

# Distribution and Role in Regeneration of N-CAM in the Basal Laminae of Muscle and Schwann Cells

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**Abstract.** The neural cell adhesion molecule (N-CAM) is a membrane glycoprotein involved in neuron–neuron and neuron–muscle adhesion. It can be synthesized in various forms by both nerve and muscle and it becomes concentrated at the motor endplate. Biochemical analysis of a frog muscle extract enriched in basal lamina revealed the presence of a polydisperse, polysialylated form of N-CAM with an average  $M_r$  of  $\sim 160,000$  as determined by SDS-PAGE, which was converted to a form of  $125,000 M_r$  by treatment with neuraminidase. To define further the role of N-CAM in neuromuscular junction organization, we studied the distribution of N-CAM in an *in vivo* preparation of frog basal lamina sheaths obtained by inducing the degeneration of both nerve and muscle fibers. Immunoreactive material could be readily detected by anti-N-CAM antibodies in such basal lamina sheaths. Ultrastructural analysis using immunogold techniques revealed N-CAM in close association with the basal lamina sheaths, present in dense accumulation at places that presumably correspond to synaptic regions. N-CAM

epitopes were also associated with collagen fibrils in the extracellular matrix. The ability of anti-N-CAM antibodies to perturb nerve regeneration and reinnervation of the remaining basal lamina sheaths was then examined. In control animals, myelinating Schwann cells wrapped around the regenerated axon and reinnervation occurred only at the old synaptic areas; new contacts between nerve and basal lamina had a terminal Schwann cell capping the nerve terminal. In the presence of anti-N-CAM antibodies, three major abnormalities were observed in the regeneration and reinnervation processes: (a) regenerated axons in nerve trunks that had grown back into the old Schwann cell basal lamina were rarely associated with myelinating Schwann cell processes, (b) ectopic synapses were often present, and (c) many of the axon terminals lacked a terminal Schwann cell capping the nerve–basal lamina contact area. These results suggest that N-CAM may play an important role not only in the determination of synaptic areas but also in Schwann cell–axon interactions during nerve regeneration.

**A**FTER injury to a peripheral nerve, motor axons regenerate and grow back to their targets to form new neuromuscular junctions. Cajal (1928) showed that damaged axons regenerate within the remnants of nerve sheath cells of the original axons. Regenerating axons elongate towards the region of former synaptic areas (Cajal, 1928) and preferentially reinnervate original endplates even if they are deflected from their former pathways (Bennett et al., 1973). Axonal elongation is followed by contact formation at the old synaptic sites and very precise reinnervation occurs at these original sites, both in the presence and absence of intact muscle fiber targets (Letinsky et al., 1976). Even in the absence of the muscle fiber itself, the muscle basal lamina sheaths that are left after muscle damage and degeneration direct the precise differentiation of regenerating axons, which show accumulation of synaptic vesicles and formation of active zones (Sanes et al., 1978).

To explain the apparent specificity of mechanisms of axonal growth and target finding, it has been proposed that both guidance cues and reinnervation cues exist. Some guidance cues are thought to be located in the extracellular matrix or basal lamina of the nerve sheath cells. Such cues would give rise to a polarity that, by some unknown molecular mechanism, directs axonal growth down the inside of the basal lamina sheaths (Ide et al., 1983; Schwab and Thoenen, 1985; Tohyama, 1985). Reinnervation cues are also provided at least in part by muscle synaptic basal lamina: active zones of regenerated axon terminals are formed in apposition to regions of basal lamina corresponding to the former secondary postsynaptic folds (Sanes et al., 1978; Glicksman and Sanes, 1983).

In the present study, we have examined the role in such processes of the neural cell adhesion molecule, N-CAM (for a review, see Edelman, 1986). N-CAM is known to accumu-

late at sites of nerve-muscle contact (Rieger et al., 1985; Covault and Sanes, 1986), and can participate in the binding of neurons to striated muscle cells in vitro (Grumet et al., 1982; Rutishauser et al., 1983; Bixby et al., 1987). After characterizing the forms of N-CAM present in a preparation of muscle basal lamina obtained from muscle after extensive detergent extraction, two means were used to assess the role of N-CAM in axonal regeneration and muscle reinnervation. (a) Frog cutaneous pectoris muscle was damaged and denervated, causing both muscle fibers and nerve terminals to degenerate, after which the animals were locally irradiated to destroy mononucleated cells (muscle satellite cells, Schwann cells, fibroblasts, etc.) in the area in order to prevent cell regeneration. The distribution of N-CAM in damaged muscle was then examined by light and electron microscopy. (b) The nerve regeneration and basal lamina reinnervation that occurred in the operated frogs were perturbed by chronic exposure in vivo to anti-N-CAM antibodies. To accomplish this, agarose implants containing anti-N-CAM-specific IgGs or Fab' fragments were inserted under the chest skin of operated animals. The pattern of reinnervation and the ultrastructure of the newly formed synaptic contacts in these animals were compared with those obtained in control animals treated with normal rabbit IgGs.

After denervation and muscle damage, N-CAM epitopes were present in muscle basal lamina sheaths, and were concentrated in the probable synaptic regions. Chronic exposure to anti-N-CAM antibodies in vivo resulted in a number of marked morphological defects including: (a) poor axon ensheathment by Schwann cells, (b) a lack of terminal Schwann cells on axon terminals that contacted the muscle basal lamina, and (c) the presence of ectopic nerve contacts in extrasynaptic regions of muscle basal lamina. These perturbations suggest that N-CAM is involved in both nerve-muscle and axon-Schwann cell interactions.

## Materials and Methods

### Animals and Basal Lamina Sheath Preparations

Experiments were performed on the paired thoracic cutaneous pectoris muscles of adult male frog, *Rana temporaria* L. with rump-to-nose lengths of 5.5–6.5 cm (body weights of 18–30 g). The in vivo experimental procedure of Sanes et al. (1978) was used to obtain naked muscle basal lamina sheaths in the motor endplate-rich region after degeneration and phagocytosis of muscle cell components and nerve terminals. On day 1, after anesthesia with tricaine methanesulfonate (0.1% MS 222; Sandoz Ltd., Basel), the right cutaneous pectoris nerve was transected and a 4–7-mm segment was either excised or crushed three times with a forceps near its entrance to the muscle. Two rectangular slabs were removed from the muscle on each side of the intramuscular nerve trunk, leaving a 1.5-mm-wide bridge of damaged muscle fibers corresponding to the motor endplate-rich region. On day 3, frogs were x-irradiated (100 kV, 8 mA; filtration: 1.7 mm Al; total dose, 1,600 rad with 127 rad/min; 30 cm from source to animal thorax), upon a restricted surface of the experimental hemithorax. For this study a total of 60 frogs were operated upon.

### Antibodies

In this study, we used two rabbit polyclonal antibodies, prepared against *Xenopus* brain N-CAM and against chicken brain N-CAM. These antibodies have been characterized previously (Fraser et al., 1984; Levi et al., 1987) and have been shown to cross react with *Rana* nerve and muscle; they do not bind significantly to epitopes other than those of N-CAM. Rabbit polyclonal antibodies prepared against *Torpedo californica* acetylcholin-

esterase (AChE; EC 3-1-1-7; a gift from Dr. P. Taylor, University of California, San Diego) which were shown to cross react with frog AChE were also used.

### Insertion of Antibody Implants

3 d after x-ray irradiation, anti-N-CAM IgGs, anti-N-CAM Fab' fragments, or preimmune IgGs incorporated in the form of agarose implants (Agarose Indubiose IBF Ville Mouve-la-Garelle, France, gel point 37–45°C) were inserted between skin and muscle. To perform the implants, the previously denervated frogs were anesthetized, the skin overlying the operated muscle was opened, and 100 µl of Ringer's solution (113.5 mM NaCl, 2 mM KCl, 1.8 mM CaCl<sub>2</sub>, 0.57 mM NaH<sub>2</sub>PO<sub>4</sub>, and 1.47 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.2–7.3) containing 3.5 mg/ml rabbit antibodies (IgG or Fab') and 1% agarose at 50–56°C were deposited on the damaged muscle with a Pipetman P-200 (Gilson, Middleton, Wisconsin). At 22–27°C, the agarose polymerized quickly and the antibody-rich implant remained in place, adherent to the muscle and covering the experimental or control regions. A total of 15 frogs were used for this study. After surgery, the skin was sutured and the frogs were kept in water tanks at room temperature (19–22°C).

