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Authors
Calof, Anne L
Reichardt, Louis F

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Motoneurons Purified by Cell Sorting Respond to Two Distinct Activities in Myotube-Conditioned Medium

ANNE L. CALOF AND LOUIS F. REICHARDT

Division of Neuroscience, Department of Physiology, School of Medicine, University of California, San Francisco, California 94143

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Spinal motoneurons from chick embryos were purified by retrograde transport and fluorescence-activated cell sorting. Growth conditions for motoneurons were studied, with experiments focused on the effects of conditioned media from chick myotubes, fibroblasts, and spinal cord dividing cells. Motoneurons rapidly extended neurites when plated onto polylysine-coated dishes that had been exposed to these conditioned media. Enzymatic analysis of the substratum-binding, neurite outgrowth-promoting activity from myotube-conditioned medium indicated that it contained heparan sulfate and protein. The neurite outgrowth-promoting activity sedimented as a peak centered at a density of 1.34 in associative cesium chloride gradients, and eluted near the void volume of a Sepharose CL-6B column. Inclusion of myotube conditioned medium in the culture medium of motoneurons also enhanced their survival over periods greater than 2 days in culture. This enhancement of survival could not be explained by myotube-conditioned medium providing motoneurons with a continuous supply of the neurite outgrowth-promoting activity. Media conditioned by spinal cord dividing cells and fibroblasts supported motoneuron survival to some extent, but this effect was not as great as that of myotube-conditioned medium.

INTRODUCTION

During embryogenesis, the vertebrate nervous system generates about twice as many motoneurons as the adult organism will possess (Hamburger, 1975). This excess of motoneurons is eliminated during the period of naturally occurring motoneuron death, whose onset coincides with the time at which characteristic motoneuron projection patterns are established (Landmesser, 1978). The factors governing motoneuron death have yet to be established conclusively, but it is clear that some interaction of the spinal motoneuron with its peripheral target tissue, skeletal muscle, is involved. Motoneurons that are destined to die have sent axons to the periphery prior to their deaths (Chu-Wang and Oppenheim, 1978b). Reduction or enlargement in the amount of target tissue available for innervation can increase or decrease, respectively, the extent of motoneuron death (Hamburger, 1958; Chu-Wang and Oppenheim, 1978a; Hollyday and Hamburger, 1976). Furthermore, a functional neuromuscular interaction must somehow be involved, since paralysis of embryos by a variety of pharmacological agents during the period of cell death can drastically decrease the number of motoneurons which die (Pittman and Oppenheim, 1979). Thus, the evidence supports the idea that epigenetic events following motoneuron-muscle contact mediate naturally occurring motoneuron death.

It is possible that the neuromuscular interaction regulating motoneuron survival involves a trophic substance (or substances) produced by muscle. Attempts to gain support for this hypothesis have been made in various tissue culture systems. The presence of muscle cells, or medium conditioned by muscle cells, has been shown to affect several aspects of the development of cultured spinal cord cells. These include survival (Bennett *et al.*, 1980), neurite outgrowth (Dribin and Barrett, 1980; Henderson *et al.*, 1981), and neurotransmitter synthesis and metabolism (Giller *et al.*, 1977; Godfrey *et al.*, 1980; Brookes *et al.*, 1980). However, in studies using whole spinal cord cultures, it is impossible to distinguish effects on motoneurons from effects on other classes of spinal cord cells. Although it is possible to identify some of the motoneurons present in mixed cultures (Bennett *et al.*, 1980), the motoneurons must, nevertheless, be maintained in the company of other cell types. Under these circumstances it is difficult to determine whether the effects of putative trophic agents on motoneurons are direct, or are instead secondary to effects on the other cells in the culture.

Until recently, it was impossible to obtain motoneurons separated from other spinal cord cells and culture them. This has been remedied by the development of a technique for purifying avian motoneurons, which utilizes the motoneurons' capacity to retrogradely transport a lectin-fluorochrome conjugate that has been injected into limb muscles *in ovo* (McPheeters and Okun, 1980; Okun, 1981). A fluorescence-activated cell sorter is used to separate the fluorescent motoneurons from the other cells of dissociated spinal cords, yielding purified populations of motoneurons for study.

Identification of factors affecting the survival and

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development of spinal motoneurons *in vitro* can be accomplished, now that pure populations of motoneurons are available for study. In the present study, a modification of the method of McPheeters and Okun (1980) has been used to obtain purified motoneurons from avian embryos at the stage just before naturally occurring motoneuron death. The first goal has been to clarify the growth conditions for purified motoneurons, and these experiments have focused on the effects of myotube-conditioned medium on motoneuron growth and survival. Two separable actions of myotube conditioned medium are reported. The first, a neurite outgrowth-promoting activity, acts by adsorbing to the culture substratum and is similar to a neurite outgrowth-promoting factor that is associated with protein and a heparan sulfate proteoglycan and known to be produced by many cell types (Lander *et al.*, 1982; 1983b). The second action of myotube-conditioned medium is to enhance the survival of motoneurons in culture over longer periods. The molecular identity and specificity of action of this second activity remain to be determined.

MATERIALS AND METHODS

Materials

Fertile White Leghorn chicken eggs (Feather Hill Farm, Petaluma, Calif.) were incubated at 99–101°F in a humidified, forced-draft incubator. Lucifer Yellow VS for pilot studies was the kind gift of Dr. Walter Stewart (NIH, Bethesda, Md.). Later, it was purchased from Aldrich Chemical Company, Milwaukee, Wisconsin. Laminin from the EHS sarcoma was purified by the method of Timpl *et al.* (1979). Antisera to nerve growth factor and laminin were prepared in this laboratory. Heparinase and heparatinase from *Flavobacterium heparinum* were purified and separated from each other and from chondroitinase on hydroxylapatite (Linker and Hovingh, 1972). Wheat germ agglutinin, trypsin (Type III), chondroitinase ABC, neuraminidase (Type V), collagenase (Type VII), and soybean trypsin inhibitor (Type I-S), were purchased from Sigma Chemical Company, St. Louis, Missouri. Iron-saturated ovotransferrin ("crude Otf fraction") was prepared according to Kimura *et al.* (1982).

Culture and Nonneuronal Cells

Myotubes. Cultures of chick myotubes that were substantially free of fibroblasts were prepared by a modification of the method of Konieczny *et al.* (1982). Leg and pectoral muscles of 11-day embryos were dissected, cleaned of skin and connective tissue, minced, and dissociated into single cells by trituration through a Pasteur pipet. Cells were diluted to 2×10^5 cells/ml in plating medium (see below), preplated for 1 hr at 37°C in tissue culture flasks to remove adherent fibroblasts, and finally decanted and plated as 25 ml per collagen-coated (calf skin collagen; Sigma), 100-mm-diameter, tissue culture dish. Myoblast plating medium consisted of Dulbecco's Modified Eagle's Medium containing 0.5% glucose (DME), supplemented with 10% horse serum (HS; Kansas City Biologicals), 2% chick embryo extract (CEE; GIBCO), glutamine (2 m*M*), penicillin (100 U/ml), streptomycin (100 μg/ml), Fungizone (Squibb and Sons, Inc., Princeton, N. J., 2.5 μg/ml), and ovotransferrin (Otf; 25 μg/ml). These, and all dissociated cell cultures, were incubated in 8% CO_2 at 37°C.

After 2 days in culture, myoblasts were washed in calcium- and magnesium-free Dulbecco's phosphate-buffered saline (CMF-PBS) and placed in fusion inhibition medium (Konieczny *et al.*, 1982), consisting of calcium-magnesium-free (CMF) DME, supplemented with 10% CMF-horse serum, 2.5% CMF-chick embryo extract, 25 μ*M* $CaCl_2$, 25 μg/ml Otf, glutamine (2 m*M*), penicillin (100 U/ml), and streptomycin (100 μg/ml). After 2 days in fusion inhibition medium, most nonmyoblast cells had detached from the dish and were removed by rinsing the cultures twice in PBS. Cultures were then placed in growth medium for 3 days, to allow fusion and maturation of myotubes, before myotube-conditioned medium was prepared (see below). Myotube growth medium consisted of DME with 10% horse serum, 25 μg/ml Otf, glutamine (2 m*M*), penicillin (100 U/ml), and streptomycin (100 μg/ml). Myotube cultures prepared in this way had few, if any, fibroblasts visible upon microscopic inspection at this stage. To maintain myotube cultures at this low level of fibroblast contamination during the several weeks for which they were maintained, cultures were fed with myotube growth medium containing 10 μ*M* cytosine-1-β-D-arabinofuranoside (Ara-C; Sigma) for several days between periods of conditioned medium preparation. After Ara-C treatment, cultures were maintained for 12–24 hr in plain myotube growth medium before another batch of conditioned medium was prepared from them.

Fibroblasts. Chick fibroblast cultures were prepared from the adherent cells obtained during the preplating step of myotube culture preparation. Adherent cells in 75-cm^2 tissue culture flasks were grown to 50% confluence in fibroblast growth medium consisting of DME supplemented with 10% fetal calf serum (FCS; Hyclone-Sterile Systems, Logan, Utah), glutamine (2 m*M*), penicillin (100 U/ml), and streptomycin (100 μg/ml). Cultures obtained in this way were passaged at least once, to remove any remaining myoblasts, and grown to near confluence prior to their use in conditioned medium preparation (see below).

