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 ultraterminal 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 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),[1] 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 medum; 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 μM Na EDTA, 5 mM Na EGTA, 100 μM leupeptin, 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., Clifton, 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 for the first two injections and in incomplete adjuvant for the third immunization. The emulsion was injected intradermally 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 fractionated by centrifugation onto a Ficoll-Hypaque cushion of 1.094 g/ml density, and then fused at a ratio of 10:1 with mouse SP2/0 mouse myeloma cells using polyethylene glycol (12). 1 d after fusion, the cells were dispersed into fourteen 96-well plates. The microcultures were subjected to selection with hypoxanthine-aminopterin-thymidine and then were screened by an enzyme-linked immunosorbent assay (ELISA) as described below.

Some hybridoma lines were subcloned in soft agarose on a feeder layer of gamma-irradiated spleen cells. Ascites fluid was prepared in nude mice (nu/nu) primed with 0.1 ml pristane injected intraperitoneally 10 d before innoculation with 2–4 × 10^6 hybridoma cells. Ochterlony analysis of rat immunoglobulin subclass was performed with Miles subclass-specific anti-rat immunoglobulin antisera.

B109.9, an IgM, was purified from ascites fluid by precipitation with 280 g/liter of AS, eluted from DE52 with a gradient of 0–0.3 M NaCl in 20 mM NaPi (pH 7.5); gel filtration over Sepharose CL 4B (Pharmacia Fine Chemicals, Piscataway, NJ) followed. B86.3, an IgG2a, was purified from ascites fluid by precipitation with 280 g per liter of AS followed by chromatography over DE52 in 5 mM NaPi (pH 8.0). Purity of both preparations was verified by SDS PAGE.

Immunoblotting

SDS PAGE and immunoblotting was performed as described previously (16). Hybridoma supernatants were buffered by addition of 1:10 vol of 0.5 M NaPi (pH 7.5) and nitrocellulose strips were incubated with 5 ml of solution overnight at 4°C. Ascites fluids were used at a dilution of 1:400 in 3% bovine serum albumin (BSA), PBS, 0.05% Tween 20. Bound rat immunoglobulin was detected with rabbit anti-rat IgG (Miles Laboratories, Inc., Elkhart, IN) used at a dilution of 1:2,000 which was then reacted with ^{125}I-labeled protein A (10^6 cpm/μg; 1.5 × 10^5 cpm/ml diluent). Autoradiography against XAR5 film was performed overnight at −70°C. Unreacted sites on the nitrocellulose were blocked with 3% gelatin in PBS and all subsequent buffer solutions contained 0.05% Tween 20.

ELISA

Flexible round-bottom micro ELISA plates (Dynatech Laboratories, Inc., Alexandria, VA) were coated overnight at 4°C with 50 μl of the 0.25 M NaCl DE52 fraction diluted to 8 μg/ml in 150 mM sodium bicarbonate buffer (pH 9.6). The wells were blocked with 0.1% BSA in the bicarbonate buffer for 1 h at 37°C, and then were reacted with 75 μl of hybridoma culture supernatant for 2 h at 37°C in an atmosphere of 5% CO_2. Bound immunoglobulin was detected with a biotinylated anti-rat IgG, avidin–horseradish peroxidase system (Vectastain) developed with *o*-phenylene diamine and hydrogen peroxide.

Two-Site Radioimmune Assay (RIA)

