Suppression of Terminal Axonal Sprouting at the Neuromuscular Junction by Monoclonal Antibodies against a Muscle-derived Antigen of 56,000 Daltons

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Abstract. After the partial denervation or paralysis of a muscle, the remaining motor axon terminals may sprout fine, neuritic processes (terminal sprouts) which escape the endplate region of the neuromuscular junction. We previously identified a muscle-derived, protein antigen of 56,000 daltons (56 kD) which plays a necessary role in terminal sprouting. A panel of monoclonal antibodies have been produced against the 56-kD antigen, some of which also partially suppress motor axon terminal sprouting. These monoclonal antibodies define at least two different epitopes upon the surface of the antigen, one of which is necessary for it to effect its biological role in vivo.

In response to partial denervation or paralysis, the motor axon terminals remaining within a muscle frequently sprout fine neuritic processes that escape the muscle fiber endplate (7). Such sprouts are called either terminal or ultra-terminal sprouts, depending on the author. Terminal sprouts can reinnervate denervated muscle fiber endplates or induce de novo neuromuscular synapses. Muscle is believed to provide a signal which is important for induction of terminal sprouting, but the molecular nature of the signal is not known. Whether released by the muscle (if soluble), exposed on the muscle surface, or inserted in the basal lamina (if substrate bound), the signal should be induced by denervation or muscle paralysis. Muscle activity per se appears to control the expression of the signal for terminal sprouting, as terminal sprouting is suppressed by direct electrical stimulation of denervated or paralyzed muscle (4, 5). After partial denervation, a second type of sprouting is also observed in muscle. The remaining motor axons will also sprout new processes from nodes of Ranvier, although nodal sprouting is not suppressed by direct electrical stimulation of denervated or paralyzed muscle (4, 5). After partial denervation, a second type of sprouting is also observed in muscle. The remaining motor axons will also sprout new processes from nodes of Ranvier, although nodal sprouting is not suppressed by direct electrical stimulation of denervated or paralyzed muscle (5, 23). Thus, the molecular requirements for nodal sprouting must differ in part from those responsible for terminal sprouting. In man, it is nodal sprouting that is primarily responsible for reinnervation of muscle in disease or after trauma to the nerve (11, 41).

The final test of a candidate “terminal sprouting factor” must be that the agent alone is sufficient to induce motor axon terminal sprouting in vivo. We do not know if such an agent should also play a role in nodal sprouting. We have pursued an immunological approach in an attempt to identify factors produced by denervated or inactive muscle that might be necessary for motor axon terminal sprouting. We have prepared a series of rabbit antisera against components of denervated muscle-conditioned medium (16). By fractionation of the conditioned medium (CM), and by producing successive rounds of antisera, we succeeded in producing antisera of limited specificity that suppressed axon terminal sprouting at the neuromuscular junction. The antisera pointed to a protein of 56,000 daltons (56 kD) as the effective antigen in the immunizations. An independent line of evidence which further implicated the 56-kD antigen in terminal sprouting was provided by work with clinical material. Sera from several neurological patients with a degenerative disease of the motor neuron, amyotrophic lateral sclerosis (ALS), were shown to suppress terminal sprouting in the in vivo assay and to contain antibodies reactive with the 56-kD antigen (17). Since none of the rabbit antisera were monospecific, it became necessary to produce monoclonal antibodies against the 56-kD antigen in order to demonstrate that it was indeed the effective antigen in the immunization.

We now describe the generation of a panel of monoclonal antibodies (mAb's) against the 56-kD antigen and show that a subset of the mAb suppresses motor axon terminal sprouting. The results obtained with the immunological reagents indicate that the 56-kD antigen plays a necessary role in motor axon terminal sprouting.

Materials and Methods

Preparation of Immunogen

Adult Sprague-Dawley rat hemidiaphragms were denervated by cutting the phrenic nerve intrathoracically and cultured as described previously (16). The

1. Abbreviations used in this paper: ALS, amyotrophic lateral sclerosis; AS, ammonium sulfate; CM, conditioned medium; ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody; N-CAM, neural cell adhesion molecule; NGF, nerve growth factor; RIA, radioimmune assay.
CM was stored frozen at −20°C until used. Before precipitation with solid ammonium sulfate (AS), the thawed CM was buffered at pH 6.5 by addition of 1:10 vol of 0.5 M NaPi, 50 mM Na EDTA, 5 mM Na EGTA, 100 μM NaPi, and 0.1 M phenylmethylsulfonyl fluoride, and centrifuged for 30 min at 13,000 rpm in a Sorvall GSA rotor at 4°C. The CM was precipitated initially at 4°C by addition of AS to 190 g/liter initial volume. After the first precipitate was collected by centrifugation, additional AS was added to bring the solution to 380 g of AS per liter of initial volume. The 190–380 g AS/liter precipitate was collected by centrifugation and then dialyzed against two changes of 4 liters 20 mM NaPi (pH 7.5). The dialyzate was passed over a 1.6 × 15 cm column of DE52 (Whatman Inc., Clinical, NJ) and eluted sequentially with steps of increasing NaCl concentration in 20 mM NaPi (pH 7.5) as indicated in Fig. 1.