Spinal cord dividing cells. Spinal cords from 6-day chick embryos (Stage 28–29, Hamburger and Hamilton, 1951) were dissected and freed of meninges and dorsal root ganglia. The cords were minced and incubated for 11 min at 37°C in 4 ml of CMF-PBS containing 0.2% trypsin. Digestion was stopped by the addition of 4 ml of 5.5 mg/ml soybean trypsin inhibitor. The spinal cord fragments were washed into fibroblast growth medium (see above), and dissociated into single cells by trituration through a Pasteur pipet. Cells were grown to 50% confluence in fibroblast growth medium, passaged at least once, and grown to near confluence in 75-cm^2 tissue culture flasks prior to their use in conditioned-medium preparation (see below).

Preparation of Motoneurons

Preparation of fluorescent conjugate. To 20 mg of wheat germ agglutinin in 2 ml of buffer (150 m*M* Na borate, pH 9.0) was added 20 mg of Lucifer Yellow VS. The mixture was incubated for 1 hr at 37°C (Okun, 1981). The fluorescent conjugate was separated from unbound dye on Bio-Gel P-2 (200–400 mesh wet; Bio-Rad Corp., Richmond, Calif.), equilibrated in 145 m*M* ammonium bicarbonate, 5 m*M* Tris–HCl, pH 7.8, and then lyophilized and stored at −20°C. Immediately before use, the conjugate was reconstituted in PBS to 20–25 mg/ml protein.

Injection and transport. Six-day chick embryos (Hamburger and Hamilton Stage 28–29) were removed from the egg, decapitated, eviscerated, and pinned out in Sylgard-coated (Dow Corning Corp., Midland, Mich.), 60-mm petri dishes containing Leibovitz L-15 medium supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml). Muscle masses in all four limbs were injected with the fluorescent conjugate, and the embryos were washed twice in L-15 and placed in 3.5 ml per dish of organ culture medium. At this point, a partial laminectomy was performed to ensure proper oxygenation of the cord during incubation. Organ culture medium consisted of DME with sodium chloride and sodium bicarbonate concentrations adjusted for pH 7.2 at equilibrium with 5% CO_2 (1.85 g/liter $NaHCO_3$; 7.68 g/liter NaCl), 10% horse serum, 2.5% chick embryo extract, and glutamine and antibiotics as with fibroblast growth medium. The embryos were incubated for 20–24 hr at 30°C on a rocking platform in a humidified chamber equilibrated with 95% O_2–5% CO_2. Embryos used for negative controls were prepared exactly as described above, except that their muscles were not injected.

Dissociation and sorting of motoneurons. Spinal cords were dissected out of the embryos, freed of meninges and dorsal root ganglia, and placed in CMF-PBS supplemented with 0.6% glucose, penicillin (100 U/ml), and streptomycin (100 μg/ml). The cords were minced and incubated for 11 min at 37°C in 0.2% trypsin. The digestion was stopped by bringing the mixture to 0.37% soybean trypsin inhibitor, and the cord fragments were collected and washed into BSA-L15 holding medium (L-15 supplemented with 0.025% bovine serum albumin (BSA; Fraction V, Sigma), 0.6% glucose, 2 m*M* glutamine, and antibiotics at usual concentrations). Cords were dissociated into single cells by gentle trituration through a Pasteur pipet and filtered through monofilament nylon mesh with 37-μm mesh openings (Small Parts, Inc., Miami, Fla.). Single-cell suspensions of 7 ml were underlaid with 2 ml of a sterile solution of 4% BSA in L-15 and centrifuged in a 15 ml conical centrifuge tube (Corning) for 10 min at 100*g* to remove small debris. Pelleted cells were resuspended in BSA-L15 holding medium and refiltered through the 37-μm mesh prior to sorting.

Cell suspensions were sorted using a Becton-Dickenson FACS IV with 18-W argon ion laser illumination (Spectra Physics, Mt. View, Calif.) and a logarithmic amplifier (Becton–Dickenson, Sunnyvale, Calif.). The 458-nm emission line of the laser (280 mW) was used for excitation. Fluorescence emission from cells was passed through a 475-nm long-pass filter and detected with a photomultiplier (EN-330KC, EMI-Gencom Inc., Planview, N. Y.) operated at 380 V.

Since all cells may autofluoresce weakly in this range, each sort was calibrated using spinal cord cells from uninjected embryos as a negative control. The negative offset of the logarithmic amplifier was adjusted so that less than 0.5% of light scatter events in the negative control showed fluorescence above a standard intensity (Channel 55 of 255). The positive sample was then analyzed with the logarithmic amplifier calibrated according to this standard. The positive sample normally contained 3–9% of scatter events with fluorescence intensities above the negative level (i.e., from channel 55 to channel 255). This fraction of the positive sample was sorted as labeled motoneurons. The sorter profile from a typical experiment is shown in Fig. 1. Samples from each sort were independently analyzed for purity by counting numbers of labeled cells in the fluorescence microscope (Zeiss Blue Violet filter set 487705: BP 400–440 nm, LP 470 nm).

Motoneuron Cell Culture

Motoneurons were cultured in multiwell plates (96-well clusters, Costar). Culture surfaces were exposed to poly-D-lysine (Sigma; 1 mg/ml in 0.1 *M* sodium borate, pH 8.4) overnight, washed with deionized distilled water, and sterilized under ultraviolet light. Cells

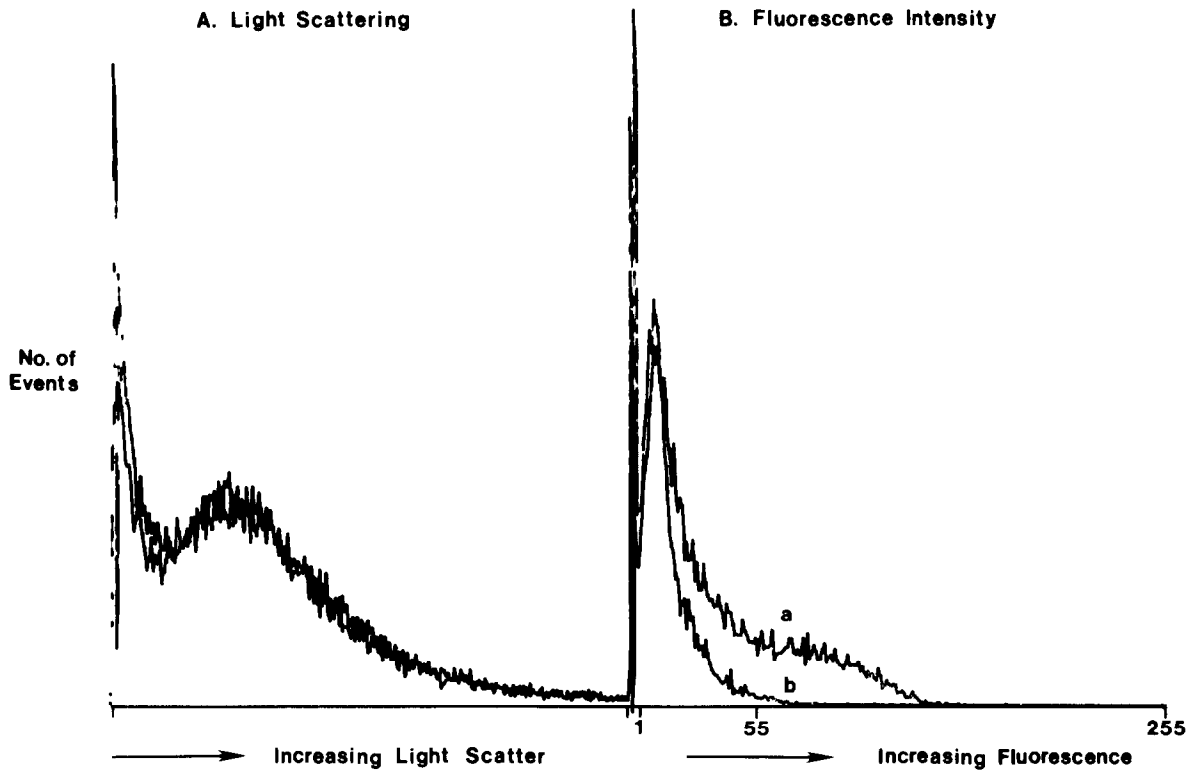

FIG. 1. Cell sorter profiles of spinal cord dissociates. Spinal cords were dissociated into single cells and analyzed with a Becton Dickenson FACS IV cell sorter equipped with logarithmic amplification, as described in the text. This figure shows superimposed profiles of dissociates from negative (uninjected) embryos and positive (injected) embryos from one experiment. (A) Light scattering profiles. Approximately 70% of all light scattering events in each sample was contained within the broad peak, which represents the cells. The light scattering profiles of positive and negative samples are essentially identical and appear superimposed. (B) Fluorescence profiles. (a) Positive sample from injected embryos; (b) negative sample from uninjected embryos. In this experiment, the positive sample (a) had 8% of its light scattering events with fluorescence intensities above that designated by channel 55. The negative sample (b) and 0.5% of its light scattering events with fluorescence in this range.

were either plated directly onto these surfaces, or onto polylysine-coated surfaces that had been further treated with solutions to be tested for neurite outgrowth-promoting activity. These solutions were applied for 8–16 hr at 4°C, after which the culture surfaces were washed three to four times in DME. Culture medium was added shortly before the cells were plated. Cells were plated at a density of 3000–5000 cells (6000 to 10,000 scatter counts) per well of 96-well plates, as estimated by hemacytometer counts. Counts of living cell cultures were made to determine the total number of cells present in each well at 24 hr after plating. Approximately 90% (range 86–93%) of the cells estimated to have been put into a well could be accounted for at 24 hr.