Microtiter wells were coated with either B130.6 ascites fluid at a dilution of 1:1,000 or with B216 ascites fluid at 1:5,000 in 150 mM bicarbonate buffer (pH 9.6) for 2 h at 37°C. After blocking unreacted plastic with 1% gelatin in PBS for 1 h at 37°C, the wells were incubated with serial dilutions of the antigen in 3% BSA, 0.05% Tween 20 in PBS for 2 h at 37°C (as indicated in Fig. 7). B130.6 and B216 mAb's were internally labeled with [^{35}S]methionine by incubating the hybridoma cells (10^5 cells per microtiter culture well) in methionine-free RPMI 1640 containing 400 μCi/ml [^{35}S]methionine (1.3 Ci/mmol; Amersham Corp., Arlington Heights, IL; SJ.204) for 4 h at 37°C in an atmosphere of 8% CO_2. Incorporation of label into the mAb was evaluated by SDS PAGE (see Fig. 7). [^{35}S]-labeled mAb was partially purified from the culture supernatant by gel filtration over AcA44 (LKB Instruments, Inc., Gaithersburg, MD; column dimensions 1.1 × 18 cm) in 0.3% BSA, PBS, 0.05% Tween 20. In the two-site RIA, 10,000 cpm of trichloroacetic acid–precipitable counts were added per well and incubated 2 h at 37°C. After rinsing with PBS containing 0.05% Tween 20, bound [^{35}S]-labeled mAb was eluted with 100 μl of 0.1 N NaOH, the solution was acidified with 100 μl of 1 N HCl, and then counted in 5 ml of Aquasol (New England Nuclear, Boston, MA) liquid scintillant.

Gluteus Muscle Assay of Ultraterminal Sprouting

Female ICR mice (20–24 g) were innoculated subcutaneously over the right gluteus muscle with ~250 pg of botulinum toxin Type A (Sigma Chemical Co., St. Louis, MO) in 5 μl of 70 mM NaPi, 0.2% gelatin buffer (pH 6.5). The percentage of gluteal motor axon terminals that develop sprouts scales linearly with toxin dose. The dose selected produced no mortality. On each of the next 6 d after injection of toxin, mice were innoculated subcutaneously over the surface of the paralyzed gluteus muscle with 0.1 ml of heat-inactivated ascites fluid (56°C, 30 min) as in Table I, with ascites fluid diluted in PBS (10 mM NaPi, 0.9% NaCl buffer, pH 7.5) to a final injection volume of 0.1 ml (see Fig. 8), or with purified mAb diluted in PBS (Table II). Groups of eight mice were exposed to each treatment. Controls were groups of mice that were innoculated with just toxin or that received toxin followed by a series of daily injections with PBS. 7 d after injection with toxin, the mice were killed, the gluteus muscle was excised, stained with zinc iodide-osmium tetroxide for 3 h in the dark at room temperature (23), and then prepared for examination as a whole mount by clearing in xylene and mounting in Permount. The terminals were left undisturbed by the dissection. Slides were coded before microscopical examination. The percentage of sprouting terminals within the zone of innervation of the superior gluteal nerve was used as the index of terminal sprouting. The length of sprouts or other indices of sprouting were not quantitated. On average, 100 terminals were scored per muscle. Data are expressed as the mean ± SEM for the number of animals (eight mice).

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 sprouting	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.