**Immunization and Hybridoma Fusion**

Male, 150–200-g Lewis rats were immunized at 14-d intervals with 50 μg protein of the 0.25 M NaCl DE52 fraction. The antigen was emulsified in Freund’s complete adjuvant and then were screened with hypoxanthine-aminopterine-thymidine and then were screened at multiple sites across the animal’s back. The serum antibody response was assayed 8 wk after the initial immunization by immunoblotting. Three more injections of antigen in phosphate-buffered saline (PBS) were delivered at 1-d intervals immediately before killing, at which time the mechanically dispersed spleen cells were centrifuged onto a Ficol-Hypaque cushion containing 0.05% Tween 20. Ascites fluid was prepared in nude mice (nu/ nu) primed with 0.1 ml pristane injected intraperitoneally 10 d before innoculation with 2–4 × 106 hybridoma cells. Ochterlony analysis of rat immunoglobulin subclass was performed with Miles subclass-specific anti-rat immunoglobulin antisera.

**Results**

**Partial Purification of the 56-kD Antigen by Anion-Exchange Chromatography**

We had available a rabbit antiserum, D10, that was raised against a preparation of the 56-kD antigen that had been partially purified by SDS PAGE. D10 suppresses motor axon terminal sprouting as described previously (16). Immunoblotting with D10 was used to follow the 56-kD antigen through Table I. Immunoglobulin Subclass and Suppression of Motor Axon Terminal Sprouting by Anti-56-kD Hybridoma Ascites Fluid

| mAb               | Class | Percent terminals | Percent inhibition |
|-------------------|-------|-------------------|--------------------|
| Toxin 1           |       | 70 ± 8            | 0 ± 8              |
| Toxin 2           |       | 74 ± 4            | 0 ± 8              |
| PBS               |       | 73 ± 5            | 0 ± 7              |
| B5                | IgG2a | 51 ± 6            | 29 ± 8             |
| B70.10            | IgM   | 57 ± 6            | 20 ± 8             |
| B86.3             | IgG2a | 58 ± 8            | 20 ± 11            |
| B109.9            | IgM   | 36 ± 8            | 50 ± 11            |
| B130.6            | IgG2b | 35 ± 8            | 51 ± 11            |
| B135              | IgM   | 36 ± 5            | 50 ± 7             |
| B216              | IgG2b |                   | 0 ± 8              |

* Determined by Ochterlony double-diffusion assay with rat immunoglobulin subclass specific antisera.

* Calculated from the average percent terminals sprouting for the toxin 1 and toxin 2 groups.

B216 ascites fluid was tested in the experimental series of Table II.
a simple fractionation of the denervated diaphragm-conditioned medium by fractional precipitation with AS and elution from DEAE-cellulose with steps of increasing ionic strength. The 56-kD antigen precipitated with a cut of 190–380 g of AS per liter, was retained by DE52, and eluted with a step from 0.15–0.25 M NaCl (Fig. 1). The 56-kD antigen remains a minor constituent of the immunogen (as illustrated in Fig. 5, left). Some 56-kD antigen remained soluble in 380 g of AS per liter, so in later experiments the amount of AS added was increased to 560 g/liter.

**Congenic Immunization Produces Antisera of Limited Specificity**

We immunized three adult male Lewis rats with the 0.25 M NaCl DE52 fraction obtained from Sprague-Dawley rat denervated diaphragm-conditioned medium. If the Lewis rats responded to the congenic antigen, we felt that the genetic relatedness of the two strains would limit the animals' immune response to serum proteins that contaminated the immunogen. The two rats that responded to the 56-kD antigen produced highly specific antisera (designated R2 and R3) despite the relative impurity of the antigen in the 0.25 M NaCl DE52 fraction (Fig. 2). The spleen from R2 was used in the first fusion, and that from R3 in the second fusion.

**Figure 1.** Partial purification of the 56-kD antigen by anion-exchange chromatography. Immunoblot analysis of the denervated diaphragm-conditioned medium (CM), fractions from the ammonium sulfate (AS) precipitation, and fractions from the DE52 anion-exchange chromatography (DE52). 50 µg total protein was analyzed in each lane. The samples were fractionated by SDS-PAGE, transferred to nitrocellulose, and the transfers were reacted with D10 (1:50 dilution) followed by 125I-labeled protein A to detect bound rabbit IgG. The 56-kD antigen (arrow) was detected in the denervated diaphragm-conditioned medium, the 190–380 g/liter and 380 g/liter AS fractions, and in the 0.25 M NaCl and 0.35 M NaCl eluates of the DE52 column. The antigen remains a minor constituent of the 0.25 M NaCl DE52 fraction as shown in Fig. 5 (left).