Motoneurons were usually grown in serum-free growth medium: DME supplemented with Otf (25 μg/ml), insulin (5 μg/ml), putrescine dihydrochloride (100 μ*M*), progesterone (20 n*M*), and selenium (30 n*M*) (modified from Bottenstein and Sato, 1979). Glutamine and antibiotics were added as with fibroblast growth medium. For some experiments, 3% horse serum was added to this medium. When growth medium was supplemented with conditioned medium, the final mixture (50% conditioned medium, 50% fresh DME) was brought to the listed concentrations of supplements.

Evaluating neurite outgrowth. For cell counting and photography, cultures were fixed in PBS containing 2.5% glutaraldehyde and 5% sucrose, and washed into 0.9% NaCl prior to counting. To determine how many cells were lost during fixation and washing, counts of the total number of cells per well were made before and after fixation (24-hr cultures were used for this determination). About 12% (range 6–17%) of cells were lost during these procedures. Therefore, because most cells remained attached to the dish, the cell counts reported here should serve as reasonable estimates of the behavior of the entire cell sample plated.

At least 200 cells in a minimum of 10 random fields were counted for each culture. Two cultures were counted for each time point in all reported tests. Cells were counted as having neurites only if they possessed

one or more processes greater than two cell diameters in length. Cells with obvious fibroblast morphology were not counted. All other cells, including dead cells, were counted. Thus, the reported percentage of cells with neurites reflects the number of intact motoneurons, alive and bearing processes, at the recorded time in culture. Using these criteria, reliable and reproducible results were obtained.

Preparation and Fractionation of Conditioned Media

Preparation of conditioned media. Cultures of myotubes, fibroblasts, or spinal cord cells were washed twice in plain DME. Serum-free conditioned media (MCM_{SF}, FCM_{SF}, and $SCCM_{SF}$, respectively) were prepared by incubating washed cultures in 7–8 ml of DME supplemented with 25 μg/ml Otf, 10 μg/ml insulin, 0.5 mg/ml polyvinyl pyrrolidone (PVP-360; Sigma), and glutamine and antibiotics as with fibroblast growth medium (adapted from Konigsberg, 1979). Serum-containing myotube-conditioned medium (MCM) was prepared by incubating washed cultures in DME supplemented with 1% horse serum, 25 μg/ml ovotransferrin, and glutamine and antibiotics as with fibroblast growth medium. After 3–5 days of incubation, conditioned media were collected, cleared by centrifugation, and stored at −20°C until used. They were filtered through a 0.22-μm pore filter prior to use.

Enzymatic treatments of MCM_{SF}. Trypsin was used at 100 μg/ml for 2.5 hr at 37°C, after which soybean trypsin inhibitor was added to a final concentration of 500 μg/ml. As a control, trypsin inhibitor was added before the trypsin. Collagenase at 75 U/ml, neuraminidase at 2.5 U/ml, and choidroitinase ABC at 5 U/ml were all used for 6 hr at 37°C. Heparatinase was used at 35 μg/ml for 6 hr at 43°C. As a control, MCM_{SF} without added enzyme was heated at 43°C for 6 hr. Heparinase was used at 20 μg/ml for 6 hr at 30°C.

Isopycnic centrifugation. MCM_{SF} (60 ml) was concentrated on 3.5 ml of DEAE cellulose (DE52, Whatman, Ltd.). The column was washed in 2 column vol of 100 m*M* NaCl/50 m*M* Tris-HCl, pH 7.4, and eluted with 9 ml of 36.9% CsCl (w/w) in 50 m*M* Tris-HCl, pH 7.4. The density of the eluate was measured and the CsCl concentration was corrected to a final concentration of 37% (w/w). Guanidinium chloride (GuHCl) was added to a final concentration of 0.4 *M*. These conditions constitute the associative gradient conditions of Sajdera and Hascell (1969). The samples were centrifuged at 5°C to equilibrium (~48 hr) at 40,000 rpm using a SW50.1 rotor (DuPont Instruments, Newton, Conn.). Gradients were eluted and the density of fractions was determined by weighing volumes in preweighed 10-μl micropipets. Fractions were dialyzed exhaustively against 100 m*M* NaCl/50 m*M* Tris-HCl, pH 7.4.

Salt fractionation and gel filtration of MCM. Saturated ammonium sulfate was added to 25 ml of MCM (containing 1% horse serum) to 45% saturation. The solution was centrifuged, and the pellet was resuspended in 1.3 ml of column buffer (200 m*M* NaCl/20 m*M* Hepes, pH 7.4/0.2 mg/ml hemoglobin (Sigma Type IV)). A column (1.5 × 8.5 cm) of Sepharose CL-6B (Pharmacia Fine Chemicals, Piscataway, N. J.) was equilibrated in column buffer at 4°C. The void volume was measured using blue Dextran 2000 (Pharmacia). The 1.3-ml sample was applied and 1-ml fractions were collected.

RESULTS

Purity of Sorted Motoneurons

The rationale of the motoneuron sorting technique requires that motoneurons are the only cells within the spinal cord that take up and accumulate the fluorescent conjugate when it is injected into limb muscles. In the present study, the extent of labeling in the spinal cords of injected embryos was evaluated by examining cryostat sections of formaldehyde-fixed spinal cords in the fluorescence microscope. A comparison of fluorescence and phase micrographs of such a section (Fig. 2) shows that the fluorescent conjugate is only found in large cells of the ventral horn, presumed to be motoneurons. Examination of sections from whole embryos has shown that some cells of the dorsal root ganglia are also labeled (data not shown), but these cells are not of concern because spinal cords are carefully stripped of meninges and dorsal root ganglia prior to dissociation and sorting. During the course of dissociation and sorting, the Lucifer Yellow-wheat germ agglutinin conjugate remains within the motoneurons, and does not appear to be transferred to other cells (McPheeters and Okun, 1980; McPheeters, 1982).

For the reasons described above, the presence of several brightly fluorescing granules inside a sorted cell qualified that cell as a motoneuron in the present study. Motoneurons retain the fluorescent label for at least 24 hr after dissociation and plating into cultures, and it does not appear to prevent their survival and development of neurites in culture. Figure 3 shows phase and fluorescence micrographs of two motoneurons grown for 24 hr in culture.

The purity of each sorted cell sample was evaluated by examining at least 200 cells in the fluorescence microscope. Before sorting, only a few of the cells in a suspension are labeled (Fig. 4). After sorting, however, almost all of the cells in the sorted motoneuron fraction are labeled. The purity of the sorted motoneuron fraction depends upon the sorting criteria and the intensity of label in a given experiment. The sorting

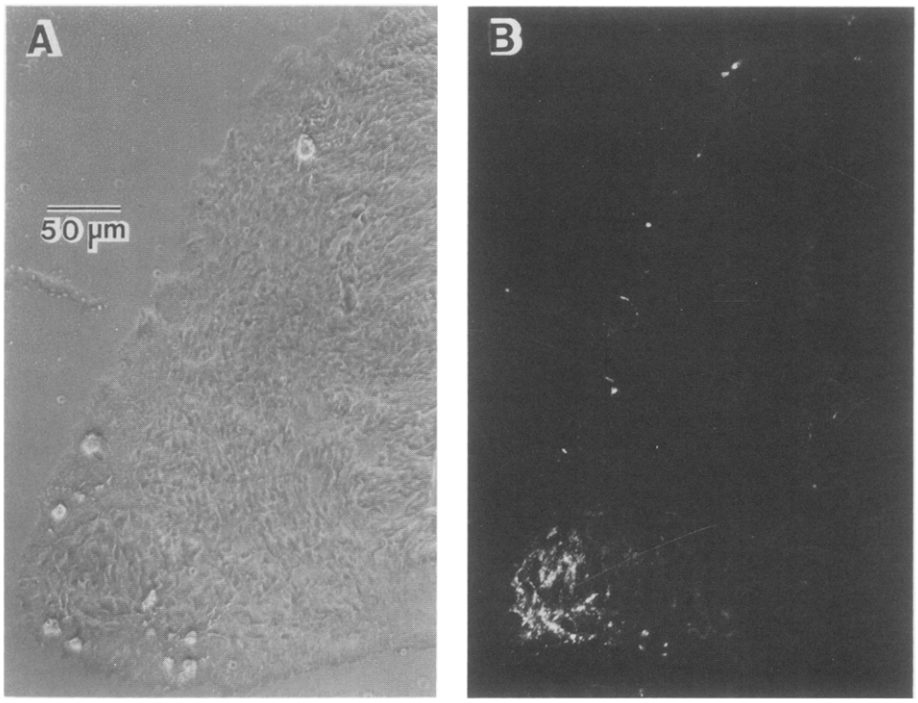

FIG. 2. Section of spinal cord labeled by retrograde transport. A stage 29 chick embryo was prepared for organ culture and its limb muscles were injected with fluorescent conjugate as described in the text. After 24 hr, the spinal cord was dissected out, fixed in 4% paraformaldehyde in 0.1 *M* Na phosphate, pH 7.4, and sectioned on a cryostat. (A) Phase contrast picture of spinal cord hemisection. (B) Fluorescence micrograph of same section.