Bridges of naked basal lamina sheaths and contralateral motor endplate-rich regions were analyzed 15 d after cutting the muscle fibers and motor nerves; at this time the degenerative processes affecting muscle and nerve are largely complete and no nerve regeneration has yet occurred (Sanes et al., 1978). Reinnervation of naked synaptic sites in the absence of regenerated muscle fibers was observed 37 d after placing the antibody implants.

### Light Microscopic Techniques

The relationships between the presynaptic nerve terminals and naked synaptic basal lamina in the course of reinnervation were determined by a combined Karnovsky's cholinesterase staining and axon silver impregnation, at 20–22°C, as detailed in Pecot-Dechavassine et al. (1979). Both operated and contralateral cutaneous pectoris muscles were carefully dissected with their sternum and skin insertions still attached and tightened upon a square rigid plastic armature at 100–105% of the normal rest muscle length and were then immersed in Ringer's solution at 4°C. After a brief fixation (5 min) in 1% neutral formalin in Ringer's solution, the synaptic basal lamina AChE was stained in Karnovsky solution for 30 min. After rinsing in Ringer's solution and postfixation in 5% neutral formalin and 80% ethanol, the subterminal and terminal axon branches were stained by silver impregnation using the Bodian technique. The preparations were subsequently mounted in toto on coverslips in a water-soluble medium. For immunofluorescent staining, the tissues were fixed 1 h (2.5% formaldehyde/0.1 M phosphate buffer [pH 7.0]), immersed for 15 min in a PBS solution containing 0.1 M glycine, washed in PBS for 15 min, and incubated overnight with the primary antibodies (10–40 µg/ml) in the presence of 4% goat serum. Second antibodies were either fluorescein- or rhodamine-conjugated goat anti-rabbit IgGs (Cappel Laboratories, Inc., Cochranville, PA). Photomicrographs were taken with a Leitz photomicroscope equipped for observation of rhodamine and fluorescein fluorescence with appropriate filters and phase contrast optics.

### Electron Microscopic Techniques

The dissected whole muscle preparations were fixed in 2.5% glutaraldehyde plus 0.5% tannic acid in 0.1 M phosphate buffer, pH 7.4, for 2 h at 4°C and conventional electron microscopy was performed. Immunogold labeling was performed on cryostat sections. Freshly dissected tissues were prefixed for 30 min with 4% paraformaldehyde solution in 100 mM phosphate buffer, pH 7.3, equilibrated with 5, 10, and 20% (wt/vol) sucrose in 100 mM phosphate buffer pH 7.3, embedded in OCT compound (Tissue-Tek, Miles Scientific, Elkhart, Indiana); frozen in isopentane cooled at –160°C with liquid nitrogen, (5 µm frozen sections were obtained at –15°C). These sections were then fixed for 15 min in 4% paraformaldehyde, washed twice in PBS/0.1 M glycine, and incubated in PBS/4% ovalbumin. After overnight incubation in primary antibody diluted in PBS/4% ovalbumin, sections were washed three times and incubated with colloidal gold particles (5 nm particles) conjugated to anti-rabbit IgGs (Janssen Pharmaceutica, Beese, Belgium) for 2 h. The sections were washed three times with PBS and fixed

1. *Abbreviations used in this paper:* AChE, acetylcholinesterase; AChR, acetylcholine receptor.

in 2.5% glutaraldehyde/0.5% tannic acid in PBS for 1 h at 4°C. After three more washes in PBS, the sections were postfixated in osmium tetroxide, dehydrated, and embedded in Epon. Ultrathin sections were cut and observed with a Phillips EM 400 electron microscope. In electron micrographs, gold particles were identified using a 10× magnifying lens by their spherical shapes and sharp and symmetric contours.

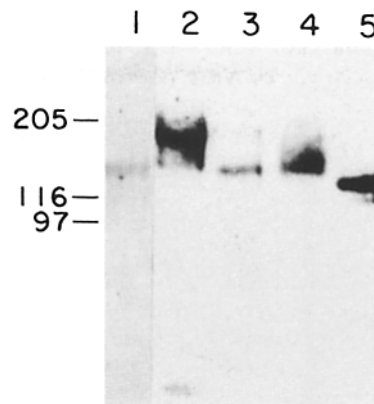
### Biochemical Procedures

Muscles were dissected from the hind legs of *Rana pipiens*. To analyze basal lamina components, we used a two-step procedure, first homogenizing the muscle in 10 ml/g wet wt of tissue in PBS containing 0.5% NP-40, a non-ionic detergent known to solubilize the N-CAM present in the plasma membrane (Cunningham et al., 1983), 1 mM EDTA, 2.5% (vol/vol) Trasylol (Mobay Chemical Corp., New York, NY), and 1 mM phenylmethylsulfonyl fluoride. After centrifuging at 100,000 g for 1 h, the pellet was washed again twice in the same buffer and finally extracted in 1 mM diethylamine, pH 11.5, and 1 mM EDTA; this extraction is known to solubilize many proteins associated with the extracellular matrix such as cytotoxin (Crossin et al., 1986). The diethylamine extract was then diluted with 10 vol of PBS and adjusted to pH 7.6. 100 µl of Sepharose 4B-protein A (Sigma Chemical Co., St. Louis, MO) saturated with a polyclonal anti-N-CAM antibody was added to the neutralized extract (24 h, 4°C) and to the NP-40 extracts of the muscle. The beads were collected, washed three times with cold PBS containing 0.5% NP-40, and boiled directly in 120 µl of SDS-PAGE sample buffer before or after being treated with neuraminidase as previously described (Hoffman et al., 1982). The immunoprecipitates were resolved by SDS-PAGE, transferred to nitrocellulose paper and sequentially reacted with 50 µg of rabbit anti-N-CAM and <sup>125</sup>I-protein A. After extensive washing, the samples were analyzed by autoradiography and molecular masses determined by comparison with the migration of standard proteins (Rieger et al., 1985).

### Results

#### N-CAM Forms in Muscle Plasma Membrane and Basal Lamina

Basal lamina was prepared as described in Materials and Methods from undamaged adult muscle. After detergent extraction to remove the plasma membrane and its associated N-CAM, the resulting insoluble material contained mainly intact basal lamina together with some cytoskeletal and myofibrillar elements as shown by ultrastructural examination (data not shown). The N-CAM associated with this fraction was then extracted with 1 mM diethylamine, pH 11.5, and analyzed as described in the Materials and Methods section; this procedure is known to solubilize proteins associated with the extracellular matrix such as cytotoxin (Crossin et al., 1986). This material was compared (Fig. 1) with N-CAM isolated from peripheral nerve and muscle plasma membranes. In contrast to the discrete form of N-CAM found in NP-40 extracts of peripheral nerves of *Rana pipiens* having an  $M_r$  of 140,000 (Fig. 1, lane 1), muscle plasma membrane N-CAM isolated from the detergent extracts appeared as a polydisperse, polysialylated form with average  $M_r$  of 170,000 that, upon neuraminidase treatment, was converted to a component of  $M_r$  of 145,000 (Fig. 1, lanes 2 and 3). The N-CAM immunoprecipitated from neutralized diethylamine extracts of basal lamina appeared as a polydisperse form with an average  $M_r$  at ~160,000 (Fig. 1, lane 4); upon neuraminidase treatment this form of N-CAM was converted to a component of 125,000  $M_r$  (Fig. 1, lane 5) that was clearly distinct from the N-CAM present in the NP-40 extracts of both the muscle (Fig. 1, lane 3, 145,000  $M_r$ ) and nerve (Fig. 1, lane 1, 140,000  $M_r$ ).

