was purified from mouse Engelbreth–Holm–Swarm sarcoma (28). Tubes were washed in PBS prior to grafting. Under chloral hydrate anesthesia, the right sciatic nerve of adult female Sprague–Dawley rats was transected at the level of the midthigh, and the distal segment of the nerve was removed. Laminin-coated nerve tubes were sutured onto the proximal segment of the transected sciatic nerve. The distal ends of the tubes were sealed with stainless steel staples to form a chamber for nerve regeneration. At various times after surgery, rats were deeply anesthetized and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2). The graft along with portions of the proximal sciatic stump was removed, and longitudinal cryostat sections (10 μm) of this tissue were picked up on gelatin-coated glass slides. Axonal regeneration into the tubes was quantified following immunohistochemical visualization of axons (see below).

The second set of experiments was designed to examine the role of the laminin/collagen receptor that is recognized by mAb 3A3 in nerve regeneration. Uncoated nerve tubes or ones coated with laminin and filled with antibodies (see below for details) were sutured to the proximal segment of the

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Abbreviations: mAb, monoclonal antibody; ECM, extracellular matrix; DRG, dorsal root ganglion.

transected sciatic nerve of adult rats as described above. Animals were divided into four groups to test the effect of mAb 3A3 on regeneration. Group 1 (control; $n = 12$) had uncoated nerve tubes and these were filled with PBS. Group 2 ($n = 12$) were grafted with identical tubes coated with laminin and also filled with PBS. Group 3 ($n = 9$) had laminin-coated tubes, but these were filled with mAb 3A3 (2 mg/ml in PBS). An additional control group, group 4, ($n = 4$) was identical to those in group 2, but the tubes were filled with JG22 (2 mg/ml in PBS). JG22, a chicken-specific anti-integrin mAb (29, 30) was used to control for noncomplementary binding of immunoglobulins to cell surfaces or ECM. mAb 3A3 was partially purified from ascites fluid with Affi-Gel Blue (Bio-Rad). Tubes were reinjected with 50 μl of the appropriate antibody solutions at the same concentrations as noted above or, in the case of controls, injected with PBS every 7 days. Reinjections were made into the distal end of the tubes after removal of the staples, and the ends were resealed as before. Twenty-five days after grafting, the rats were perfused with fixative, and longitudinal cryostat sections of the nerve tubes were obtained as described above.

Immunohistochemistry. For neurofilament immunohistochemistry, cryostat sections were blocked overnight with 3% (wt/vol) ovalbumin at 4°C, incubated with mAb RT97 (31) overnight, and then incubated with rhodamine-labeled goat anti-mouse IgG (HyClone) for 2 hr. Sections were then incubated with 0.001% nuclear yellow for 1 min. Slides were mounted in sodium carbonate-buffered glycerol (pH 9) and viewed with an epifluorescence microscope (Leitz).

For immunohistochemical localization with mAb 3A3, sciatic nerves from adult rats were removed and placed in Ca^{+2}/Mg^{+2}-free Hanks' balanced salt solution containing collagenase (final concentration of 0.002%) for 1 hr. The tissue was then rinsed in fresh Hanks' balanced salt solution and teased with fine dissecting forceps. The teased nerves were placed on gelatin-coated glass slides and incubated with antibodies as follows: mAb 3A3 (10 μg/ml) or JG22 for 30 min, rhodamine-labeled goat anti-mouse IgG (HyClone; 1:100) for 30 min, and 0.001% nuclear yellow for 1 min.

RESULTS AND DISCUSSION

To establish conditions for assaying nerve regeneration *in vivo*, we assessed (*i*) neurite outgrowth in culture on the substrata that were to be used as tubes for nerve regeneration *in vivo* and (*ii*) the rate of sciatic nerve regeneration through the tubes. In the first set of experiments, dissociated dorsal root ganglion (DRG) cells from chicken embryos were seeded onto the collagen substrata synthesized by methods identical to those used for the nerve tubes. DRG neurons attached poorly to these substrata but well to culture dishes coated with crude rat-tail collagen or laminin (Fig. 1*A*), suggesting that the collagen had been inactivated during synthesis of the substrata. Coating these substrata with laminin dramatically improved neurite outgrowth (Fig. 1*B*), approximating the extensive growth seen on culture substrata coated with laminin (10, 11, 32–34). On the basis of these observations, nerve tubes were similarly coated with laminin (400 μg/ml) to evaluate the rate of nerve regeneration *in vivo* into these modified tubes. The extent of axon growth through the tubes was quantified following immunocytochemical labeling of axons with anti-neurofilament antibodies (Fig. 2). Immunohistochemical studies of rat cervical sympathetic trunk with mAb RT97, which binds to neurofilaments, indicated that axons could be visualized readily in this largely unmyelinated nerve by fluorescence microscopy. Therefore, as an alternative to counting myelinated axons in cross-sections of regenerating nerves (21–24), we charted the progress of the growing nerve immunohistochemically. Quantification of regeneration in this way proved relatively simple and reliable (Fig. 3). Nerve regeneration into these tubes showed a lag of about 2 days after which the nerve increased in length monotonically for up to 30 days (Fig. 3). This rate of regeneration (≈0.33 mm/day) is within the range observed by others who have used silicone, polyethylene, or other tubes with or without inclusion of the distal stump (21–24). The finding that the tube material supports very little neurite outgrowth *in vitro*, whereas the tubes *in vivo* do permit axonal regeneration, implies that these tubes, like the silicone and polyethylene ones, may become modified *in vivo*, possibly by the fibrin clot and/or by cells (21–24).