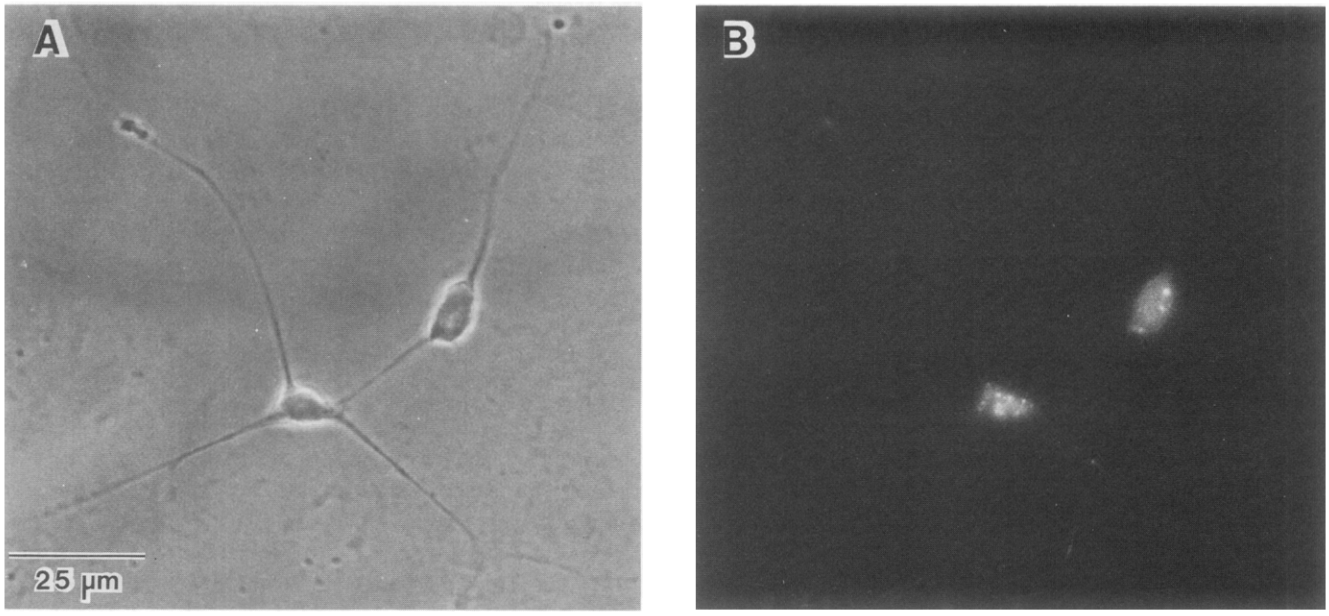

FIG. 3. Sorted motoneurons grown in culture. Motoneurons were purified by retrograde transport and fluorescence-activated cell sorting as described in the text. Sorted motoneurons were plated onto coverslips upon which spinal cord dividing cells had been grown to confluence and hypotonically lysed. Motoneurons were grown for 24 hr in 3% horse serum growth medium supplemented with 50% myotube-conditioned medium (see text), then fixed in 10% formalin in PBS and photographed. (A) Phase contrast; (B) Fluorescence. Note bright fluorescent granules in cell bodies of motoneurons.

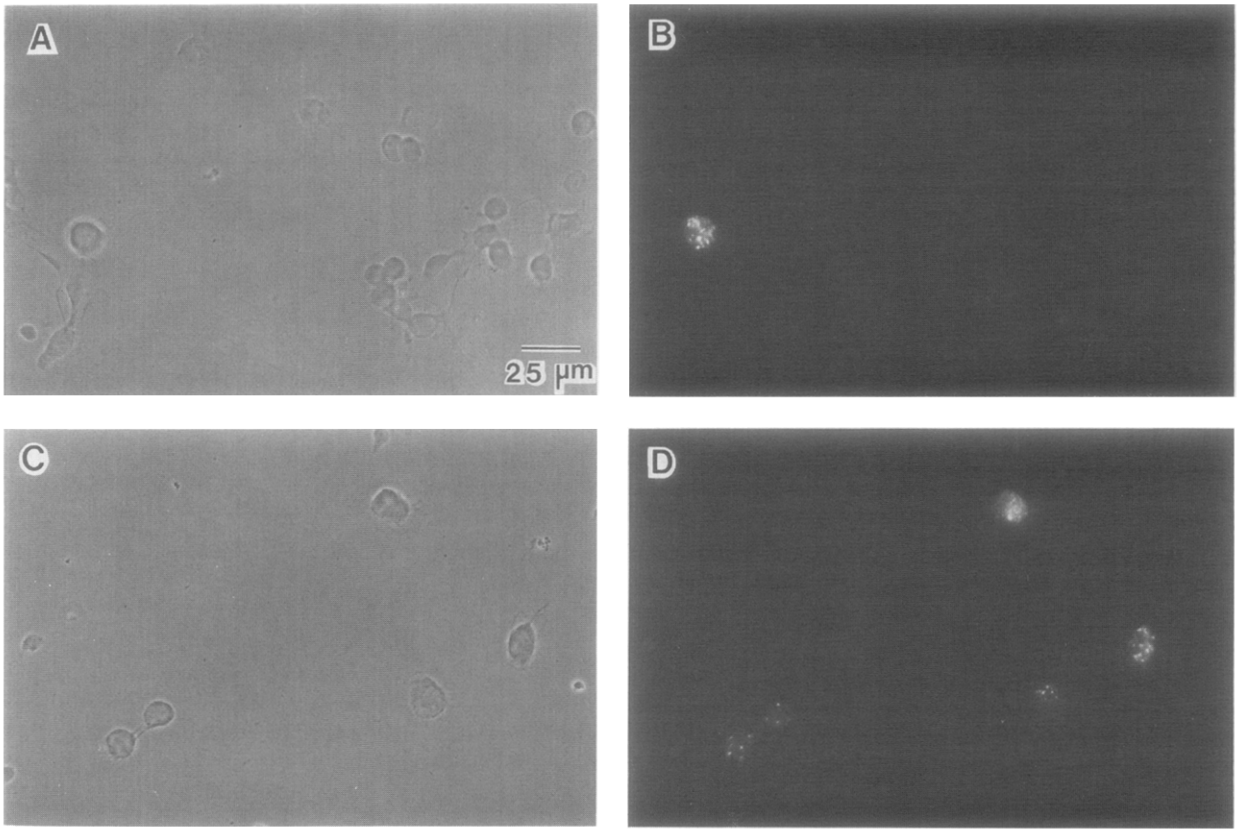

FIG. 4. Spinal cord cells before and after cell sorting. Dissociated spinal cord cells were prepared from injected embryos as described. Aliquots of cell suspensions, before and after sorting, were plated onto polylysine-coated glass coverslips and fixed in absolute methanol at −20°C. (A) Phase-contrast picture of cells before sorting. (B) Fluorescence micrograph of same field. Very few cells are labeled. (C) Phase contrast of cells after sorting. (D) Fluorescence of same field. All cells are labeled.

criteria described under Materials and Methods routinely gave purities of greater than 80%. With stricter criteria, higher purities—but poorer yields—can be obtained.

About $1.5\text{–}2 \times 10^6$ cells were obtained from each dissected spinal cord by the initial dissociation. Approximately 10% of these cells were lost during the BSA sedimentation that preceded cell sorting. Sorter analysis of these cell samples indicated that about 70% of all light scattering events were in one peak, corresponding to the cells. Of the scatter events within the peak, from 3 to 9% showed fluorescence above negative control levels. This number agreed well with the extent of labeling seen in unsorted samples evaluated with the fluorescence microscope. Sorter yields were 20,000–50,000 positive scatter events per embryo. Only about half this number was recovered as cells. It was assumed that this apparent loss was due mainly to two factors: (1) Sorting of erroneous light scattering events. For example, pieces of cells or cell processes, from labeled cells that were broken during the process of dissociation and sorting, could fall within the "cell" range of light scattering events and be sorted as positively labeled cells. (2) Failure to recover cells from the sorter. Problems of this sort include sticking of cells to the sides of tubes in which they are recovered, and loss of cells due to misdirection of the cell-containing fluid stream as it is deflected during the sorting process.

Effect of Myotube-Conditioned Medium on Motoneuron Neurite Outgrowth

Factors that bind to polyornithine- or polylysine-treated surfaces and enhance neurite outgrowth are known to be produced by many cell types, including the mouse skeletal muscle cell line C_2 (Adler *et al.*, 1981; Lander *et al.*, 1982). It was suspected that the same kind of factor might be produced by primary cultures of chick myotubes. A test was performed to see whether myotube-conditioned medium could exert a neurite outgrowth-promoting effect when applied to a polylysine substratum. Serum-free medium was con-

ditioned by 4 days of exposure to cultures of chick myotubes. Polylysine-coated tissue culture dishes were exposed overnight to this conditioned medium (MCM_{SF}), and washed thoroughly. Motoneurons plated onto MCM_{SF}-treated substrata and examined after 24 hr in culture had extended long neurites, but motoneurons plated onto polylysine alone had few or no processes (Fig. 5). This indicates that MCM_{SF} can exert a short-term, neurite outgrowth-promoting effect on motoneurons when applied to the substratum alone; it does not have to be present in the cell culture medium to exert this effect.

When sympathetic neurons are grown on polylysine substrata treated with medium conditioned by bovine corneal endothelial cells (BCE), they respond by rapidly extending neurites. The BCE factor responsible for neurite outgrowth from sympathetic neurons has been purified in this laboratory (Lander *et al.*, 1982; 1983a). Experiments performed in the present study confirm the similarity of the motoneuron neurite outgrowth-promoting activity from MCM_{SF}, with the BCE factor.

MCM_{SF} promotes neurite outgrowth from sympathetic neurons (data not shown), and the BCE factor promotes neurite outgrowth from motoneurons. Thus, both factors promote neurite outgrowth from both neuronal types. Figure 6 shows results from motoneurons grown in serum-free culture conditions either on polylysine alone, or on polylysine treated with various conditioned media. After being grown for 24 hr on substrata treated with MCM_{SF}, over 50% of cells have extended one or more neurites greater than two cell diameters in length. The number of responding cells is less on substrata treated with BCE conditioned medium or with chick fibroblast-conditioned medium, but still five to six times greater than that of cells plated on polylysine alone.