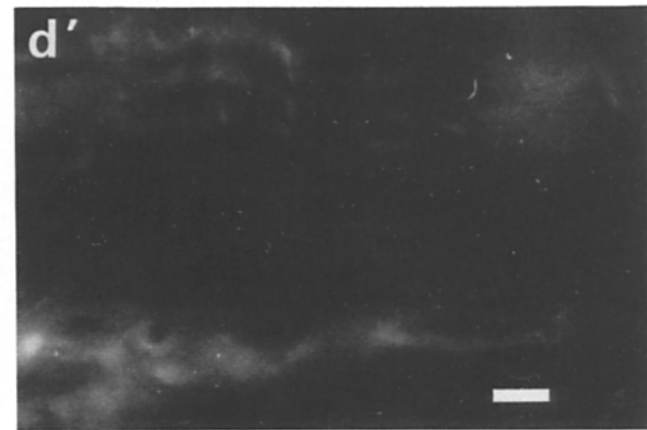
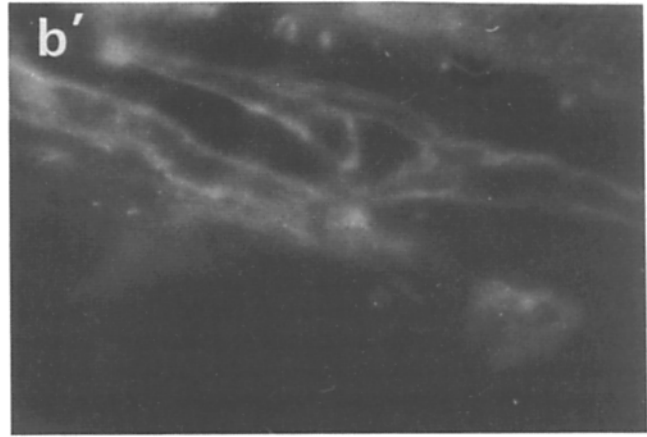
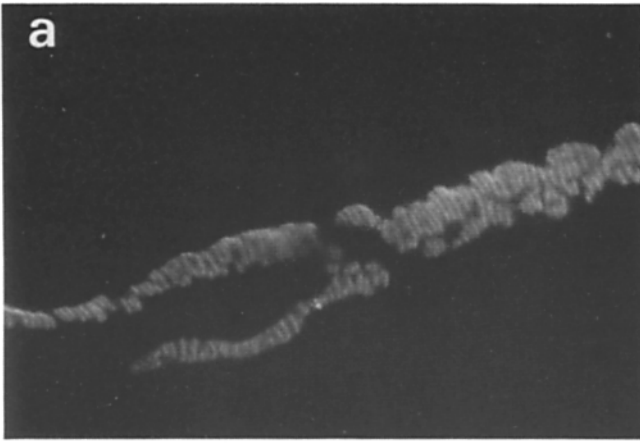


**Figure 1.** Forms of N-CAM in plasma membrane and basal lamina. The plasma membrane of frog (*Rana pipiens*) peripheral nerve (10 mg) (lane 1) or muscle (10 g) (lane 2 and 3) was extracted with 0.5% NP-40; muscle basal lamina and extracellular matrix extracts (lanes 4 and 5) were prepared by treating the pellet obtained after NP-40 extraction with diethylamine, pH 11.5. N-CAM was immunoprecipitated from the neutralized extracts using an immobilized rabbit polyclonal anti-N-CAM antibody; the resulting immunoprecipitates were analyzed by immunoblotting as described in the Materials and Methods section. Particular fractions (lanes 3 and 5) were treated with neuraminidase. Molecular mass markers are at 205,000, 116,000, and 97,000  $M_r$ , respectively.

#### Immunocytochemical Detection of N-CAM in Basal Lamina Sheaths after Denervation and Muscle Injury

In normal cutaneous pectoris muscle, the motor endplates are located in the central portion of the muscle and can be visualized by staining using fluorescent  $\alpha$ -bungarotoxin, which irreversibly and specifically binds to the acetylcholine receptor (AChR; Fig. 2, a and b). AChR staining is found concentrated on the edges of the synaptic gutters of the motor endplates and on crossbands which correspond to the crests of the junctional folds (Letinsky et al., 1976). N-CAM staining outlined the motor endplate ramifications (Fig. 2 a') in a manner similar to that of the AChR although crossbands could not be observed. In this respect, N-CAM staining resembled the AChE staining (Fig. 2 b'; see also Letinsky et al., 1976; McMahan et al., 1978). After a total section of the cutaneous pectoris nerve with damage to the corresponding muscle fibers and destruction of mononucleated cells, the old synaptic areas lost all AChR staining (Fig. 2, c and d). However, these synaptic areas remained positive for N-CAM, as shown in double-staining experiments using a rabbit polyclonal antibody that recognizes epitopes present in all three polypeptide forms components of N-CAM in *Xenopus* brain (Levi et al., 1987) (Fig. 2 c'). In parallel experiments (Fig. 2 d'), AChE staining with polyclonal rabbit anti-AChE antibodies showed patterns that appeared essentially similar to that obtained for N-CAM; both presented the overall distribution and morphological features characteristic of old synaptic regions (see Letinsky et al., 1976).

The observations at the light microscope level suggested that a substantial portion of junctional N-CAM is associated with muscle basal lamina. To verify this conclusion and to localize N-CAM more precisely in the muscle basal lamina, a detailed electron microscopic study was carried out using



anti-N-CAM IgG and colloidal gold particles (5 nm) coupled to anti-rabbit IgG. Colloidal gold particles or small aggregates of particles were scattered all along the muscle basal lamina (Fig. 3 *a*) as well as in the extracellular matrix material (Fig. 3 *a* and see below). N-CAM immunoreactive material was often concentrated in specific regions of the basal lamina possibly corresponding to the areas of old synaptic contacts as suggested by a high degree of membrane folding (Fig. 3 *b*). The immunoreactive material was not associated with remnants of plasma membrane, which are very rarely found in this preparation (Nicolet et al., 1986). Control experiments with nonimmune rabbit IgG showed very low or no labeling of the muscle basal lamina. A careful examination at high resolution of the distribution of N-CAM in the extracellular matrix-rich spaces (Fig. 3, *c* and *d*) showed that particles were not distributed at random but that most were closely associated with collagen fibrils, often in an orderly fashion, attached in aggregates of ~5–10 particles to particular armlike structures disposed along the longitudinal collagen fibrils.

#### ***Alteration of the Pattern of Reinnervation of Basal Lamina Sheath by Anti-N-CAM Antibodies***

A few weeks after denervation and muscle damage, axon regeneration and subsequent reinnervation of the cutaneous pectoris muscle basal lamina sheaths occurred in the operated animals. To determine the influence of N-CAM on the process of axonal regeneration and muscle reinnervation, the course taken by the regenerating axons in the presence of agarose implants containing anti-N-CAM antibodies was compared with that of control implants containing nonimmune rabbit IgG. 5–6 wk after surgery, the preparations were stained by the method of Pecot-Dechavassine et al. (1979), in which the motor nerve terminals and the regenerating preterminal axons that have not yet become myelinated are silver impregnated and muscle synaptic areas are AChE positive (Fig. 4). Normally fasciculated axon bundles, with classical primary branching, were observed in both normal IgG and anti-N-CAM-treated tissues (data not shown). In control animals treated with normal rabbit IgG agarose, the regenerated axons contacted the AChE-positive muscle basal lamina all along the old synaptic gutter (Fig. 4 *a*). When anti-N-CAM IgGs or Fab' fragments were applied to the muscles, however, the regenerated axons did not contact the

synaptic basal lamina sheaths in a normal way (Fig. 4 *b*). In such preparations, the axon made only localized contacts with the AChE-positive regions and not all along their length. Axon terminal branching and intense terminal sprouting with numerous growth cones were observed in anti-N-CAM-treated muscle (Fig. 4, *d* and *e*) in all fields examined, in sharp contrast to control muscle.