On the basis of the above data, animals in the four groups designed to test the effect of mAb 3A3 on nerve regeneration were sacrificed 25 days after grafting. Fig. 4 shows that regeneration into tubes coated with laminin was 23% greater (4.6 mm) than in uncoated tubes (3.7 mm; $P < 0.01$). mAb 3A3 inhibited regeneration by 32% (3.1 mm) in laminin-coated tubes ($P < 0.01$) and by 27% compared with identical tubes containing mAb JG22 ($P < 0.05$). mAb JG22 had no significant effect (4.3 mm) on growth in laminin-coated tubes. In these experiments, the growing front was visible in longitu-

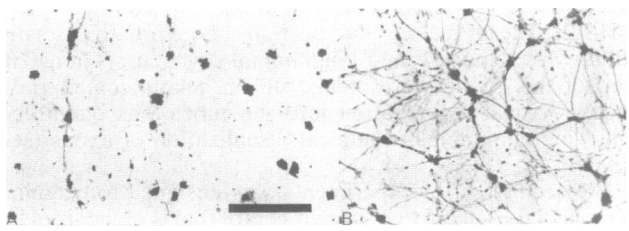

FIG. 1. Neurite growth on synthetic substrata. (*A*) Chicken DRG cells cultured on a synthetic substratum of nerve tube material. After 24 hr, small clusters of neurons have attached poorly and have extended neurites occasionally. (Bar = 50 μm.) (*B*) In contrast to this, DRG neurons attached much more extensively to the same substratum coated with laminin and elaborated an extensive network of neurites.

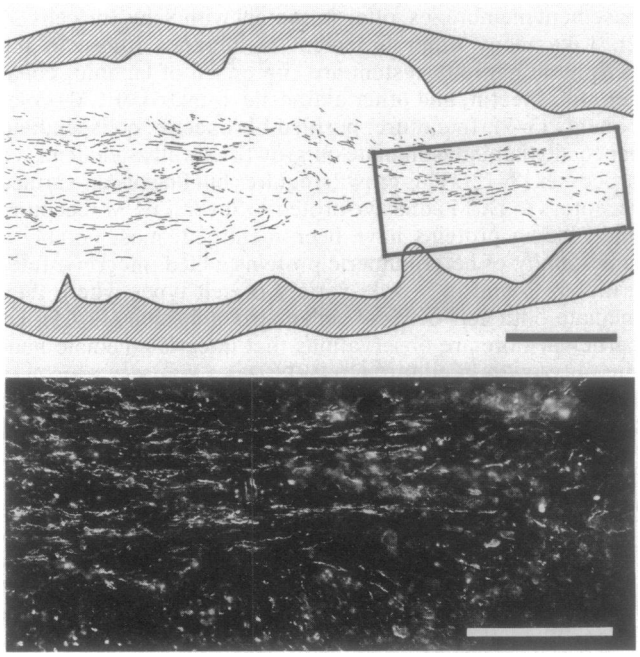

FIG. 2. Immunohistochemical localization of regenerating axons in nerve tubes. (*Upper*) Line drawing of a single longitudinal cryostat section through the graft, showing the distribution of regenerated axons that have been visualized with anti-neurofilament antibodies. The tip of the growing front is at the extreme right. This graft consisted of a nerve tube filled with PBS and was obtained 25 days after grafting. The hatched regions represent the walls of the nerve tube. (Bar = 500 μm.) (*Lower*) The area outlined within the box in *Upper* is shown. (Bar = 250 μm.)