**Figure 2.** Congenic immunization elicits antisera of limited specificity as assayed by immunoblotting. Three rats were immunized with the 0.25 M NaCl DE52 eluate. A sample of the immunogen (100 µg protein) was fractionated by SDS-PAGE and transferred to nitrocellulose. The replicate transfers were reacted with sera from the immunized rats (R1–R3) as indicated at a dilution of either 1:50 or 1:100. Bound rat Ig was detected with I-125-labeled goat anti-rat IgG (Cappel Laboratories, Cochranville, PA). The sera obtained from R2 and R3 reacted highly specifically with an antigen of 56 kD (arrow) at a dilution of 1:100. A representative protein stain of the 0.25 M NaCl DE52 immunogen is illustrated in Fig. 5 (left).
On the left, the figure compares the general appearance of R1 and R3. Immunization of R3 elicited an antiserum of high titer against an antigen of 56 kD (Fig. 2). When the photo was taken, R1 weighed 440 g while R3 weighed 273 g. R3 was markedly thinner than R1 along its flanks and throughout its hind legs. R3’s tail noticeably kinked, and discoloration by fecal material of both the fur on top of its head and its tail indicated impaired grooming. R3 was noticeably weak in forepaw grip (tested by pulling the rat over a wire grid) and easily exhausted. In the center photo, R3 has been pulled across a flat surface by its tail and then released. It was slow to resume a normal body posture with both hind and fore legs tucked beneath its body. In c, histology of the gastrocnemius muscle from the affected hind limb is normal. The muscle was frozen sectioned at 15 μm and stained with hematoxylin and eosin. Had muscle denervation been present, atrophic muscle fibers of small diameter should have been visible.

By 4–6 mo into the immunization, both R2 and R3 showed loss of weight, apparent muscle wasting, and motor deficits, while R1, a rat that developed a weak response to the 56-kD antigen, remained healthy. The forepaw grip of R2 and R3 seemed weak compared to R1 or unimmunized rats, and when their back legs were extended, R2 and R3 showed little tendency to resume a normal posture (Fig. 3). Superficially, the appearance of the rats corresponded to what might be expected in an animal model of ALS, however, no signs of muscle denervation were noted in the gastrocnemius muscle from one of the affected hindlimbs of R3, nor was loss of ventral horn neurons noted in the lumbar spinal cord or medulla. (The histology was performed by K. Steffanson, M.D., Department of Neurology and Pathology, The University of Chicago.)

Fusion and Screening of Hybridomas for mAB against the 56-kD Antigen

Blast cells from an immunized rat’s spleen were collected by centrifugation onto a cushion of Ficoll-Hypaque (1.094 g/ml) and were then fused with mouse SP2/0 myeloma cells by using polyethylene glycol. After selection of the hybrids in hypoxanthine-aminopterin-thymidine, we performed a two-step screen of the antibody secreted by the hybridoma lines. Since the animals’ response to irrelevant antigens present in the immunogen was limited, we initially screened the hybridoma supernatants for binding in an ELISA with the 0.25 M NaC1 DE52 fraction. As we did not have purified 56-kD antigen available, we could not use the ELISA to demonstrate specificity. To select specific, anti-56-kD mAb, ELISA-positive hybridomas were grown up to flasks and then the supernatants were rescreened by immunoblotting.

In two fusions of spleen cells obtained from R2 and R3 with mouse SP2/0 myeloma cells, we generated 1118 hybridoma lines that were screened by ELISA. 188 of them were positive in the ELISA. Of these, 109 hybridomas were re-screened by immunoblotting (79 of 109 lines from the first fusion were discarded) and seven hybridomas were obtained that were positive by immunoblotting for anti-56-kD mAb. All seven were obtained in the second fusion with spleen cells from R3.

To establish the ELISA, we compared binding of antibody in immune serum from R2 and R3 with preimmune sera to microtiter wells coated with varying concentrations of the 0.25 M NaCl DES2 fraction. As the wells were coated with increasing amounts of antigen (Fig. 4, top), both R2 and R3 showed greater binding than preimmune serum. A concentration of 8 μg protein/ml of the immunogen was chosen for coating the microtiter wells for the ELISA screen.

In the screen, wells varied from twice background to saturation of the ELISA. After anti-56-kD mAb's were selected by immunoblotting, we sought to validate retrospectively the initial ELISA screen. Serial dilutions of the anti-56-kD hybridoma supernatants were reacted with wells coated with 8 μg protein per milliliter of the 0.25 M NaCl DES2 fraction. All anti-56-kD mAb tested reacted positively in the ELISA (Fig. 4b).