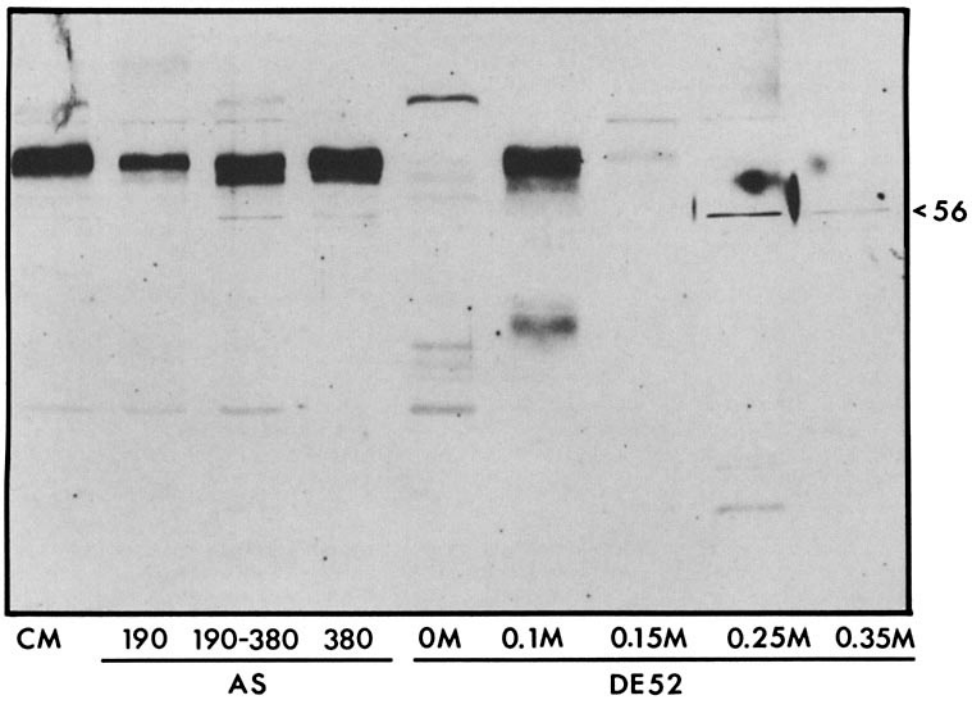
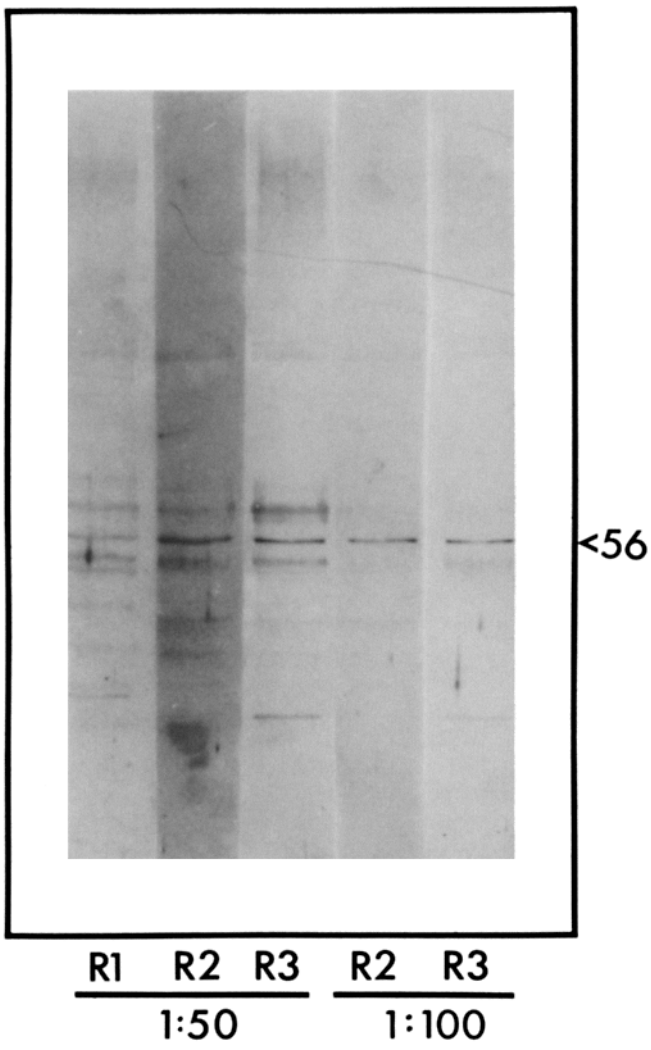


Figure 1. Partial purification of the 56-kD antigen by anion-exchange chromatography. Immunoblot analysis of the denervated diaphragm–conditioned medium (*CM*), fractions from the ammonium sulfate (*AS*) precipitation, and fractions from the DE52 anion-exchange chromatography (DE52). 50 μg total protein 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 ^{125}I-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*).



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 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*).