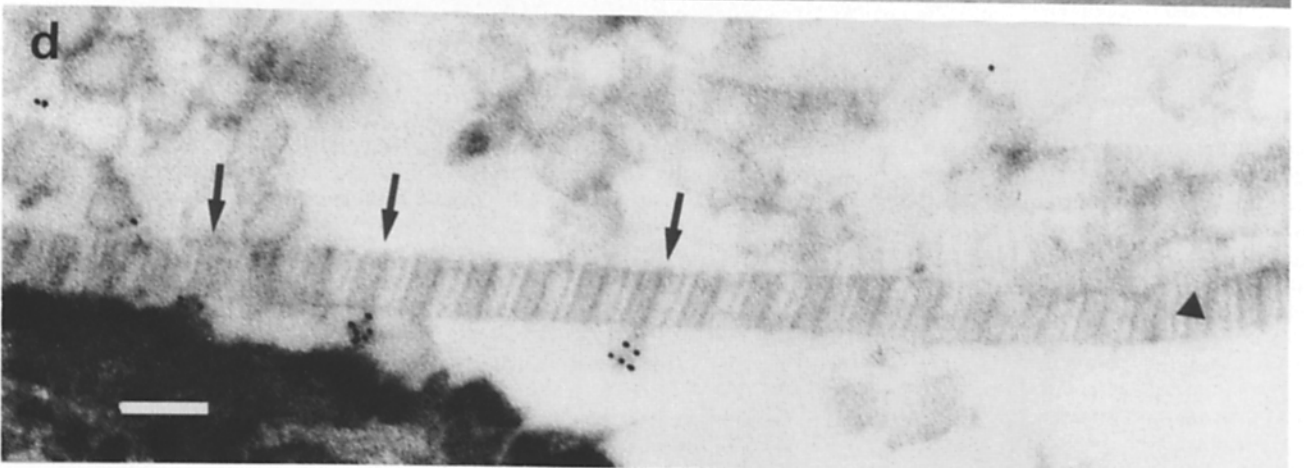
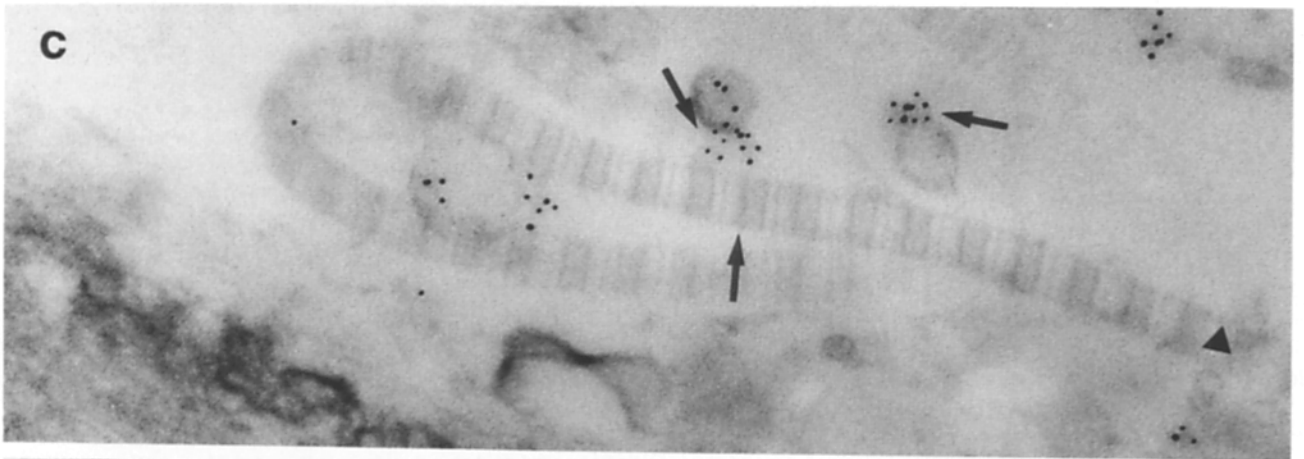
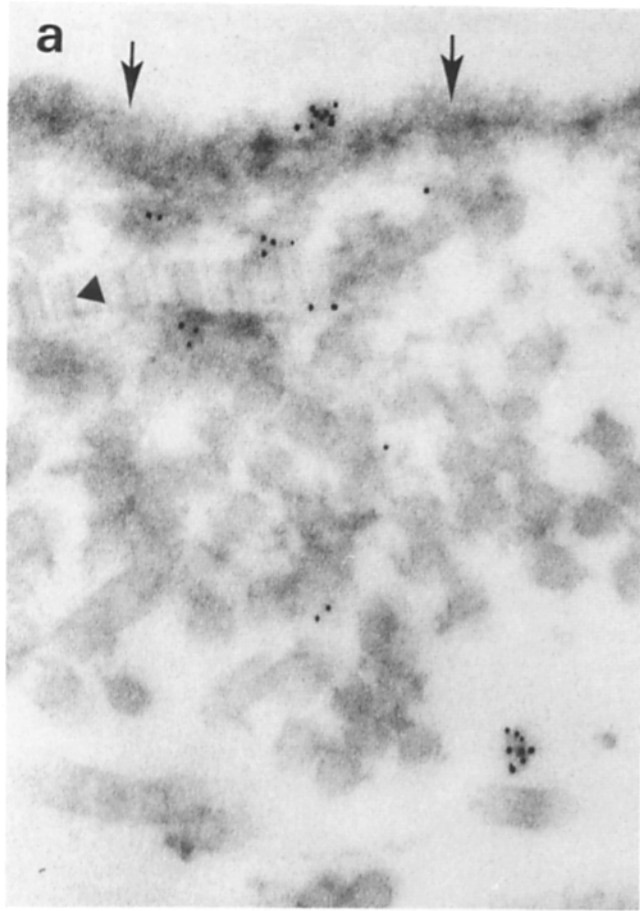
#### ***Ultrastructural Examination of Anti-N-CAM-treated Basal Laminae: Defective Axon Ensheathment by Schwann Cells***

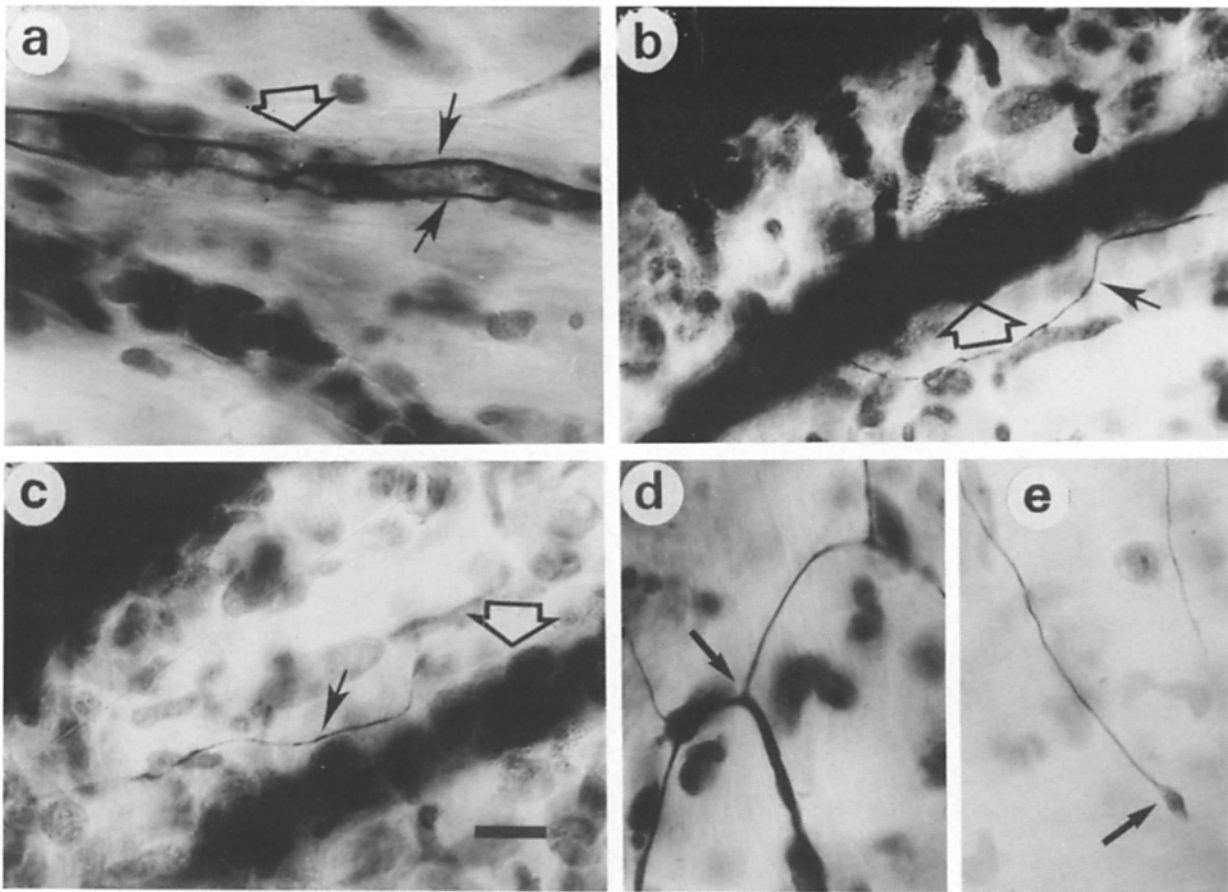
In normal peripheral nerve, N-CAM is present on both axon and Schwann cell surfaces (Rieger et al., 1986). To evaluate the influence of chronic exposure to anti-N-CAM antibodies on regenerating axons and associated Schwann cells, longitudinal sections of the regenerated nerve to the cutaneous pectoris muscle were examined at the electron microscopic level. By 37 d after denervation and damage to the muscle, all axons were completely ensheathed by Schwann cells in control preparations (Fig. 5 *a*) although myelin had not yet been deposited. In contrast, in anti-N-CAM-treated animals and in virtually all electron microscopic fields examined, regenerated axons presented long stretches of cell surface without any apposed Schwann cell process (Fig. 5 *b*). Only old Schwann cell basal laminae were present in these naked regions, possibly providing guiding structures for regenerating axons.