Most of the hybridoma supernatants failed to react with any antigen on the nitrocellulose transfers. Immunoblotting with the panel of anti-56-kD mAb was illustrated in Fig. 5. Each reacts with a band of 56 kD.

Four of the seven hybridomas were subcloned in soft-agarose (B86.3, B70.10, B109.9, and B130.6). Ascites fluid was prepared from each of the cell lines by passage in nude mice. The subclass of antibody being secreted by each line was characterized by immunodiffusion in Ochterlony plates.
Figure 4. ELISA with rat sera and anti-56-kD hybridoma culture supernatants. In the top panel, the microtiter wells were coated with serially decreasing concentrations of the 0.25 M NaCl DE52 eluate and then reacted with the immune sera obtained from R2 and R3 and with the preimmune serum from R2 (preR2). Both R2 and R3 reacted more strongly than the preimmune serum in the ELISA. Hybridoma culture supernatants were initially screened with the ELISA and then rescreened by immunoblotting as in Fig. 5. Of 82 hybridoma supernatants which were positive in the ELISA, seven were found to contain immunoglobulin that reacted with an antigen of 56 kD on nitrocellulose transfers of the 0.25 M NaCl DE52 eluate as fractionated by SDS PAGE (Fig. 5). In the bottom panel, serial dilutions of the anti-56-kD hybridoma culture supernatants were reacted with microtiter wells coated with a constant amount of the 0.25 M NaCl DE52 eluate at 8 μg protein/ml. All showed good binding in the ELISA. Bound rat Ig was detected with biotinylated goat anti-rat IgG followed by strepavidin: biotinylated horseradish peroxidase conjugate. The wells were developed with o-phenylenediamine and optical density at 450 nm (OD450) was determined with a Titertek dual wavelength ELISA plate reader.

**Suppression of Motor Axon Terminal Sprouting at the Neuromuscular Junction by the Anti-56-kD mAb**

The gluteus is a thin sheet of muscle in the mouse and only 3–5 muscle fibers thick through its midsection (22). The muscle sheet is innervated by two branches of the gluteal nerve which enter the muscle at its posterior and anterior margins and join across its center. The number of terminals that developed ultraterminal sprouts was quantitated within the thin, central region of the muscle (16). Botulinum toxin paralyzes a muscle by preventing neurotransmitter release (acetylcholine) from the motor axon nerve terminal. We injected the toxin subcutaneously across the surface of the muscle. The dose chosen was nonlethal and caused 74 ± 8% of the gluteal nerve terminals to develop ultraterminal sprouts. Histological examination of the muscle was conducted “blind,” at least 100 terminals per muscle were scored, and generally 3–4 treatment groups of eight mice each were coded randomly and read as a group before breaking the code. The zinc iodide-osmium tetroxide stain clearly demonstrates the unmyelinated portion of the preterminal axon and details of the nerve terminal morphology. Any fine process growing from the normally smooth contour of the nerve terminal that “escaped” the endplate was classified as an ultraterminal sprout (Fig. 6). Indices of terminal remodeling within the endplate region such as the size of the endplate or the number of branches made by the claw-like terminal within the endplate region were not quantitated (see reference 30). The latter are suppressed by anti-acetylcholine receptor antibody in myasthenic sera or by alpha-bungarotoxin, but myasthenic sera do not suppress botulinum toxin-induced ultraterminal sprouting in the mouse.