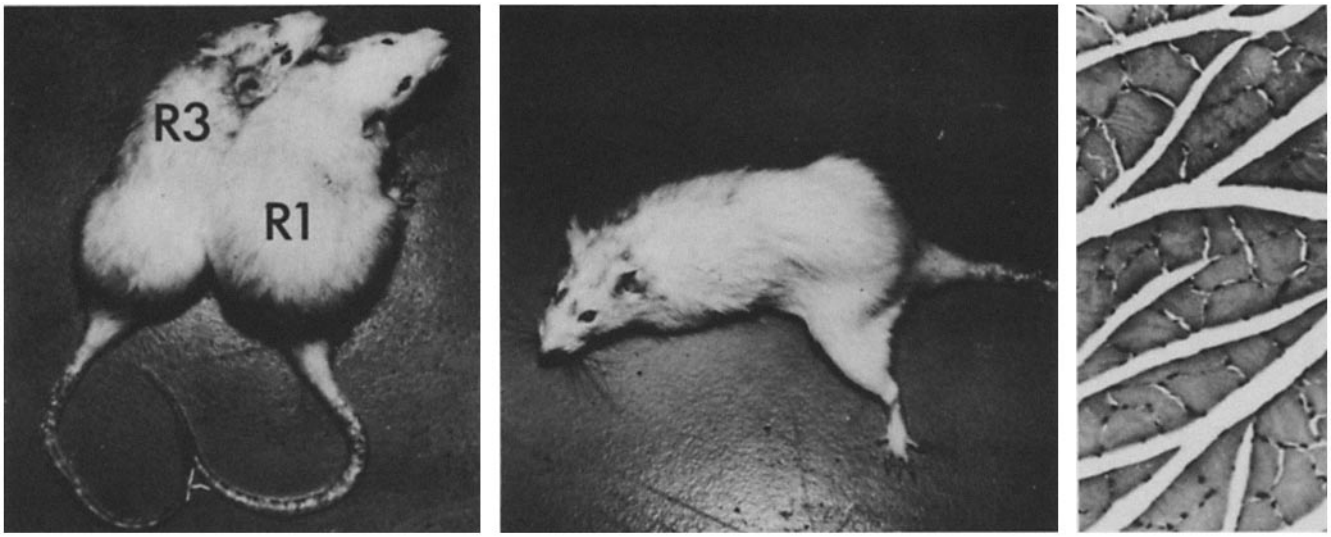


Figure 3. On the left, the figure compares the general appearance of R1 and R3. Immunization of R3 elicited an antiserum of high titer against an antigen of 56 kD (Fig. 2). When the photo was taken, R1 weighed 440 g while R3 weighed 273 g. R3 was markedly thinner than R1 along its flanks and throughout 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 NaCl 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 rescreened 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 DE52 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 DE52 fraction. All anti-56-kD mAb tested reacted positively in the ELISA (Fig. 4*b*).

Most of the hybridoma supernatants failed to react with any antigen on the nitrocellulose transfers. Immunoblotting with the panel of anti-56-kD mAb is 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