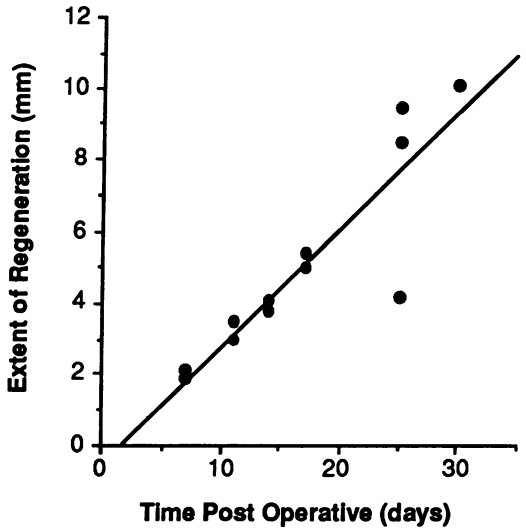

FIG. 3. Rate of sciatic nerve regeneration into laminin-coated nerve tubes. Nerve tubes modified by incubation with laminin (400 μg/ml) were sutured onto the proximal segment of the transected sciatic nerve. Survival times after surgery are indicated on the abscissa. The length of axon growth into the tubes was quantified in frozen sections by measuring the distance from the proximal end of the collagen tube to the distal tip of the regenerating axons (ordinate).

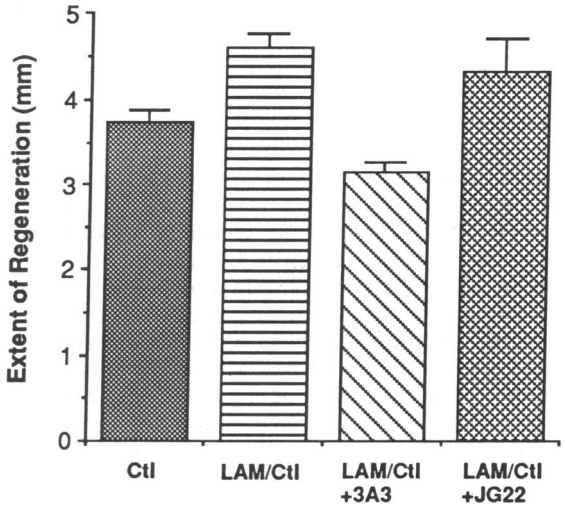

FIG. 4. Inhibition of nerve regeneration by mAb 3A3. Animals with nerve tube implants were divided into four groups. Group 1 (Ctl; $n = 12$) had uncoated tubes filled with PBS. Group 2 (LAM/Ctl; $n = 12$) had tubes coated with laminin and filled with PBS. Groups 3 (LAM/Ctl + 3A3; $n = 9$) and 4 (LAM/Ctl + JG22; $n = 4$) had tubes identical to those in group 2 but filled with mAbs 3A3 (2 mg/ml) and JG22 (2 mg/ml), respectively. The results shown are from three separate experiments in which each experimental group displayed the same trends as the aggregate data. The histogram shows the length of axon regeneration into the chamber. Bars on each histogram show the standard error of the mean; data were analyzed statistically by analysis of variance.

dinal sections at multiple levels taken through the nerve tubes and was similar in appearance in all experimental groups. No obvious differences were observed in the density of regenerating axons in the four groups. In addition, nuclear yellow labeling failed to demonstrate any pronounced cellular infiltration into the tubes in any of the grafts, as would be expected if an immune response had been mounted against the tubes or their contents.

The distribution of the 3A3 antigen in the adult rat sciatic nerve was examined in frozen sections. We were unable to detect the binding of mAb 3A3 to these cryostat sections by immunofluorescence labeling, probably because this antibody binds to a sparsely distributed antigen in plasma membranes. In contrast, embryonic rat DRG neurons and Schwann cells in culture are labeled by this antibody (S.C. and N. Tawil, unpublished results). We therefore examined the distribution of the 3A3 antigen in the adult rat sciatic nerve *in vivo* by immunofluorescence staining of teased preparations of the sciatic nerve. The teased preparations permit labeling of intact cells and visualization of the entire cell surface. In these studies, mAb 3A3 labeled the teased nerve fibers (Fig. 5). The pattern of the 3A3 labeling suggests that it is likely to be labeling Schwann cells, which ensheath both unmyelinated and myelinated axons. Labeling of axons *per se* cannot be determined with certainty in these preparations because the antibody does not have easy access to the axonal plasma membrane except at the nodes of Ranvier. Labeling was however observed along the entire length of the teased nerve fibers, including the nodal regions, suggesting that axons may also be labeled by this antibody.