A pair of mAb's, B130.6 and B216, exemplify the results obtained with the in vivo assay. B130.6 suppresses terminal sprouting by 50%, whereas B216 has no effect in the assay. Both of the mAb's are of the same subclass, IgG2b, as shown by Ochterlony assay and by the molecular mass of their heavy chains (55-kD), which is also characteristic of rat IgG2b (32). The antigen-binding domains of the two mAb's are distinct as their light chains differ substantially in molecular mass (Fig. 7). Since the two mAb's share the same heavy chain effector domains, the difference in their ability to suppress terminal sprouting must reside in the antigen-binding domain. Either each mAb recognizes a different epitope on the 56-kD antigen, in which case B130.6 must define a domain of the 56-kD protein which is functionally important for terminal sprouting, or both recognize the same epitope but the affinity of B216 for that epitope is much less than that of B130.6. To address this question, we determined if the two mAb's could form a sandwich with the 56-kD antigen in a two-site RIA. To perform the RIA, mAb's from the two dilutions of the anti-56-kD hybridoma culture supernatants were reacted with microtiter wells coated with a constant amount of the 0.25 M NaCl DE52 eluate at 8 μg protein/ml. All showed good binding in the ELISA. Bound rat Ig was detected with biotinylated goat anti-rat IgG followed by strepavidin: biotinylated horseradish peroxidase conjugate. The wells were developed with o-phenylenediamine and optical density at 450 nm (OD450) was determined with a Titertek dual wavelength ELISA plate reader.
Figure 5. Immunoblot of anti-56-kD mAb. The 0.25 M NaCl DE52 eluate was fractionated by SDS PAGE, transferred to nitrocellulose, and then transfers of individual lanes were reacted with different immunochemical reagents as indicated. The results of three separate experiments are illustrated. (Left) Replicate transfers have been stained for protein with Amido black or reacted with D10. D10 identifies an antigen of 56 kD (arrow). (Center) Replicate transfers have been incubated without monoclonal antibody (no mAb), with ascites fluid prepared from the SP2/0 myeloma fusion partner, or with MCB-1, an anti-NGF rat IgG2b mAb (42), at 10 µg IgG2b per milliliter as controls, or with B109.9 or B216 ascites fluid (1:400). B109.9 and B216 identify an antigen of 56 kD (arrow). (Right) Replicate transfers have been reacted with ascites fluids prepared from the panel of mAb. All react with an antigen of 56 kD (arrow). Reactivity with an antigen of 67 kD is also observed, as well as with some low molecular mass material. The antigen of 67 kD is thought to be rat serum albumin. Because the preparation of the 56-kD antigen was not highly purified, serum protein which was washed out of the cultured hemidiaphragm provides the bulk of contaminating protein. Rat IgG2b was variably present in the 0.25 M NaCl DE52 fraction and proved to be especially troublesome as its heavy chain has a relative mobility on SDS PAGE (55 kD) which is similar to that of the 56-kD antigen.

Figure 6. Histology of zinc iodide-osmium tetroxide-stained motor axon terminals in normal muscle (normal) or in muscle paralyzed with botulinum toxin and exposed to B216 ascites fluid (B216) or B130.6 ascites fluid (B130.6). Terminal axonal sprouting was induced by botulinum toxin; the mice were then injected with 0.1 ml of ascites fluid subcutaneously over the botulinum-intoxicated gluteus muscle on each of the next 6 d, and the muscle was excised and stained with zinc iodide-osmium tetroxide 7 d after injection with toxin. B130.6 maximally suppressed terminal sprouting by ~50% while terminal sprouting was unaffected by exposure to B216. With the exception that fewer terminals had sprouts in muscles treated with B130.6 as compared to B216, no other histological differences were noted. No sprouts are seen in the normal muscle. Terminals with sprouts escaping the endplate region in the botulinum-treated muscles are indicated by the arrows.

hybridoma lines were internally labeled with [35S]methionine. Plastic microtiter wells were coated with unlabeled B130.6 or B216 in ascites fluid, incubated with serial dilutions of the immunogen which contained the 56-kD antigen, and then were incubated with one of the internally labeled mAb's. The results obtained were consistent with recognition by the mAb of two different epitopes on the 56-kD antigen, as illustrated in Fig. 7.

Are There Two Signals for Terminal Sprouting?
The maximum suppression of terminal sprouting achieved was obtained with ascites fluid prepared from B130.6, B109.9,
and B135 (the latter two are IgM's). Each of the three suppressed sprouting by 50% (Table I). B109.9 IgM, when purified from ascites fluid, also suppressed sprouting by 50% (Fig. 8 and Table II). Since none of the mAb suppressed terminal sprouting completely, we considered two hypotheses. Perhaps there are two or more signals for sprouting, only one of which is blocked by the mAb, or perhaps there is only one signal, but it is blocked incompletely.

To begin to distinguish between the two hypotheses, we determined a titration curve for the suppression of terminal sprouting by B135. The trend of the relationship between the amount of B135 injected and the suppression obtained (Fig. 8) suggests that the mAb is unlikely to block terminal sprouting completely. A 10-fold increase in the amount of B135 injected increases the suppression obtained by only twofold. Although it is not clear that the titration curve has reached a plateau, despite further increases in the amount of mAb injected, it appears that the degree of suppression obtained should be asymptotic with a value between 50 and 60% suppression.

Lack of complete inhibition might be due to poor penetration of the mAb reagent into the muscle or into the cleft of the neuromuscular junction; that remains a possibility, although the two IgM's (B135 and B109.9) suppress terminal sprouting to the same extent as the IgG2b (B130.6) despite the substantial differences in their size. The incomplete inhibition obtained may also be simply due to rapid clearance of the injected mAb from the gluteus muscle such that the concentration of mAb near the endplates is not as great as believed. We demonstrated previously that immune sera must be delivered over the surface of the paralyzed muscle; delivery via the circulation after intraperitoneal injection was not sufficient to prevent terminal sprouting (16). That observation indicates that the concentration of the injected antibody must achieve a sufficient level within the paralyzed muscle in order for suppression of terminal sprouting to occur.