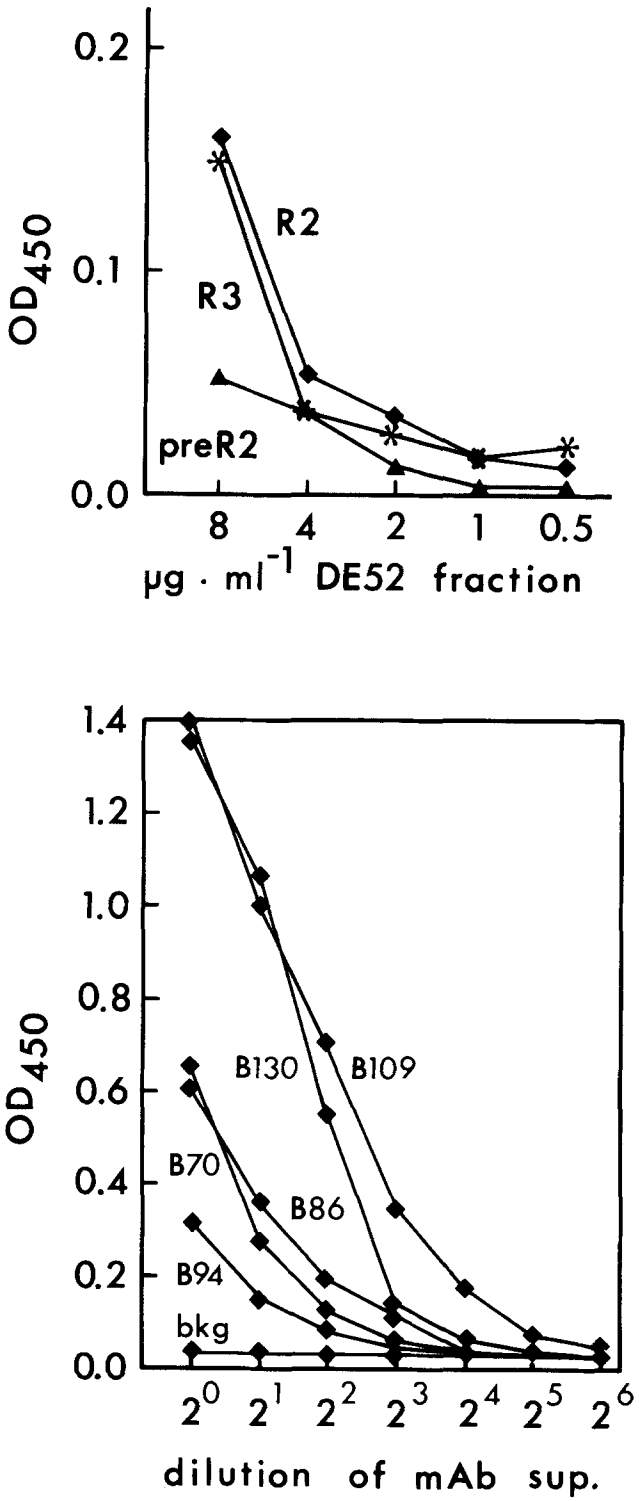


Figure 4. ELISA with rat sera and anti-56-kD hybridoma culture supernatants. In the top panel, the microtiter wells were coated with serially decreasing concentrations of the 0.25 M NaCl DE52 eluate and then reacted with the immune sera obtained from R2 and R3 and with the preimmune serum from R2 (*preR2*). 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 (OD_{450}) was determined with a Titertek dual wavelength ELISA plate reader.

against rat immunoglobulin subclass specific antisera purchased from Miles Laboratories, Inc. (Table I).

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

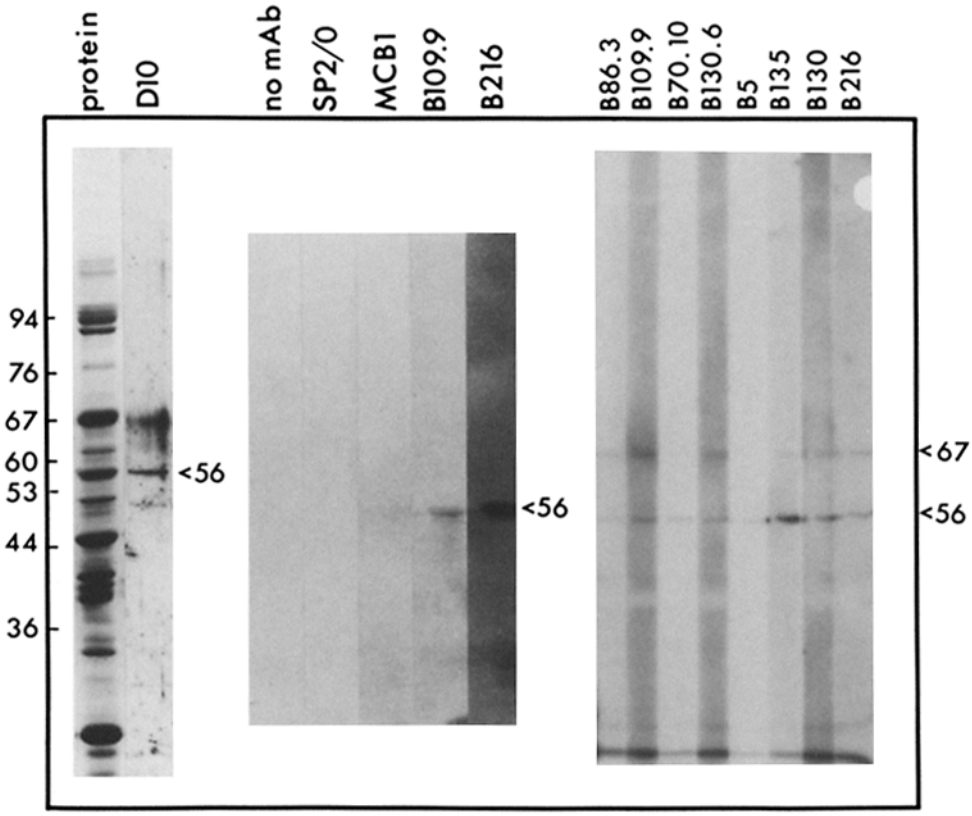


Figure 5. Immunoblot of anti-56-kD mAb. The 0.25 M NaCl DE52 eluate was fractionated by SDS PAGE, transferred to nitrocellulose, and then transfers of individual lanes were reacted with different immunochemical reagents as indicated. The results of three separate experiments are illustrated. (*Left*) 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.