It is generally agreed that both cell–cell adhesion and cell–matrix adhesion can mediate neurite outgrowth in culture. Although ECM from nerve or muscle appears to support peripheral nerve regeneration (1, 35), there is some controversy regarding the relative efficacy of cell–cell and cell–matrix interactions *in vivo* (36–38). In our studies, coating nerve tubes with laminin significantly enhanced regeneration (Fig. 4; cf. ref. 21). Consistent with this, Sandrock and Matthew (20) have shown that a mAb to a laminin–heparan sulfate complex inhibits reinnervation of the rat iris. Laminin is apparently responsible for the stimulation of nerve fiber growth by this molecular complex (33). It is noteworthy that in both instances inhibition of regeneration is partial. The

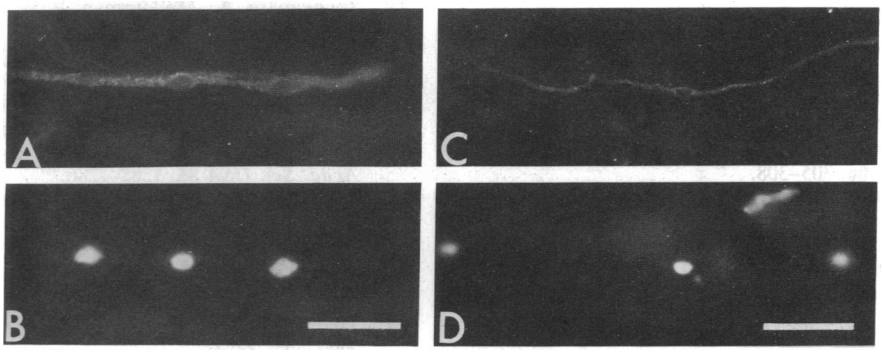

FIG. 5. Immunohistochemical localization of the laminin/collagen receptor recognized by mAb 3A3 in teased nerve fibers of adult rat sciatic nerve (*A* and *C*). The regularly spaced nuclei along the nerve fibers, visualized by nuclear yellow staining (*B* and *D*), suggest that these are Schwann cells. mAb 3A3 appears to label Schwann cells since this labeling follows the contours of the cells (*A* and *C*). Labeling is also observed along the entire length of the nerve, including nodal regions. Control sections were unlabeled. (*B*, bar = 30 μm; *D*, bar = 50 μm.)

reason for this partial inhibition *in vivo* is unclear (20). In our experiments, intermittent application of the antibody or some leakage from the tubes may contribute to the partial inhibition. Alternatively, multiple adhesive systems, both cell–cell and cell–matrix, that are present on neurons, Schwann cells, and fibroblasts (17, 18) may be responsible. As in cell culture, inhibition of only one adhesive mechanism results in partial inhibition of growth. Our data, together with those of others (20), implicate laminin in these several adhesive systems.

Although the effects of mAb 3A3 can be ascribed to its inhibition of a laminin/collagen receptor, it is unclear whether it acts *in vivo* solely or primarily on axonal extension. Nerve fiber growth may be inhibited directly by 3A3, as it is in culture (25), but it is also possible that 3A3 alters Schwann cell proliferation or migration into nerve tubes. Schwann cells potently stimulate nerve fiber growth in culture (17, 39) and possibly *in vivo* (36–38).

In principle, laminin-dependent regeneration may be mediated by one or more of the several identified laminin-binding proteins on cell surfaces. These include members of the integrin family (16, 40–42) and two non-integrin laminin-binding proteins (43–46). The integrins mediate adhesion in culture in cells as diverse as neurons, monocytes, and fibroblasts (13–15). They have been identified relatively recently; hence, the structural relationships among the different members are still being detailed. The smaller subunit of the 3A3 antigen cross-reacts in Western blots with several polyclonal antisera to the $\beta 1$ subunit of the integrin superfamily (19, 26, 47). More recently, immunoaffinity purification of the α subunit (26) has made it possible to sequence the N-terminal 26 amino acid residues. These data identify the receptor unequivocally as a homolog ($\alpha 1$, $\beta 1$) of one of the very late antigens (VLA 1) (S.C., N. J. Tawil, M. Houde, R. Blacher, F. Esch, L. F. Reichardt, and D. C. Turner, unpublished data), which was first described in human lymphocytes and more recently in human cell lines (48, 49). Inhibition of nerve regeneration by mAb 3A3 suggests that the laminin/collagen integrin recognized by this antibody is functional during nerve regeneration *in vivo*.

We thank Dr. J. Wood for generously providing the neurofilament antibody, N. J. Tawil for preparation and purification of ascitic fluids, and Dr. A. Masi for advice on statistical analysis. We also thank Drs. G. Bray and D. C. Turner for their constructive criticisms of this manuscript. Finally, we are especially grateful to Dr. S.-T. Li (American Biomaterials) for supplying us with collagen nerve conduits. This work was supported by grants to S.C. from the Medical Research Council (MRC) (MA 1082) and the Spinal Cord Research Foundation and to S.D. from MRC (MA 8723) and the Multiple Sclerosis Society of Canada. B.T. was supported by a Fonds pour la Formation de Chercheurs et l'aide a la Recherche studentship, S.C. is the recipient of a Chercheur-boursier from the Fonds de la Recherche en Santé du Québec, and S.D. is the recipient of an MRC scholarship.

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