**Discussion**

Results obtained with the anti-56-kD mAb confirm and extend our previous work with antisera from rabbits immunized with partially purified 56-kD antigen and with the sera from patients with ALS (16, 17). Suppression of botulinum toxin-induced motor axon terminal sprouting was obtained with certain of the anti-56-kD mAb, and furthermore, the results obtained with the pair of IgG2b, B130.6 and B216, indicate that only certain epitopes upon the 56-kD antigen are functionally important in the gluteus muscle bioassay. The result again underscores our observation that the suppression of terminal sprouting that we obtain with the various immunochemical reagents is highly specific. We previously demonstrated that polyclonal rabbit antisera directed against dozens of other antigens present in the denervated muscle-conditioned medium have no effect on terminal axonal sprouting; Pestronk and Drachman (30) have similarly shown that myasthenic sera directed against the acetylcholine receptor do not suppress ultraterminal sprouting in mice. Thus, the suppression of sprouting that we obtain is unlikely to be
due to nonspecific deposition of immune complexes within the synaptic cleft or upon the muscle fiber surface. Instead, the data argue that the 56-kD antigen, and indeed, only a limited domain upon the surface of the protein, is functionally important for motor axon terminal sprouting.

Perturbation of axon terminal sprouting with the anti-56-kD mAb does not reveal the biological role of the antigen in vivo. Muscle-conditioned medium, our source of the 56-kD antigen, has been reported to contain a number of factors that are active upon cultured spinal neurons (1, 3, 10, 13, 20, 39). Among these are factors that influence neuron attachment to and extension of neurites upon the culture substrate, factors that influence neuron survival, and a factor or factors that influence neuronal expression of choline acetyltransferase (40). The in vivo experiments do not allow us to identify the 56-kD antigen with one or another of these biological activities.

Of the soluble, neuroactive survival factors, nerve growth factor (NGF) remains the best characterized (25, 40). NGF has both trophic and tropic activity. Sympathetic neurons require NGF for growth and survival in culture, and exogenous NGF can be shown to increase the survival of sympathetic neurons in vivo (25, 40). Two experiments demonstrate the tropic activity of NGF. First, the growing tips of neuritic processes from sensory neurons will orient towards local increases in NGF concentration in vitro (15), and second, peripheral sympathetic afferents will grow into a site of exogenous NGF infusion within the brain (28).

With the realization that only certain populations of neurons respond to NGF grew the belief that NGF might be only one of many neuronal growth factors. Destruction of sympathetic neurons in vivo with antisera against NGF (the most persuasive demonstration that NGF functions in vivo; see reference 26) further suggested that target tissues might supply trophic factors which specifically maintained their own innervation. Thus, the death of spinal cord motor neurons that results from limb bud extirpation (19), and the trophic effects of culture medium conditioned by muscle for dissociated spinal neurons or spinal cord explants cultured in vitro (1, 3, 10, 13, 20, 39) suggested the existence of a muscle-derived survival factor for spinal motor neurons (35-38). Several groups have provided evidence that these activities are modulated by muscle denervation (21, 29, 37). Motor axon terminal sprouting at the neuromuscular junction, a local response to partial denervation in muscle (6, 36), similarly seems to depend upon a factor derived from muscle, and indeed, can be viewed as a tropic action of the hypothesized motor neuron growth factor (7, 35, 38). Our preliminary results associate the 56-kD antigen with a spinal neuron survival activity assayed in culture (18).

Most studies of ultraterminal sprouting in vivo indicate that the signal for sprouting travels at most a few muscle fiber diameters (2, 6, 36). Since the biological response is the outgrowth of new neuritic processes, perhaps factors which directly influence neurite extension in culture assays will be most relevant to the situation in vivo. Two components of the muscle surface or extracellular matrix, N-CAM (neural cell adhesion molecule) and laminin (9, 34), respectively, are active on spinal neurons in culture (8, 24, 27, 31). No evidence that laminin is regulated by denervation has yet been published, whereas N-CAM shows a dramatic redistribution upon the muscle fiber surface after denervation (9). In normal muscle, N-CAM is localized to the endplate region. Denervation or paralysis induces appearance of N-CAM across the muscle fiber surface and accumulation within interstitial spaces. Upon reinnervation, extrajunctional N-CAM expression is suppressed. Since F(ab)2 fragments of anti-N-CAM antibody disrupt adhesion of neurites from spinal cord explants to skeletal myotubes in culture (14, 33), modulation of N-CAM expression may be highly important for ultraterminal sprouting. The hypothesis should be testable in vivo by application of F(ab)2 fragments of anti-mammalian N-CAM antibody to a preparation such as the botulinum-intoxicated mouse gluteus muscle.