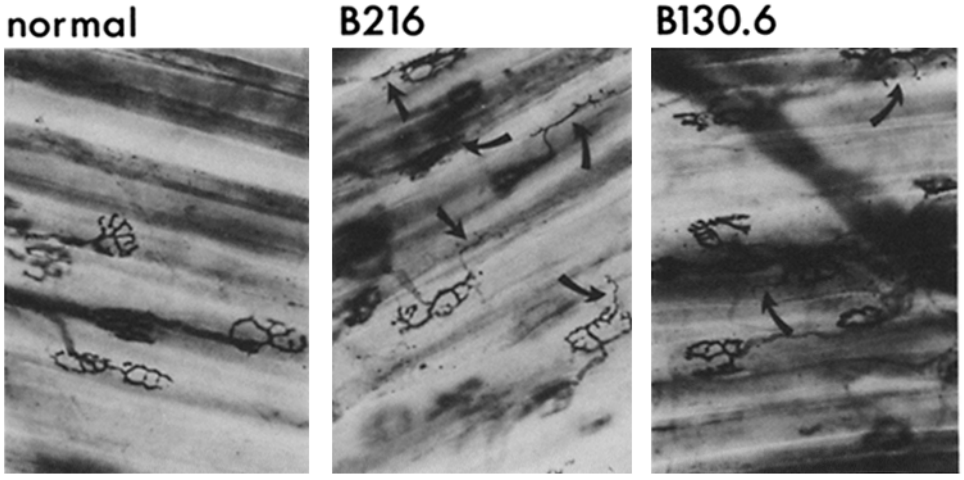


Figure 6. Histology of zinc iodide-osmium tetroxide-stained motor axon terminals in normal muscle (*normal*) or in muscle paralyzed with botulinum toxin and exposed to B216 ascites fluid (*B216*) or B130.6 ascites fluid (*B130.6*). Terminal axonal sprouting was induced by botulinum toxin; the mice were 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 [^{35}S]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,