Characterization of the MCM_{SF} Substratum Factor

The substratum-bound, neurite outgrowth-promoting activities of MCM_{SF} and the BCE factor are also similar in their susceptibilities to enzymes. Table 1 lists the responses of motoneurons plated onto polylysine substrata treated with MCM_{SF} that had been subjected to a variety of enzymatic treatments. The neurite outgrowth-promoting activity of MCM_{SF} is sensitive to trypsin, heparatinase, and heparinase, but insensitive to collagenase, neuraminidase, and chondroitinase ABC. This suggests that both protein and heparan sulfate are important on the substratum-binding, neurite outgrowth-promoting activity of MCM_{SF}. This characterization corresponds to that of the BCE factor, which is known to consist of a complex containing protein and a heparan sulfate proteoglycan (Lander *et al.*, 1982, 1983a).

The substratum-binding, neurite outgrowth-promoting activity in MCM_{SF} has a buoyant density similar to that of the BCE factor. This is illustrated in Fig. 7. MCM_{SF} was concentrated on DEAE cellulose and sedimented in a cesium chloride gradient containing 0.4 *M* guanidinium chloride. Fractions from the gradient were dialyzed and applied to polylysine substrata. The

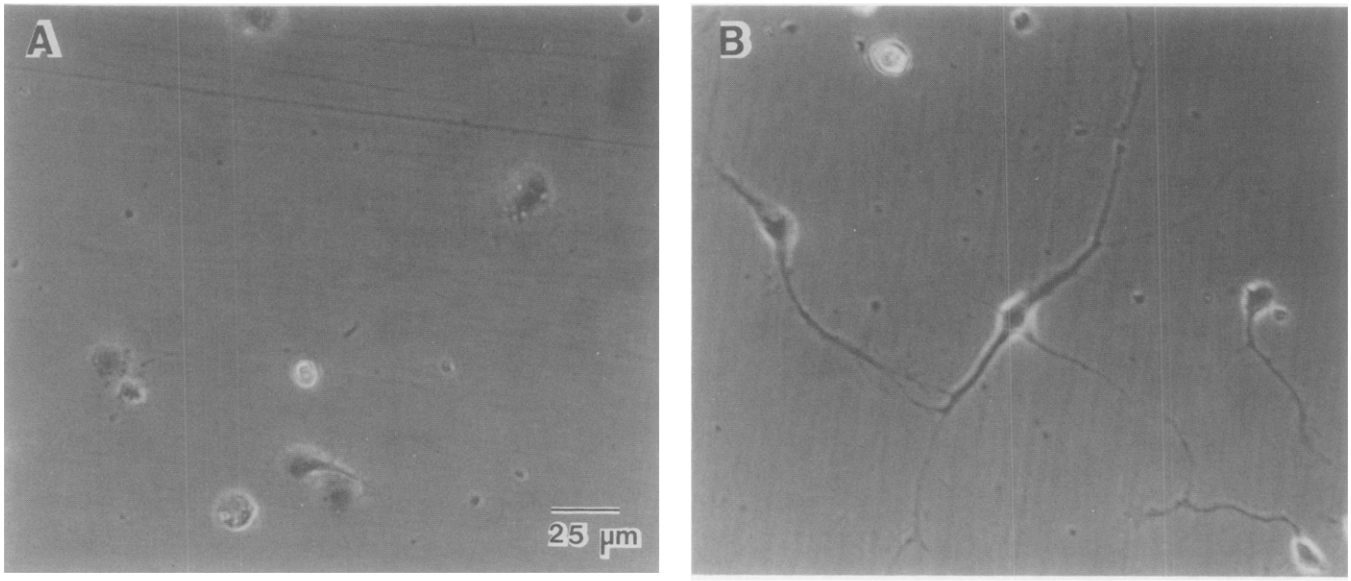

FIG. 5. Effect of myotube-conditioned medium on motoneuron neurite outgrowth. Sorted motoneurons were grown for 24 hr in serum-free growth medium, either on polylysine substrata, or on polylysine substrata that had been exposed to myotube-conditioned medium and then washed. (A) Motoneurons on polylysine substratum. (B) Motoneurons on substratum treated with conditioned medium.

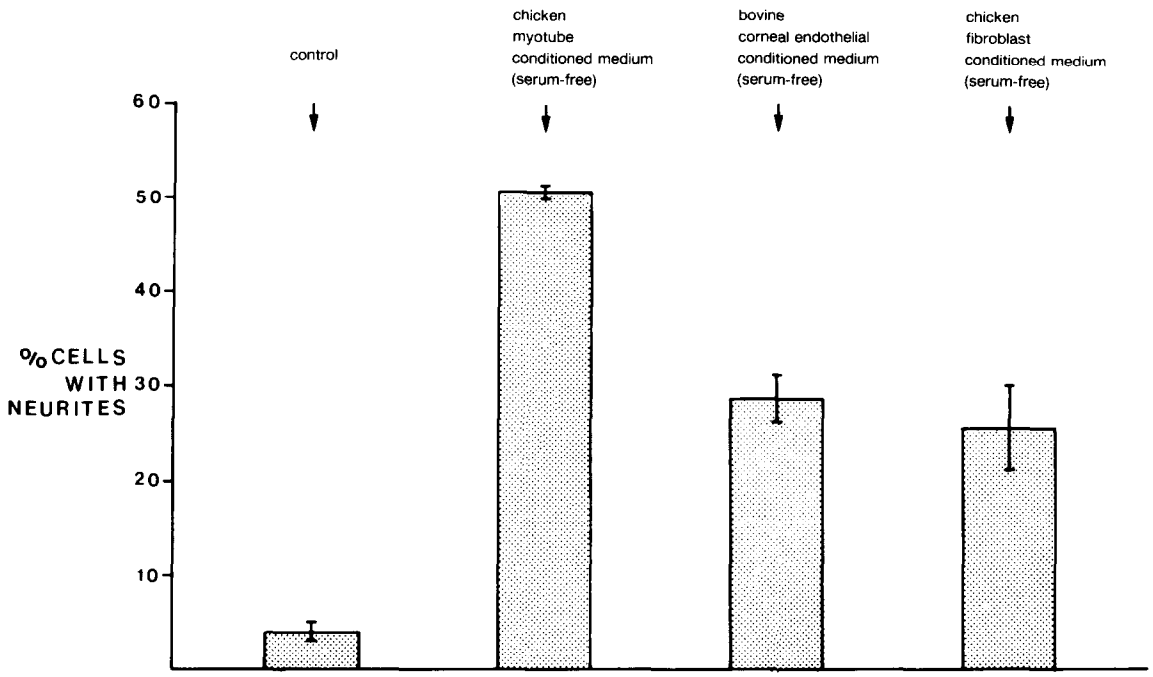

FIG. 6. Motoneuron neurite outgrowth on substrata treated with different conditioned media. Serum-free conditioned media were applied to polylysine substrata for 8–16 hr at 4°C, and then the substrata were washed and plated with sorted motoneurons. Motoneurons were grown for 24 hr in serum-free growth medium, fixed, and cells with neurites were counted in duplicate cultures. Error bars show means ± 1 SD.

treated substrata were washed, plated with motoneurons, and analyzed for their ability to promote neurite outgrowth. The peak of neurite outgrowth-promoting activity eluted at a density of about 1.34. This is between the densities of pure proteins and polysaccharides, and close to the density of the BCE factor (Lander *et al.*, 1982). This is consistent with the presence of glycosaminoglycan as well as protein in the MCM_{SF} neurite outgrowth-promoting factor, as was indicated by enzymatic tests.

TABLE 1
SENSITIVITY OF SUBSTRATUM-BINDING, NEURITE OUTGROWTH-PROMOTING ACTIVITY TO ENZYMATIC DIGESTION

Enzymatic treatment	% Cells with neurites	(% of control)
Trypsin	1 ± 0	(3)
Heparatinase	10 ± 1	(32)
Heparinase	7 ± 1	(23)
Soybean trypsin inhibitor + trypsin	28 ± 2	(90)
Collagenase	24 ± 1	(77)
Neuraminidase	25 ± 3	(81)
Chondroitinase ABC	25 ± 1	(81)
Untreated	31 ± 1	(100)
43°C for 6 hr	25 ± 3	(81)

Note. MCM_{SF} was subjected to enzymatic treatments as described in the text. Treated MCM_{SF} (or control, untreated MCM_{SF}) was applied to polylysine substrata for 12 hr at 4°C, and the substrata were then washed and plated with sorted motoneurons. Motoneurons were grown for 24 hr in serum-free growth medium, fixed, and cells with neurites were counted in duplicate cultures. Numbers in second column show means ± SD. Numbers in parentheses indicate neurite outgrowth expressed as percentage of control response on untreated MCM_{SF}.

Virtually all the neurite outgrowth-promoting activity from MCM can be precipitated in 45% saturating ammonium sulfate. When the supernatant from such a precipitation was concentrated to less than the original volume of the starting material (15 versus 25 ml), dialyzed against PBS, applied to a polylysine substratum, and plated with motoneurons, it proved to be negative in the neurite outgrowth test (Fig. 8). The precipitate was fractionated on a column of Sepharose CL-6B, and the peak of neurite outgrowth-promoting activity eluted near the void volume of the column. (The exclusion limit of Sepharose 6B is $\sim 4 \times 10^6$ Da for globular proteins and $\sim 10^6$ Da for polysaccharides). The void volume fraction, containing the partially purified substratum factor, was designated "NOPA" (for "neurite outgrowth-promoting activity"). The salt fractionation and chromatographic properties of the NOPA from MCM correspond to those of the neurite outgrowth-promoting factor from BCE (Lander *et al.*, 1982, 1983b).