Table II. Suppression of Motor Axon Terminal Sprouting by Purified mAb

| mAb          | Percent terminals sprouting | Percent inhibition* |
|--------------|----------------------------|---------------------|
| Toxin 3      | 62 ± 4                     | 0 ± 8               |
| Toxin 4      | 60 ± 8                     | 2 ± 13              |
| B86.3 IgG2a  | 39 ± 6                     | 36 ± 10             |
| B109.9 IgM   | 31 ± 4                     | 49 ± 6              |
| Rat serum IgG2a | 56 ± 5                  | 8 ± 9               |
| B216 ascites | 61 ± 5                     | 0 ± 8               |

*Calculated from the average of percent terminals sprouting for the toxin 3 and toxin 4 groups.
The present study does not resolve the question of whether the 56-kD antigen is a sprouting factor, and therefore, sufficient in and of itself to induce terminal axonal sprouting, or whether it is a necessary co-factor for sprouting. Complete suppression was not achieved with any of the immunoneurochemical reagents, possibly due to limitations of the in vivo assay, such as incomplete penetration of the mAb into the muscle or variable affinity of the mAb. We feel that the most likely alternative, however, is that there are a number of requirements for motor axon terminal sprouting among which we would include the soluble, muscle-derived antigen that is identified by the monoclonal antibodies described in this report.

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References

1. Bennet, M. R., K. Lai, and V. Nurcombe. 1980. Identification of embryonic motoneurons in vitro: their survival is dependent on skeletal muscle. Brain Res. 190:537–542.

2. Betz, W. J., J. H. Caldwell, and R. R. Ribchester. 1980. Sprouting of active nerve terminals in partially inactive muscles of the rat. J. Physiol. 303:281–297.

3. Brookes, N., D. R. Burt, A. M. Goldberg, and G. G. Bierkamper. 1980. The influence of muscle-conditioned medium on cholinergic maturation in spinal cord cell cultures. Brain Res. 186:474–479.

4. Brown, M. C., and R. L. Holland. 1979. A central role for denervated tissues in causing nerve sprouting. Nature (Lond.) 282:724–726.

5. Brown, M. C., R. L. Holland, and R. Irons. 1980. Nodal and terminal sprouting from motor nerves in fast and slow muscles of the mouse. J. Physiol. 306:493–510.

6. Brown, M. C., R. L. Holland, W. G. Hopkins, and R. J. Keynes. 1980. An assessment of the spread of the signal for terminal sprouting within and between muscles. Brain Res. 210:145–151.

7. Brown, M. C., R. L. Holland, and W. G. Hopkins. 1981. Motor nerve sprouting. Annu. Rev. Neurosci. 4:17–42.

8. Calof, A. L., and L. F. Reichardt. 1984. Motoneurons purified by cell sorting respond to two distinct activities in myotube conditioned medium. Dev. Biol. 106:194–210.

9. Covault, J., and J. R. Sanes. 1985. Neural cell adhesion molecule (NCAM) accumulates in denervated and paralyzed skeletal muscles. Proc. Natl. Acad. Sci. USA. 82:4544–4548.

10. Dribin, L. B., and J. N. Barrett. 1982. Conditioned medium enhances neuritic outgrowth from rat spinal cord explants. Dev. Biol. 74:184–195.

11. Edds, Jr., M. V. 1953. Collateral nerve regeneration. Q. Rev. Biol. 28:260–276.

12. Galfré, G., and C. Milstein. 1981. Preparation of monoclonal antibodies: strategies and procedures. Methods Enzymol. 73:1–45.

13. Gilmer, Jr., E. L., J. H. Neale, P. N. Bullock, B. K. Schrier, and P. G. Nelson. 1977. Choline acetyltransferase: activity of spinal cord cell cultures increased by co-culture with muscle and by muscle conditioned medium. J. Cell Biol. 74:16–29.

14. Grumet, M. U., R. Sheuer, and G. M. Edelman. 1982. Neural cell adhesion molecule is on embryonic muscle cells and mediates adhesion to nerve cells in vitro. Nature (Lond.) 295:693–695.

15. Gunderson, R. W., and J. N. Barret. 1979. Neuronal chemotaxis: chick dorsal-root axons turn toward high concentrations of nerve growth factor. Science (Wash. DC). 206:1079–1080.

16. Gurney, M. E. 1984. Suppression of sprouting at the neuromuscular junction by immune sera. Nature (Lond.) 307:546–548.

17. Gurney, M. E., A. C. Belton, N. Cashman, and J. P. Antel. 1984. Inhibition of terminal axonal sprouting by serum from patients with amyotrophic lateral sclerosis. N. Engl. J. Med. 311:933–939.

18. Gurney, M. E., and B. R. Apatoft. 1984. Activity of a muscle-derived growth factor for spinal neurons in vitro and in vivo. Soc. Neurosci. Abstr. 10:1051.