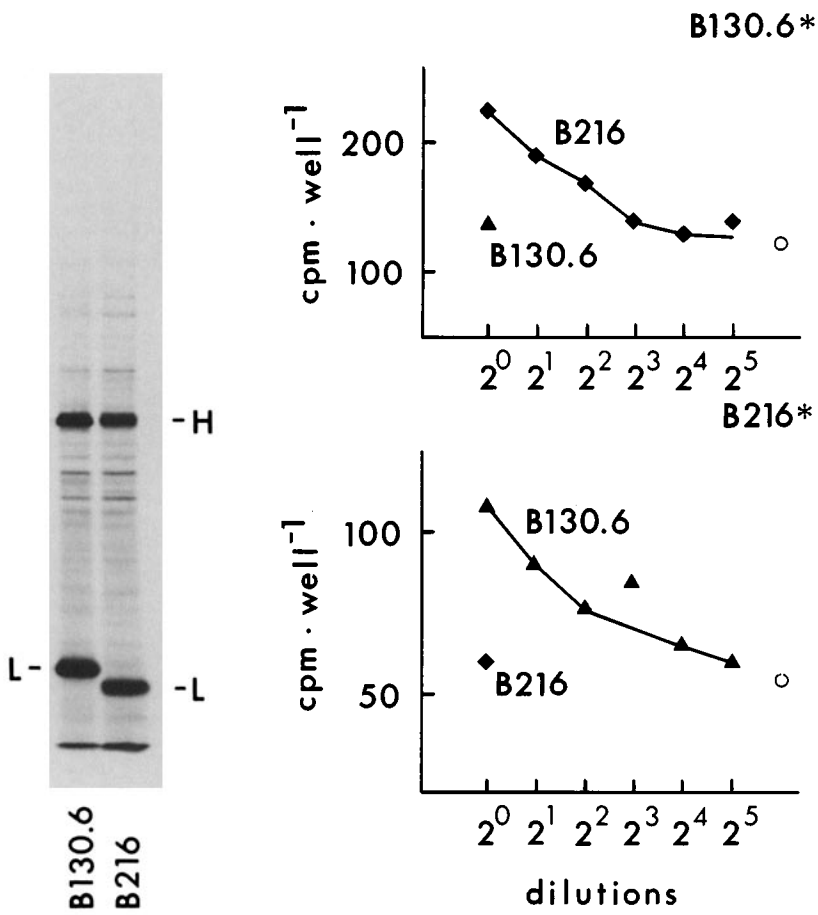


Figure 7. Two-site RIA with B130.6 and B216. (*Left*) [^{35}S]Methionine-labeled supernatants from B130.6 and B216 cultures were analyzed by SDS PAGE. The mAb synthesized by each hybridoma has a heavy chain of 55 kD which is characteristic of rat IgG2b (32), yet the size of their light chain differs. For the two-site RIA (*right*), microtiter wells were coated with the indicated mAb (B130.6 [▲] or B216 [◆]) in ascites fluid. The wells were blocked and then incubated with serial dilutions of the 0.25 M NaCl DE52 fraction (2° dilution = 10 μg protein/ml) followed by incubation with 10,000 cpm of trichloroacetic acid–precipitable [^{35}S]-labeled mAb (*B130.6** [*top*]; *B216** [*bottom*]). Binding of B130.6* was observed in wells that had been coated with B216, but not in those coated with B130.6. Correspondingly, binding of B216* was observed in wells coated with B130.6, but not in those coated with B216. No binding of either [^{35}S]-labeled mAb was observed in the absence of antigen (○). Thus, the antigen is monovalent and the two mAb's bind to distinct epitopes upon the surface of the antigen.

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 penetrance 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

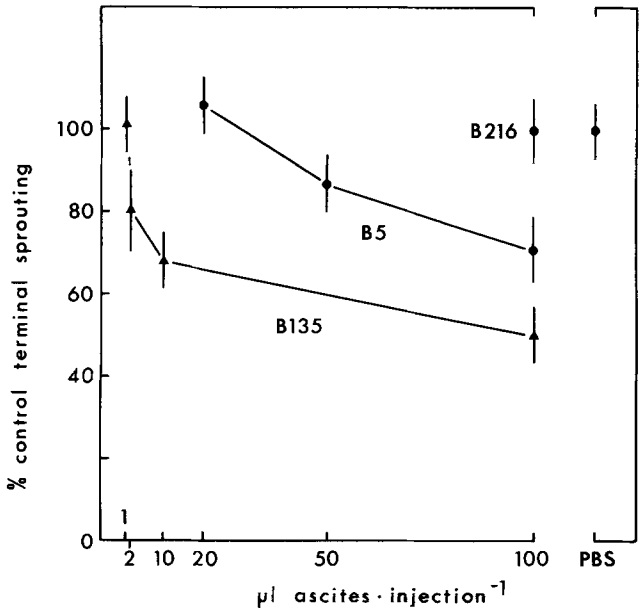


Figure 8. Titration of anti-56-kD mAb ascites fluid suppression of botulinum toxin-induced terminal axonal sprouting. Groups of eight mice were injected subcutaneously over the surface of the gluteus with botulinum toxin and then received 0.1 ml of ascites fluid (or the indicated amount of ascites fluid diluted in 0.1 ml PBS) by subcutaneous injection on each of the next 6 d. B135 ascites fluid contained from 1 to 2 mg per milliliter of IgM as estimated by ELISA. The mice were killed 7 d after the toxin injection, the gluteus was excised, and stained with zinc iodide-osmium tetroxide. The mean and SEM of percent terminals sprouting normalized to the percent terminals sprouting in a group of toxin-only controls are graphed for each group. The dose of toxin used induced 74 ± 6% (mean ± SEM) of the gluteal axon terminals to sprout in the toxin-only control.

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.

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 influences 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 fiber surface or extracellular matrix, N-CAM (for 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.

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 immunochemical reagents, possibly due to limitations of the in vivo assay, such as incomplete penetrance 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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