#### ***Ectopic Synapse Formation and Absence of Terminal Schwann Cells after Reinnervation in the Presence of Anti-N-CAM Antibodies***

Axonal regeneration is followed by reinnervation of denervated damaged muscle fibers precisely at the original synaptic sites. In accord with earlier observations (Sanes et al., 1978) we found that: (*a*) contacts between axon and muscle basal lamina sheaths systematically occurred in the old synaptic areas; (*b*) synaptic vesicles and active zones were present in the axon terminals in apposition to synaptic basal lamina sheaths; and (*c*) terminal Schwann cell processes generally covered the axon terminals (Fig. 6 *a*). When the damaged muscle was treated with agarose implants containing nonimmune rabbit IgG or Fab' fragments, the portions of the axons in contact with the basal lamina sheaths differen-

*Figure 2.* Immunofluorescence detection of N-CAM in synaptic basal lamina sheaths. (*a*, *a'* and *b*, *b'*) Double staining of AChR and N-CAM (*a*, *a'*) and AChR and AChE (*b*, *b'*) in normal muscle. (*a* and *b*) The motor endplates in normal adult frog cutaneous pectoris muscle were visualized by an immunofluorescence procedure using rhodamine-conjugated  $\alpha$ -bungarotoxin, which irreversibly and specifically binds to the AChR concentrated at the crests of the postsynaptic folds. The crests appear as strongly fluorescent crossbands. (*a'*) N-CAM was stained in the double immunocytochemical staining procedure together with the AChR (*a*) using a rabbit polyclonal N-CAM antibody, revealed by indirect immunofluorescence with fluorescein-conjugated goat anti-rabbit IgG. The N-CAM staining outlines the contours of the gutter-like structures of the motor endplates. AChE was stained (*b'*) in the double immunocytochemical staining procedure together with the AChR (*b*) using specific rabbit polyclonal antibodies raised against the post-synaptic protein AChE purified from *Torpedo californica* electric organs which cross react with the frog enzyme. (*c*, *c'* and *d*, *d'*) Double staining of AChR and N-CAM (*c*, *c'*) and AChR and AChE (*d*, *d'*) in denervated and damaged muscle. (*c* and *d*) There is no staining for AChR in the region of those synaptic basal lamina sheaths which remain after denervation and muscle fiber damage. However, positive staining for N-CAM by anti-*Xenopus* N-CAM polyclonal antibodies is observed in these areas (*c'*). The overall pattern of N-CAM staining (*c'*) is very similar to the AChE staining (*d'*; see also Letinsky et al., 1976). Note the often wavy appearance (*c'*, *d'*) of the basal lamina sheaths, which indicates that they were localized close to the site of muscle injury. Staining intensity for N-CAM is generally decreased compared to that in intact muscle suggesting that a significant portion of N-CAM staining at the intact motor endplate corresponds to synaptic plasma membrane N-CAM. Bar, 10  $\mu$ m.