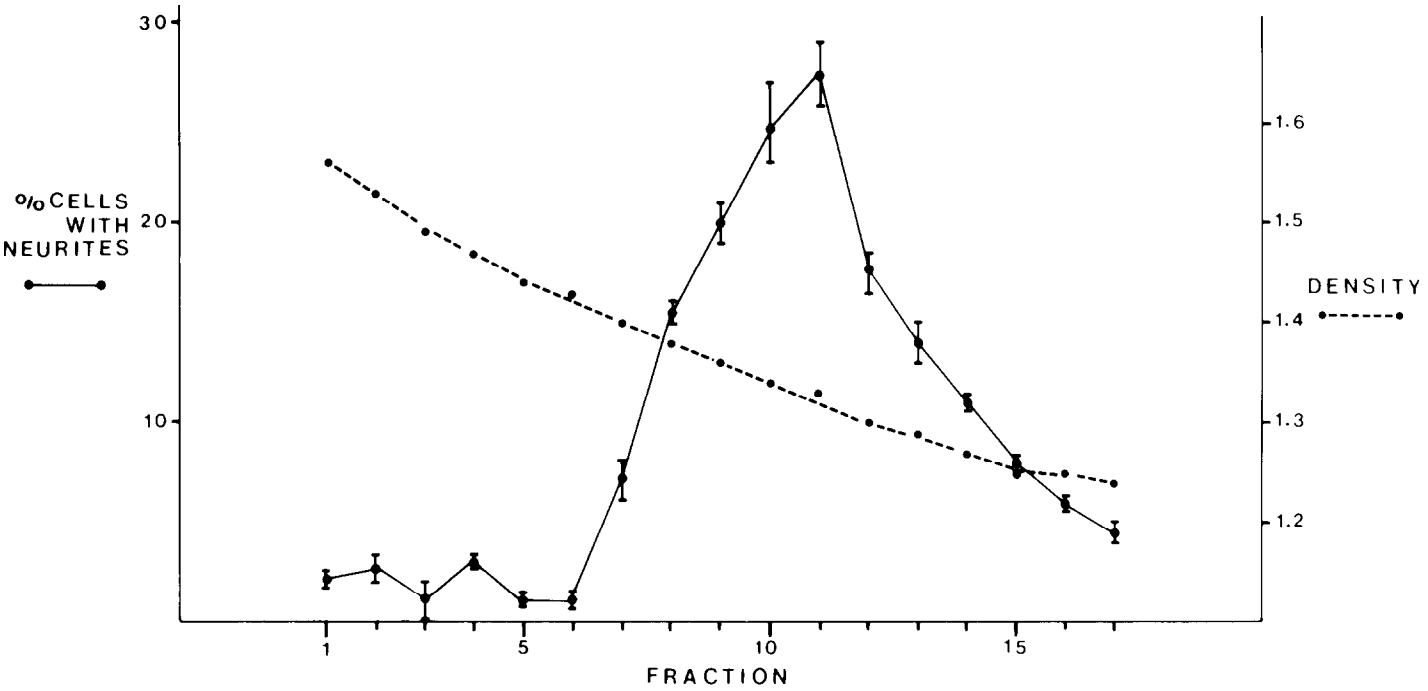

FIG. 7. Isopycnic sedimentation in associative cesium chloride gradient. MCM_{SF} was concentrated and centrifuged in CsCl containing 0.4 *M* GuHCl, as described. The densities of fractions were measured, and fractions were dialyzed and applied to polylysine substrata. Treated surfaces were washed and plated with motoneurons. After 24 hr, duplicate cultures were fixed and cells with neurites were counted. Error bars show means ± 1 SD.

Effects of Antibodies to Laminin and Nerve Growth Factor on Motoneuron Neurite Outgrowth

Conditioned medium is not the only substance that can bind to a substratum and promote neurite outgrowth by motoneurons. The basement membrane glycoprotein laminin is also effective when applied to a polylysine substratum at a concentration of 10 μg/ml (Fig. 9). The experiments shown in Fig. 9 demonstrate that laminin's neurite outgrowth-promoting effect can be blocked by anti-laminin antibodies present in the culture medium. These same antibodies do not block the neurite outgrowth-promoting effect of the MCM_{SF} factor. Similar results have been obtained with anti-laminin antiserum treatment of cultures of rat sympathetic neurons: the antibodies block neurite outgrowth on laminin but not on the BCE factor (Lander *et al.*, 1983b). Preimmune serum and antiserum to nerve growth factor, both present in the culture medium, had no effect on motoneuron neurite outgrowth.[1]

MCM_{SF} in the Culture Medium Allows Motoneurons to Survive

Although purified motoneurons survive and extend neurites 1 to 2 days when grown on substrata treated with MCM_{SF}, they begin to deteriorate unless conditioned medium is also included in their culture medium. This is illustrated in Fig. 10, which shows the numbers of healthy cells with neurites in motoneuron cultures grown for 4 days. Motoneurons grown in serum-free medium on polylysine substrata never develop neurites to any appreciable extent. Motoneurons grown on substrata that have been treated with MCM_{SF} extend neurites and remain healthy for about 2 days in culture, but by Day 4 have deteriorated, as shown by the decrease in the number of healthy neurons with neurites in the cultures. However, if serum-free myotube-conditioned medium is included in the culture medium, as well as being present on the substratum, motoneurons grow and remain healthy for at least 4 days in serum-free culture.

This apparent trophic activity of myotube conditioned medium could be explained in several ways: (1) Myotube-conditioned medium could contain a particular trophic factor, which is necessary for motoneuron survival in culture; or (2) motoneurons might take up and use up the substratum-bound factor from myotube conditioned medium. If so, they might require a continuous supply of the factor. This could be provided by the conditioned medium that had been added to the culture medium. Finally, (3) the suvival-promoting effect of myotube-conditioned medium may not be due

[1] See *Note added in proof.*

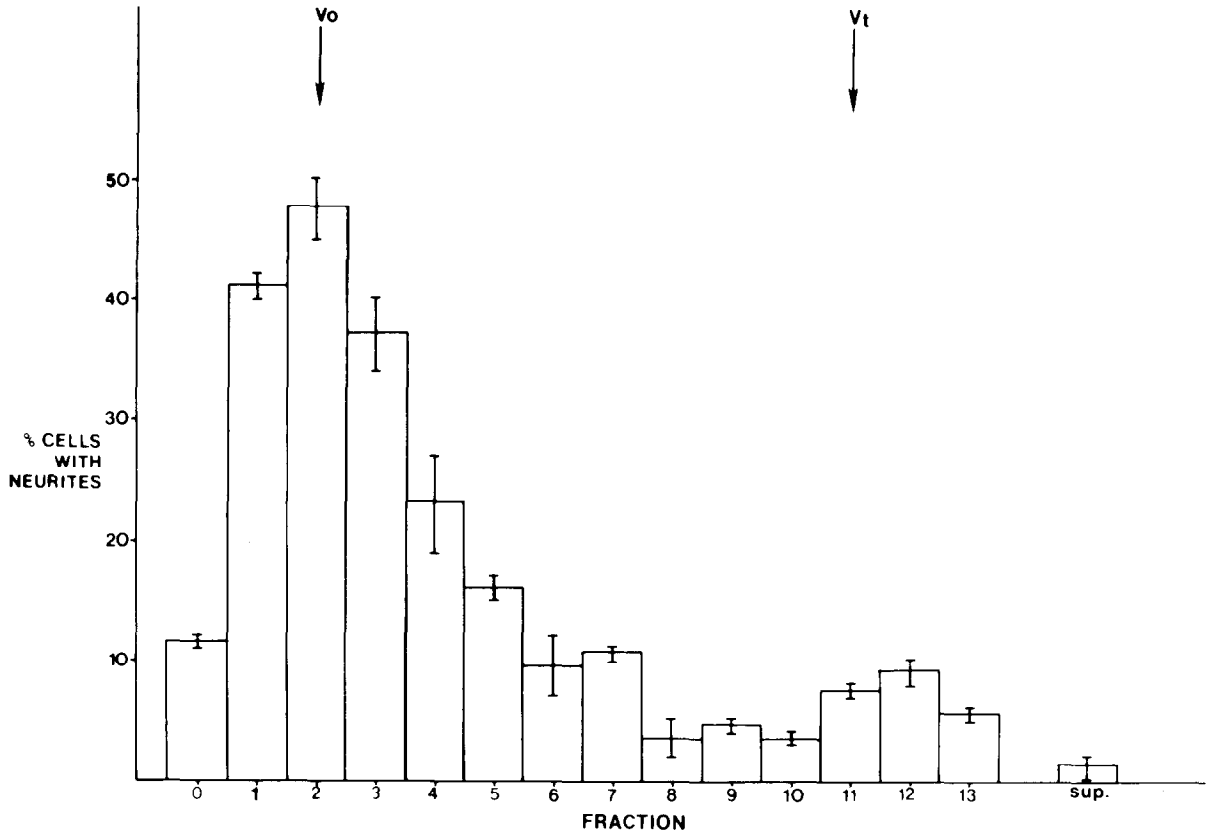

FIG. 8. Ammonium sulfate precipitation of MCM: fractionation of precipitate on Sepharose CL6B. MCM was precipitated with 45% saturated ammonium sulfate as described. The pellet was resuspended in column buffer and applied to a Sepharose CL6B column (see text). The supernatant was concentrated threefold by dialysis against polyethylene glycol (Aquacide III, Calbiochem. Behring Corp.), and dialyzed against PBS. Samples of the column fractions, as well as a sample of the concentrated supernatant, were applied to polylysine substrata and tested for neurite outgrowth-promoting activity. Motoneurons were grown for 24 hr, fixed and counted. sup., concentrated ammonium sulfate supernatant; V_0, void volume; V_t, total included volume. Error bars show means ± 1 SD.