19. Hamburger, V. 1958. Regression versus peripheral control of differentiation in motor hypoplasia. Am. J. Anat. 102:365–410.

20. Henderson, C. E., M. Hutchet, and J-P. Changeux. 1981. Neurite outgrowth from embryonic chicken spinal nerves is promoted by medium conditioned by muscle cells. Proc. Natl. Acad. Sci. USA. 78:2625–2629.

21. Henderson, C. E., M. Hutchet, and J-P. Changeux. 1983. Denervation increases a neurite-promoting activity in extracts of skeletal muscle. Nature (Lond.) 302:609–611.

22. Hopkins, W. G., and J. R. Slack. 1981. The sequential development of nodal sprouts in mouse muscles in response to nerve degeneration. J. Neurocytol. 10:537–556.

23. Ironont, R., M. C. Brown, and R. L. Holland. 1978. Stimuli to intra- muscular nerve growth. Brain Res. 156:351–354.

24. Lander, A. D., D. K. Fuji, and L. F. Reichardt. 1985. Laminin is associated with the "neurite outgrowth-promoting factors" found in conditioned medium. Proc. Natl. Acad. Sci. USA. 82:2183–2187.

25. Levi-Montalcini, R. 1966. The nerve growth factor: its mode of action on sensory and sympathetic cells. Harvey Lect. 60:217–259.

26. Levi-Montalcini, R., and B. Booker. 1961. Destruction of the sympathetic ganglia in mammals by an antisemun to a nerve-growth hormone. Proc. Natl. Acad. Sci. USA. 46:384–391.

27. Manthorpe, M., E. Engvall, E. Ruoslahti, F. M. Longo, G. E. Davis, and S. Varon. 1983. Laminin promotes neurite regeneration from cultured peripheral and central neurons. J. Cell Biol. 97:1882–1890.

28. Menesini-Chen, M. G., J. S. Chen, and R. Levi-Montalcini. 1978. Sympathetic nerve fibers ingrowth in the central nervous system of neonatal rodent upon intracerebral NGF injections. Arch. Ital. Biol. 116:53–84.

29. Nurcombe, V., M. A. Hill, K. L. Eagle, and M. R. Bennett. 1984. Motor neuron survival and neuritic extension from spinal cord explants induced by factors released from denervated muscle. Brain Res. 291:19–28.

30. Pestronk, A., and D. B. Drachman. 1985. Motor nerve terminal outgrowth and acetylcholine receptors: inhibition of terminal outgrowth by alpha-bungarotoxin and anti-acetylcholine receptor antibody. J. Neurosci. 5:751–758.

31. Rogers, S. L., P. C. Letourneau, S. L. Palm, J. B. McCarthy, and L. T. Furch. 1983. Neurite extension by peripheral and central nervous system neurons in response to substratum-bound fibronectin and laminin. Dev. Biol. 82:728–220.

32. Rousseaux, J., M. T. Pique, H. Bazin, and G. Buette. 1981. Rat IgG subclasses: differences in affinity to protein A-Sepharose. Mol. Immunol. 18:639–645.

33. Rutishauser, U., M. Grumet, and G. M. Edelman. 1983. Neural cell adhesion molecule mediates initial interactions between spinal cord neurons and muscle cells in culture. J. Cell Biol. 97:145–152.

34. Sanes, J. R. 1984. Laminin, fibronectin, and collagen in synaptic and extrasynaptic portions of muscle fiber basement membrane. J. Cell Biol. 93:442–451.

35. Sanes, J. R. 1984. More nerve growth factors? Nature (Lond.) 307:500.

36. Slack, J. R., and S. Pockett. 1981. Terminal sprouting is a local response to a local stimulus. Brain Res. 217:368–374.

37. Slack, J. R., and S. Pockett. 1982. Motor neurotrophic factor in denervated adult skeletal muscle. Brain Res. 247:138–140.

38. Slack, J. R., W. G. Hopkins, and S. Pockett. 1983. Evidence for a motor nerve growth factor. Muscle & Nerve. 6:243–252.

39. Tanaka, H., M. Sakai, and K. Obata. 1982. Effects of serum, tissue extract, conditioned medium, and culture substrate on neurite appearance from spinal cord explants of chick embryo. Dev. Brain Res. 4:303–312.

40. Thoenen, H., and D. Edgar. 1985. Neurotrophic factors. Science (Wash. DC). 229:238–242.

41. Wohlfart, G. 1958. Collateral nerve regeneration in partially denervated muscles. Neurology. 8:175–180.

42. Zimmerman, A., A. Suter, and E. M. Shooter. 1981. Monoclonal antibodies against B-nerve growth factor and their effects on receptor binding and biological activity. Proc. Natl. Acad. Sci. USA 78:4611–4615.