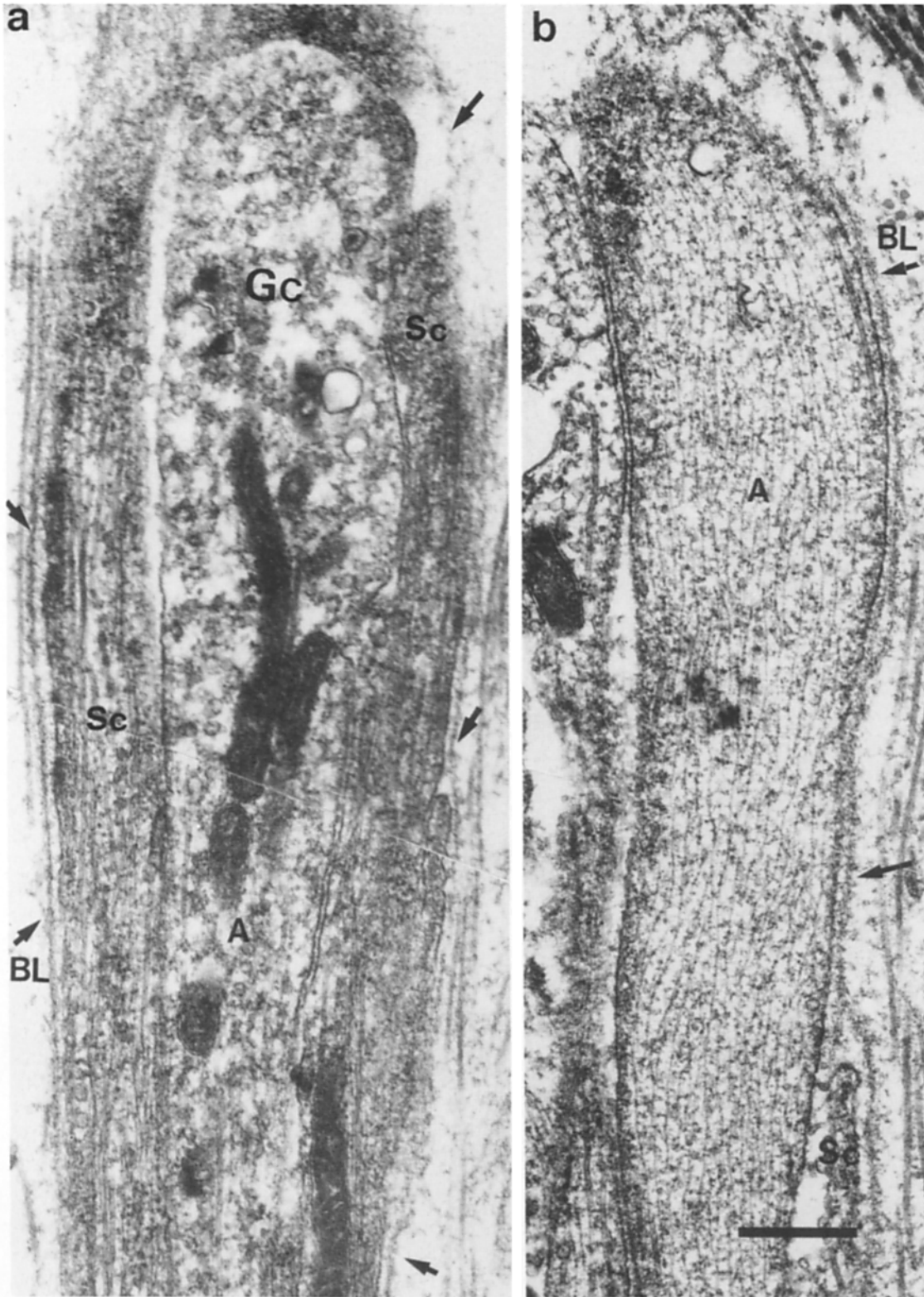


**Figure 4.** Effects of anti-N-CAM on muscle reinnervation after denervation and muscle damage. (a) In muscles treated with nonimmune rabbit IgGs, reinnervation occurs with axons running along the synaptic basal lamina sheaths, characterized by their AChE staining. In this micrograph, two axons (arrows) run along the basal lamina remnants of a myofiber, slightly stained for AChE (open arrowhead). The nuclei stained by the AChE-silver nitrate procedure essentially belong to macrophages, which are invading the damaged muscle, as evidenced by electron microscopy (data not shown; see Nicolet et al., 1976). (b and c) In anti-N-CAM exposed muscles, axons (arrows) do not grow along the myofiber ghost. The basal lamina remnants of the two myofibers shown in both micrographs (open arrowheads) are rather intensely labeled by the AChE procedure, as recognized at the microscope by their distinctive red-brown staining. Staining intensities vary widely from one region of the muscle to the next and differences between the stainings in a compared with b or c are only incidental. The axons, which sometimes contact the myofiber ghost, do not seem to have any privileged association with the old synaptic sites. (d and e) Commonly found aspects of axonal branching (d; arrow), sprouting and growth, and an example of very frequently observed growth cones (e; arrow) in anti-N-CAM-treated muscle. Chronic anti-N-CAM Fab' fragment treatment was performed using agarose implants as described in Materials and Methods. Bar, 10  $\mu$ m.

tiated into nerve terminals that were similar by all morphological criteria to nerve terminals in intact muscle. In striking contrast, muscles exposed to implants containing anti-N-CAM antibodies (IgG or Fab' fragments) showed abnormal contacts and patterns of reinnervation. In most cases, there

was no terminal Schwann cell isolating the portion of differentiated axon in contact with the muscle basal lamina (Fig. 6 b) and contacts of axons with basal lamina were frequently found in extrasynaptic regions (Fig. 7, a and b). Table I summarizes the effects of anti-N-CAM antibodies on the pattern

**Figure 3.** Ultrastructural localization of N-CAM in muscle basal lamina sheaths after denervation and muscle damage. (a and b) Electron micrographs of muscle basal lamina and extracellular spaces 15 d after nerve section and muscle damage. In samples exposed to anti-N-CAM antibodies, colloidal gold particles (5 nm) are predominantly associated with the muscle basal lamina (arrows). Control sections treated with nonimmune rabbit IgGs (not shown) show an extremely low particle background distributed uniformly on the section. The electron micrograph displayed in a shows clusters of gold particles associated with the continuous, typical, fuzzy material which constitutes the basal lamina sheaths (see Nicolet et al., 1986 for a more detailed morphological characterization of basal lamina). Particles are also present at low density in the extracellular matrix, often in close proximity to collagen fibrils which are disposed in longitudinal or transverse orientations (arrowheads; and see below). The electron micrograph in b shows the intimate relationship of N-CAM immunoreactive material with the basal lamina sheaths (arrows). High density labeling such as the one demonstrated on the micrograph is very often found in restricted regions of basal lamina sheaths characterized by the presence of folds. These regions may be the old synaptic regions and such basal lamina may correspond to the old postsynaptic basal lamina. (c and d) After reaction with anti-N-CAM antibodies and anti-rabbit IgG-colloidal gold, clusters of particles can be found distributed in a rather orderly fashion along collagen fibrils seen in a longitudinal orientation (arrowheads), in apparent association with structures protruding laterally from the fibrils (arrows). Bar, 0.5  $\mu$ m.



**Figure 5.** Effect of anti-N-CAM Fab' on Schwann cell interaction with regenerating axons. 37 d after denervation and nerve damage. (a) Nonmyelinated axon in a nerve fascicle regenerating over basal lamina sheaths treated with nonimmune IgGs. Normal ultrastructure with Schwann cell processes (Sc) completely encircling the axon and its growth cone (Gc) was observed. The Schwann cell has a continuous basal lamina (BL; arrows). (b) Nonmyelinated axon in a nerve fascicle regenerating over basal lamina sheaths treated with anti-N-CAM Fab' fragments. The region of the axon (A) displayed in this micrograph is essentially not surrounded by a Schwann cell, but otherwise the axon looks normal. The axon is directly in contact with a continuous basal lamina (BL; arrows) from the old Schwann cell. Bar, 0.5  $\mu\text{m}$ .



of reinnervation of muscle basal lamina sheaths. The great majority (86%) of nerve terminals on basal laminae treated with nonimmune IgG possessed an associated Schwann cell whereas two-thirds (65%) of the nerve terminals on basal laminae treated with anti-N-CAM IgGs were devoid of terminal Schwann cells. An even greater proportion of terminals (~90%) were devoid of these cells in basal laminae exposed to anti-N-CAM Fab' fragments. A major proportion (84%) of the nerve terminals in control animals were found to be in areas recognized morphologically as synaptic areas by the presence of characteristic junctional fold remnants and also basal lamina density (Fig. 6, *a* and *b*). In animals exposed to anti-N-CAM IgG or anti-N-CAM Fab' fragments, however, most of the nerve terminals were found in extrasynaptic areas of the basal lamina sheaths (53 and 64%, respectively). Most of the ectopic contacts found in anti-N-CAM-treated animals were also devoid of a terminal Schwann cell (Fig. 7, *a* and *b*). This widespread perturbation of regenerative patterns by antibodies to N-CAM suggests a key role for N-CAM not only in Schwann cell-axon interactions in nerves but also in Schwann cell-axon-muscle interactions at the motor endplate.

## Discussion

We report here the results of a study of the distribution and role of N-CAM in the amphibian neuromuscular system. The main conclusions of this study are as follows. (*a*) A muscle extract enriched in basal lamina from normal frog muscle contained a polydisperse form of N-CAM that, upon neuraminidase treatment, was converted to a component 125,000  $M_r$  different from detergent-extracted N-CAM of both nerve and muscle. (*b*) N-CAM immunoreactive material was readily detected in basal lamina sheaths obtained after nerve section and muscle damage both at the light and at the electron microscopic level. N-CAM was found distributed on the basal lamina sheaths and is generally often concentrated in areas which apparently correspond to old synaptic sites. N-CAM was also found in the extracellular matrix; N-CAM immunoreactive material often appeared in close association with type I collagen fibrils in an orderly fashion. (*c*) When agarose implants containing anti-N-CAM-specific antibodies were applied to the damaged muscles, a number of abnormalities were induced in the newly formed nerve-basal lamina contacts; these abnormalities were not observed in animals treated with preimmune rabbit IgGs. In the presence of anti-N-CAM antibodies, the regenerating axon lacked a continuous sheath of myelinating Schwann cell processes, synaptic contacts often occurred at ectopic extrasynaptic sites, and terminal Schwann cells were mostly absent from the nerve terminal.