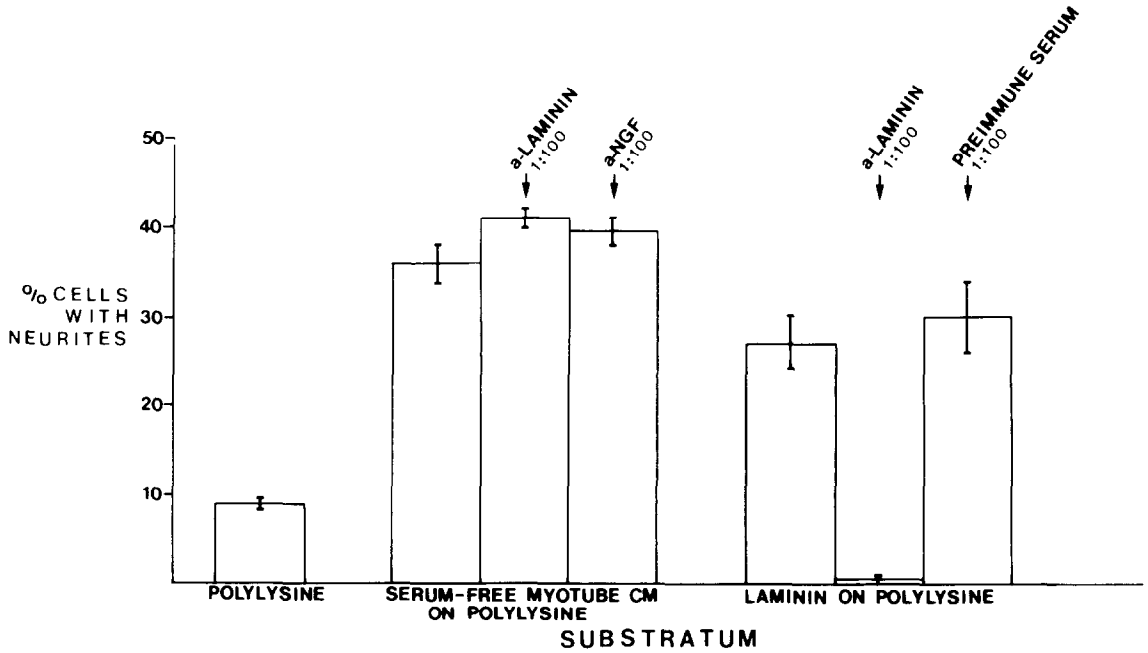

FIG. 9. Effects of antisera on motoneuron neurite outgrowth. Sorted motoneurons were plated onto polylysine substrata that had been treated with MCM_{SF} or laminin (10 μg/ml). Motoneuron growth medium contained 3% horse serum and was supplemented as indicated with preimmune or immune rabbit sera diluted 100-fold. Motoneurons were fixed at 24 hr and the neurite outgrowth in duplicate cultures was evaluated. Error bars represent means ± 1 SD. The same results were obtained with sera diluted 50-fold (data not shown).

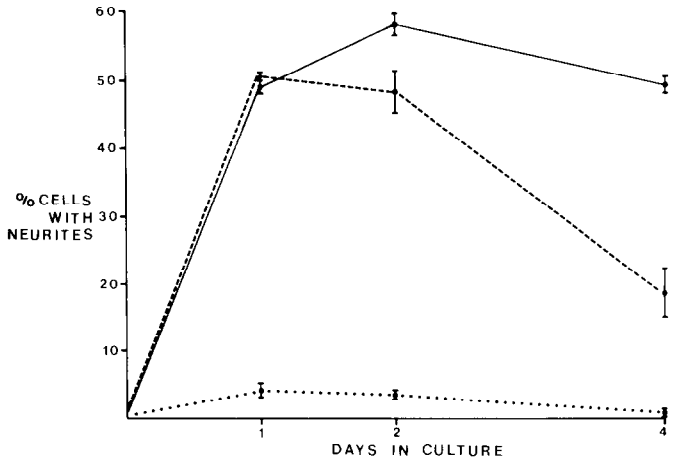

FIG. 10. Effect of MCM_{SF} added to motoneuron culture medium. Motoneurons were grown under different substratum and medium conditions. Duplicate cultures were fixed at 1, 2, and 4 days. The percentage of healthy cells with neurites was determined. (· · ·) untreated polylysine substratum, unsupplemented serum-free growth medium; (- - -) polylysine substratum treated with MCM_{SF}, unsupplemented serum-free growth medium; (——) polylysine substratum treated with MCM_{SF}, serum-free growth medium supplemented with MCM_{SF} (to 50%). Error bars represent means ± 1 SD.

to the presence of any specific trophic factor, but may instead be due to a general medium conditioning effect.

Several experiments were performed to test these hypotheses. In order to determine whether the addition of MCM_{SF} to the culture medium benefits motoneurons simply because it provides a continuous supply of the substratum-binding factor, the following was done: the substratum-binding factor was partially purified from MCM by ammonium sulfate precipitation and gel filtration as shown in Fig. 8. This partially purified fraction (NOPA) was then added back to the medium of motoneuron cultures every 2-3 days for 6 1/2 days to see whether it could enhance motoneuron survival. The results are shown in Fig. 11. While the addition of MCM_{SF} to motoneuron growth medium promotes survival, the addition of the partially purified substratum-binding factor (NOPA) has no effect. It is unlikely that this lack of effect reflects toxic or inhibitory substances that are present in the NOPA fraction, since the addition of *both* MCM_{SF} and NOPA to cultures is as effective as MCM_{SF} alone. Instead, it must be concluded that MCM_{SF} supplies motoneurons with something more than just the substratum-binding factor.

The extent of the difference between motoneurons grown in the presence and absence of MCM_{SF} is reflected dramatically in cell body size and neurite morphology. This is shown in Fig. 12. At short times (1-2 days) in culture, neurons grown on the partially purified neurite outgrowth-promoting factor (NOPA) look similar, whether or not MCM_{SF} is included in the culture medium (Figs. 12D, G). However, by 4 days in culture, cells on the NOPA substratum still have neurites, but appear to be deteriorating (Fig. 12H). This is especially apparent when these cells are compared with motoneurons grown for 4 days on NOPA in the presence of MCM_{SF} (Fig. 12E). These motoneurons have larger cell bodies and larger numbers of longer, thicker neurites. By 6 1/2 days in culture these differences in cell morphology and survival are even greater (Figs. 12F, I) and are reflected in the graphs of Fig. 11. Since changes in morphology indicating deterioration were noticed before this deterioration was advanced enough to show in cell counts, the graphs in Fig. 11 are probably delayed with respect to the real time course of motoneuron survival under these conditions.

Another possible explanation of the apparent trophic activity of myotube-conditioned medium is a general medium conditioning effect, such as the secretion by other cells of nutritive substances which the motoneurons do not themselves make in culture, or which they make in insufficient quantities for their own utilization (e.g., Ham, 1973). If the survival promoting effect of myotube-conditioned medium is due to such a general conditioning effect, then media conditioned by other cell types might be able to substitute for MCM_{SF}. To test this possibility, motoneurons were plated onto the partially purified NOPA substratum and grown for 6 1/2 days in growth medium supplemented with serum-free conditioned medium from myotubes (MCM_{SF}), spinal cord dividing cells ($SCCM_{SF}$), or fibroblasts (FCM_{SF}) (Fig. 13A). Survival of motoneurons over 6 1/2 days in culture is enhanced to some extent by the presence of any of these conditioned media. However, motoneurons grown in MCM_{SF} show initial neurite outgrowth greater than that of motoneurons grown in the other conditioned media. Furthermore, the percentage of healthy motoneurons at 6 1/2 days in culture is greater in cultures supplemented with MCM_{SF} than in cultures supplemented with the other conditioned media.