Immunoblot analysis of extracts of frog basal laminae suggested that the molecule present in muscle basal lamina is a polydisperse, polysialylated form of N-CAM with an average  $M_r$  of 160,000 and a desialylated  $M_r$  of 125,000. The unusual molecular mass of the polypeptide, 125,000  $M_r$  instead of 145,000  $M_r$  found for N-CAM extracted from muscle with detergent, raises the possibility that this component differs from other forms of N-CAM found so far in the neuromuscular system (Rieger et al., 1985; Covault and Sanes, 1986). In its desialylated form this component is similar to

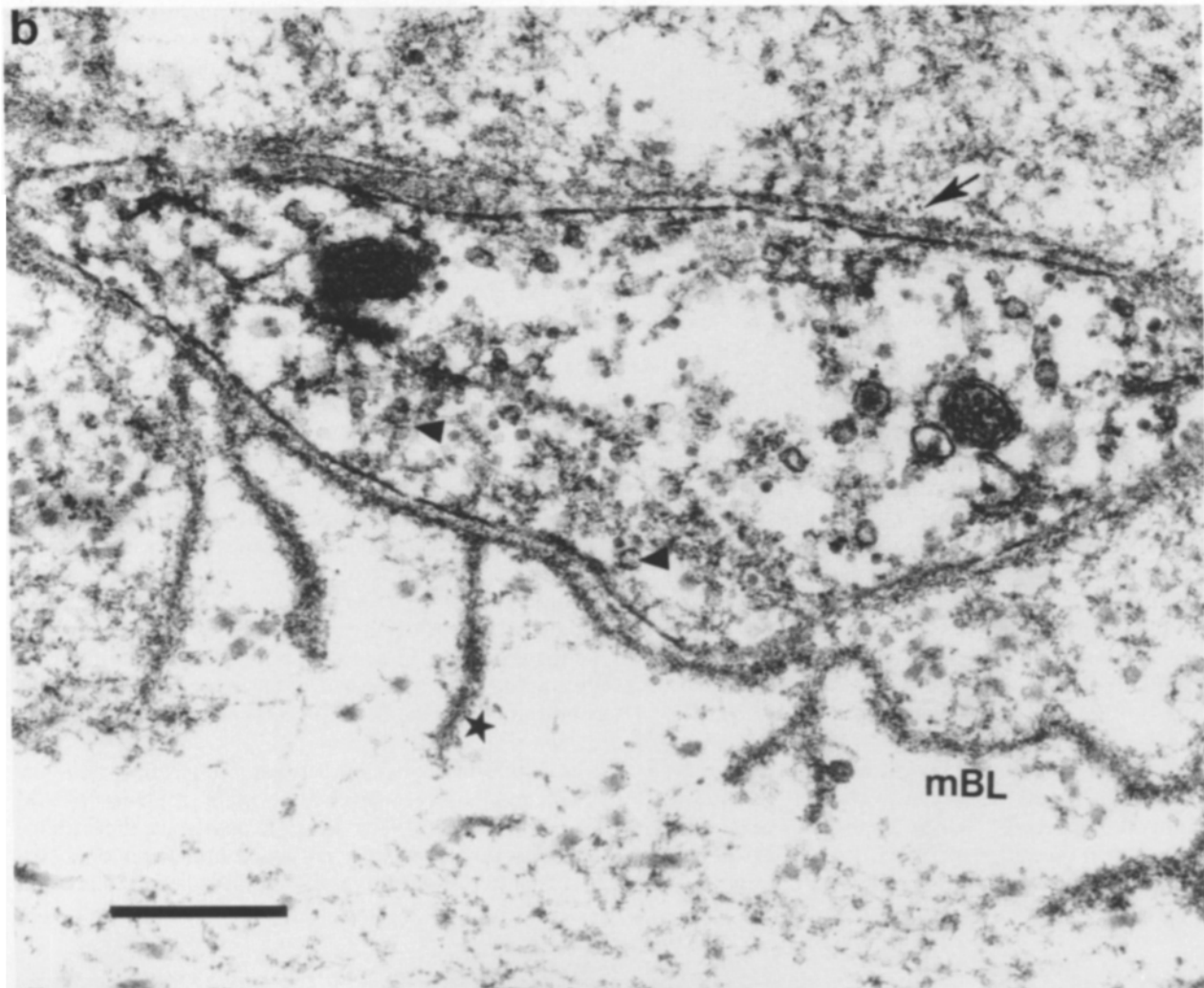
an N-CAM form of muscle recently reported by Dickson et al. (1987) and Moore et al. (1987). Like other N-CAM polypeptides, it may arise by a form of alternative RNA splicing (Cunningham et al., 1987) that is specific to muscle and possibly to Schwann cells. It will be particularly important to determine whether N-CAM in the muscle basal lamina interacts directly with the muscle plasma membrane and, if so, whether it is anchored to the membrane by a phospholipid moiety as seen for the ssd polypeptide of N-CAM in brain (Hemperly et al., 1986).

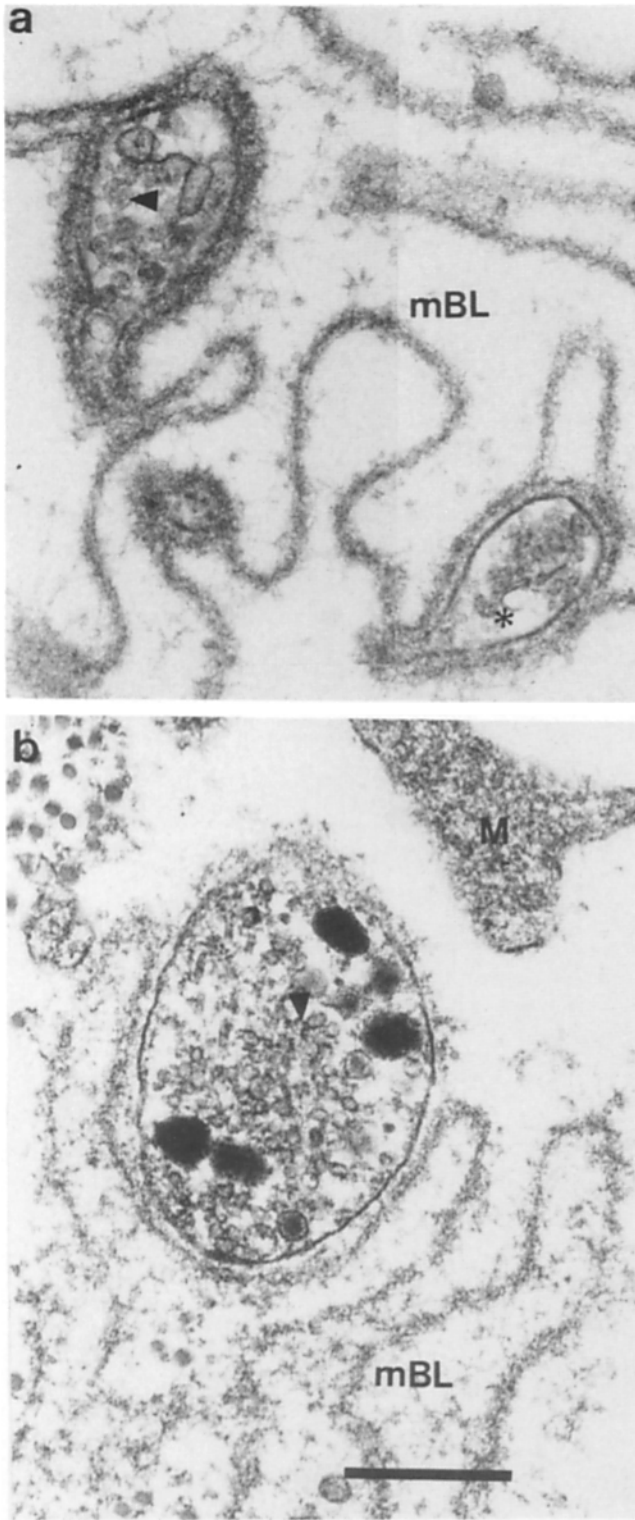
When muscle and nerve fibers are damaged in frog cutaneous pectoris muscle, the orientations of the basal lamina sheaths are preserved (Sanes et al., 1978) and the sites where synapses had been situated can be identified at the light (Letinsky et al., 1976) as well as at the electron microscopic level (Marshall et al., 1977; Nicolet et al., 1986). After nerve and muscle lesions, the axons start growing back towards territories containing the muscle basal lamina sheaths (Letinsky et al., 1976) and axons interact with Schwann cells that, early during the regenerative processes, completely enwrap the axons (Ide et al., 1983). Subsequently, regenerating axons make their contacts preferentially at the old synaptic sites (Sanes et al., 1978) and the areas of contact induce regenerating growth cones to differentiate into nerve terminals with active zones and synaptic vesicles (Glicksman and Sanes, 1983).

Several observations have suggested that N-CAM, which binds by an homophilic mechanism (Hoffman and Edelman, 1983; Edelman, 1986) and is present on both axons and Schwann cells (Rieger et al., 1986), might be involved in various steps of this regenerative process. Antibodies directed against N-CAM can disrupt axon distributions *in vitro* (Buskirk et al., 1980) and *in vivo* (Fraser et al., 1984). N-CAM is also present in high amounts on embryonic motor axons and it may be responsible in part for the formation and maintenance of nerve trunks (Covault and Sanes, 1986).

In the present studies, we found that nerve fasciculation was normal in the presence of anti-N-CAM suggesting that if N-CAM has a role in this process, it is either shared with other adhesion molecules such as Ng-CAM (Edelman 1986; Hoffman et al., 1986), or is not affected by the presence of anti-N-CAM antibodies. As we have shown, however, in the presence of such antibodies, Schwann cells did not associate properly with regenerated axons and wide areas at the ultrastructural level were devoid of any apposed Schwann cell processes. This effect of the antibodies may have been due to a cytotoxic action on Schwann cells, although this seems unlikely; in treated tissues, Schwann cells were frequently observed at the electron microscopic level. It is more probable that the migratory activity or adhesivity of the Schwann cells onto axons during regeneration was impaired by the antibodies. This conclusion is in agreement with previous results suggesting a critical role for N-CAM in the formation, stabilization, and maintenance of the interactive cellular system constituted by the Schwann cell and the axon (Rieger et al., 1986).

The region of apposition between the terminal Schwann cell cap and the nerve terminal normally contains N-CAM (Covault and Sanes, 1986). In our experiments, the terminal Schwann cell was generally not found in the area of nerve-muscle contact when anti-N-CAM antibodies were present,





**Figure 6.** Ultrastructural aspects of muscle reinnervation on control and anti-N-CAM-treated basal lamina sheaths. (a) Reinnervated basal lamina sheaths treated chronically with nonimmune rabbit IgGs. 37 d after the operation, axon terminals have contacted the basal lamina sheaths. Most generally the contact occurs in old synaptic regions as shown by the characteristic remnants of secondary folds (*star*). A higher concentration of synaptic vesicles is present close to the presynaptic membrane (*arrowheads*). Schwann cell processes (*Sc*) completely encircle the axon terminal. (b) Reinnervated basal lamina sheaths treated chronically with anti-N-CAM Fab' fragments. In contrast to *a*, the nerve terminals are constituted only by the axon terminal surrounded by a basal lamina, which probably corresponds to the original Schwann cell basal lamina (*arrow*), and are in tight apposition to the muscle basal lamina sheaths (*mBL*) with remnants of postsynaptic secondary folds (*b; star*). Numerous synaptic vesicles are seen inside the axon terminal, accumulated close to the presynaptic membrane. Bar, 0.4  $\mu\text{m}$ .

**Table I.** Effect of Anti-N-CAM Antibodies on Schwann Cell Association with Nerve Terminals and on Ectopic Synapse Formation after Reinnervation of Frog Basal Laminae\*

Treatment	Percent of nerve terminals on old synaptic sites with no associated Schwann cell <sup>‡</sup>	Percent of ectopic contacts <sup>‡</sup>	Percent of ectopic contacts with no associated Schwann cell <sup>‡</sup>
Nonimmune IgG	14 (3/22)	16 (3/19)	0 (0/3)
Anti-N-CAM IgG	65 (22/34) <sup>§</sup>	53 (18/34) <sup>§</sup>	78 (14/18)
Anti-N-CAM Fab'	91 (10/11) <sup>§</sup>	64 (7/11) <sup>§</sup>	86 (6/7)

\* Serial sections of naked basal lamina sheaths were examined by electron microscopy 37 d after placing the implants containing the antibodies. Basal laminae from three different animals were examined for each determination. The presence of Schwann cell processes capping the nerve terminals was considered as evidence for terminal Schwann cells.

<sup>‡</sup> Numbers in parenthesis represent number of positives scored/number of total contacts examined.

<sup>§</sup> Significantly different from results obtained after nonimmune IgG treatment ( $P < 0.01$  in a Chi-square test).

suggesting that contacts between axon and basal lamina can be made in the absence of Schwann cells and that these contacts differentiate into axon terminals. If Schwann cells play a guiding role in innervation at specific sites, this role is diminished or abolished in the presence of anti-N-CAM antibodies.

After exposure of muscle to anti-N-CAM antibodies, there was no strict target specificity for reinnervation. Old synaptic sites, identified by the remnants of the basal lamina folds, could be reinnervated as well as sites in the extrasynaptic regions of muscle basal lamina sheaths. These observations

**Figure 7.** Muscle reinnervation in the presence of anti-N-CAM antibodies and formation of ectopic axon-muscle contacts on basal lamina sheaths after denervation and muscle damage. (a and b) 37 d after denervation and muscle damage, the axons regenerating in the presence of anti-N-CAM Fab' often form differentiated contacts (*asterisk*) in extrasynaptic regions of the muscle basal lamina (*mBL*). These contacts have essentially the same presynaptic features as those encountered in old synaptic areas (Fig. 6 b) with a high density of synaptic vesicles (*arrowheads*). In the region of the ectopic contact, the basal lamina very often shows small folds such as those seen on both micrographs. These folds are essentially different from the postsynaptic fold remnants that look like fused lamellae structures (see Fig. 6). They may originate from mechanochemical constraints imposed by the nerve as it contacts the basal lamina. The extrasynaptic basal lamina displays here a significantly diminished electron opacity compared with synaptic basal lamina (Fig. 6, a and b) characteristic of tannic acid treatment. Note that, generally, the ectopic nerve contacts do not have an accompanying Schwann cell but do possess a basal lamina. *M*, macrophage. Bar, 0.5  $\mu\text{m}$ .

suggest that N-CAM present in the basal lamina may be involved in determining which sites will become reinnervated during nerve regeneration, possibly as a "stop signal" for further local axonal extension. In the presence of anti-N-CAM antibodies, selective adhesivity at synaptic sites may be decreased and innervation may occur in synaptic as well as extrasynaptic regions.

In the ultrastructural studies, N-CAM epitopes were also found associated with collagen and often disposed in an orderly manner along type I collagen fibrils in small aggregates that seemed to be spatially apposed to armlike structures. This observation raises the possibility that collagen and N-CAM may interact directly or through an intermediate ligand. They also raise the possibility that N-CAM in extracellular matrix is involved in the process of specific reinnervation, sharing this role with other extracellular matrix proteins such as those designated J1 (Sanes et al., 1986) or cytotactin (Daniloff, J. K., K. L. Crossin, M. Pinçon-Raymond, M. Murawsky, F. Rieger, and G. M. Edelman, manuscript submitted for publication) both of which are concentrated in interstitial spaces near denervated sites.

Motor axons can recognize components of the basal lamina present in the synaptic cleft that accumulate upon synapse formation (Sanes et al., 1978; Chiu and Sanes, 1984; Powell et al., 1984; Rieger et al., 1984). As shown in this and previous studies, N-CAM appears to be one of the key molecules involved in cell-cell interactions in the neuromuscular system as shown by its binding characteristics and its presence at critical sites determined by such interactions including the node of Ranvier, the areas of contact between terminal Schwann cell and nerve terminal, and the neuromuscular junction. Given the presence of the thick 50-nm layer of basal lamina in the synaptic cleft, direct homophilic interactions between axonal N-CAM and muscle basal lamina N-CAM are topographically possible. The presence of N-CAM in muscle synaptic basal lamina provides evidence for a function of N-CAM in synapse formation and stabilization. The perturbation experiments described here suggest that, along with other molecules, N-CAM may act in key recognition events during formation and regeneration of the neuromuscular system.

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