The results at 1 day in several experiments indicated that motoneurons grown in $SCCM_{SF}$ and FCM_{SF} had their initial outgrowth depressed relative to that of cells grown in MCM_{SF} or even in the absence of any conditioned medium supplement (Fig. 13A). To see whether this apparent effect might be due to the presence of toxic or inhibitory substance(s) in the $SCCM_{SF}$ and FCM_{SF}, motoneurons were grown in these media combined with small amounts of MCM_{SF} (final medium: 50% $SCCM_{SF}$ or FCM_{SF}, 20% MCM_{SF}). Motoneurons grown in the combined conditioned media showed initial neurite outgrowth and long-term survival that equaled or exceeded that seen in medium supple-

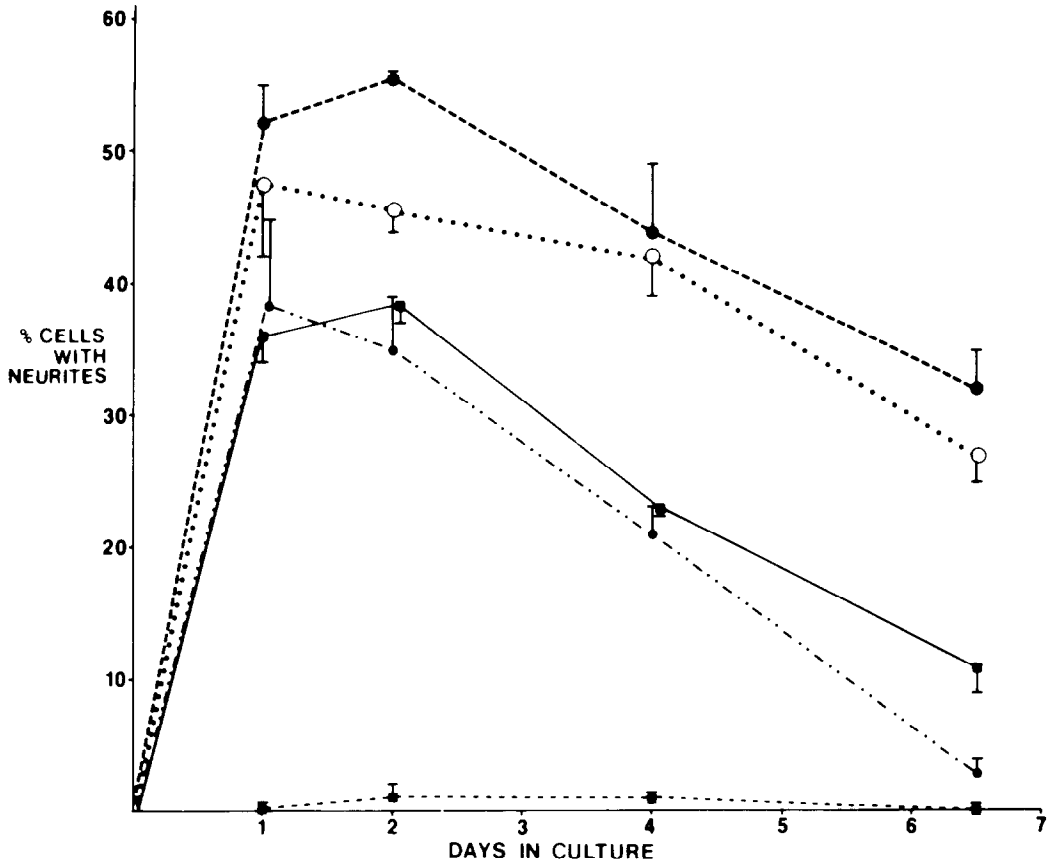

FIG. 11. Motoneuron survival is not enhanced when the substratum-binding factor is added back to the medium. Partially purified substatum-binding factor (NOPA) was prepared from 25 ml of MCM by ammonium sulfate precipitation and gel filtration. It was recovered in a final volume of 5 ml, in a buffer containing 200 m*M* NaCl/20 m*M* Hepes, pH 7.4/0.2 mg/ml hemoglobin. Motoneurons were grown under the following conditions and fixed and evaluated at the indicated times: (---■---) untreated polylysine substratum, unsupplemented serum-free growth medium; (—■—) NOPA-treated substratum, unsupplemented serum-free growth medium; (---●---) NOPA-treated substratum, serum-free growth medium supplemented with 50% MCM_{SF}; (-···●-···) NOPA-treated substratum, serum-free growth medium supplemented with 20% NOPA fraction and sufficient distilled water to readjust the salt concentration to 145 mOsm; (···○···) NOPA-treated substratum, serum-free growth medium supplemented with *both* MCM_{SF} (50%) and NOPA (20%) plus distilled water as above. The percentage of healthy cells with neurites is given as the means of duplicate cultures ± 1 SD.

mented with standard amounts (50%) of MCM_{SF} (Figs. 13B, C). Since the initial depression of neurite outgrowth seen when motoneurons are grown in $SCCM_{SF}$ and FCM_{SF}-supplemented media can be overcome by the addition of low levels of MCM_{SF}, it is unlikely that large amounts of toxic or inhibitory substances are present in these media.

DISCUSSION

Evidence has been presented that myotube-conditioned medium (MCM) exerts at least two, separable actions on motoneurons in culture. First, MCM contains a neurite outgrowth-promoting factor which has been shown to be effective when adsorbed to the culture substratum alone. This factor appears to be similar to the neurite outgrowth-promoting factor (BCE factor) purified by Lander and colleagues from bovine corneal endothelial cell conditioned medium, and known to be present in media conditioned by many other cell types (Lander *et al.*, 1982, 1983a,b). Its effects are evident at short times (24 hr), it is susceptible to the same enzymes, has about the same buoyant density in cesium chloride, and behaves similarly in salt fractionation and gel filtration experiments. In the same manner as the BCE factor, the substratum-binding, neurite outgrowth-promoting activity from MCM can be duplicated by conditioned media from other cell types, and can act upon different neurons. The factor appears to contain protein and heparan sulfate.[2]

Several investigators have reported that myotube conditioned medium or muscle extracts can enhance neurite outgrowth from spinal cord cells (Dribin and Barrett, 1980, 1982; Henderson *et al.*, 1981; Obata and Tanaka, 1980). The results of these studies are difficult

[2] See *Note added in proof.*

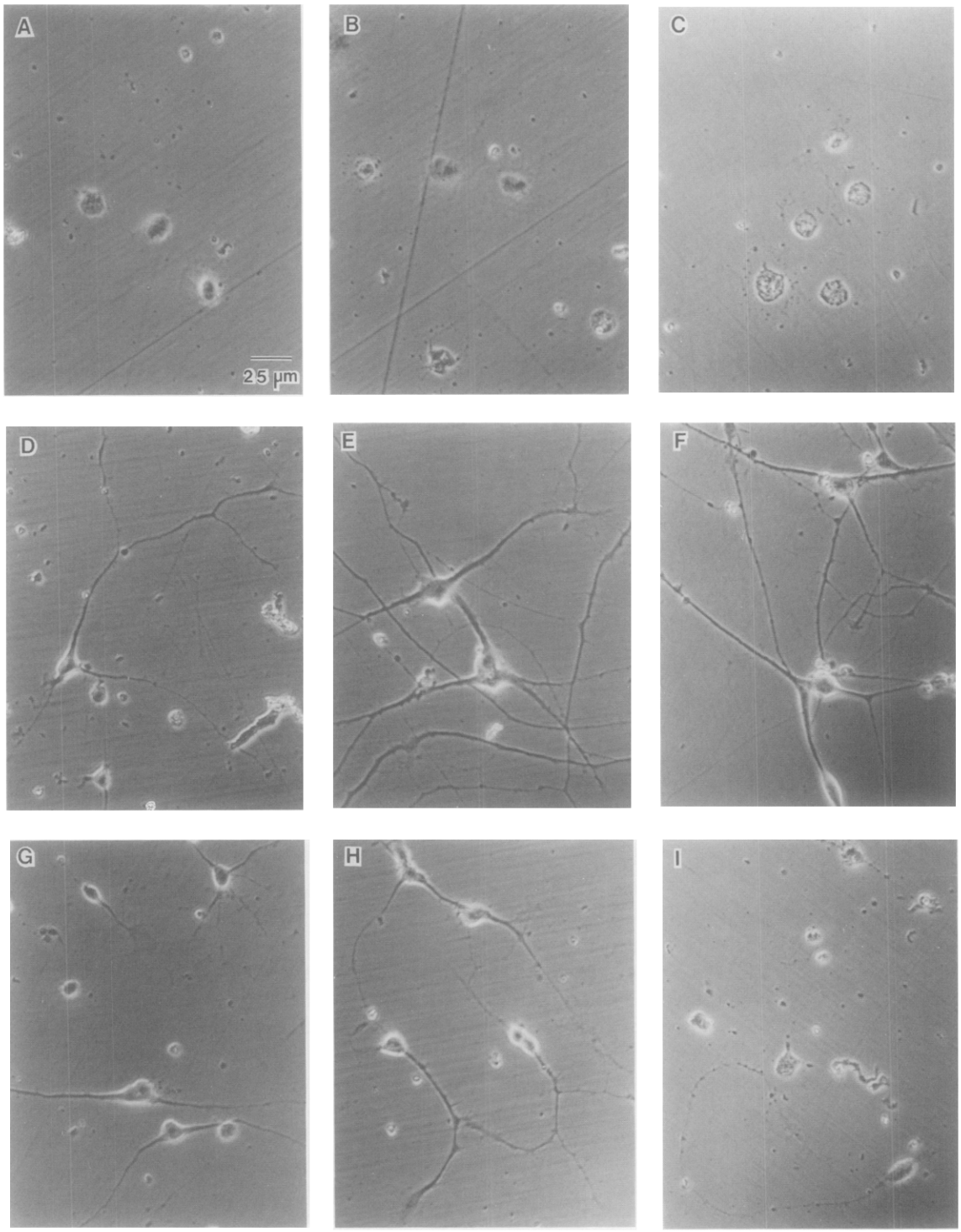

FIG. 12. Motoneurons grown under conditions described in Fig. 10. (A–C) untreated polylysine substratum, unsupplemented serum-free growth medium, cells fixed at 1 day (A), 4 days (B), and 6 1/2 days (C). (D–F) NOPA-treated substratum, serum-free growth medium supplemented with 50% MCM_{SF}; cells fixed at 1 day (D), 4 days (E), and 6 1/2 days (F). (G–I) NOPA-treated substratum, serum-free growth medium supplemented with 20% NOPA as described in Fig. 11; cells fixed at 1 day (G), 4 days (H), and 6 1/2 days (I).

to compare with each other and with the present study. There are differences in test conditions, differences in embryonic ages of cultured cells, and the further difficulty of identifying responding cells in the spinal cord cultures other investigators have employed. However, Dribin and Barrett (1982) have reported a neurite outgrowth-promoting activity that acts by adsorbing to the culture substratum. That a substratum-binding

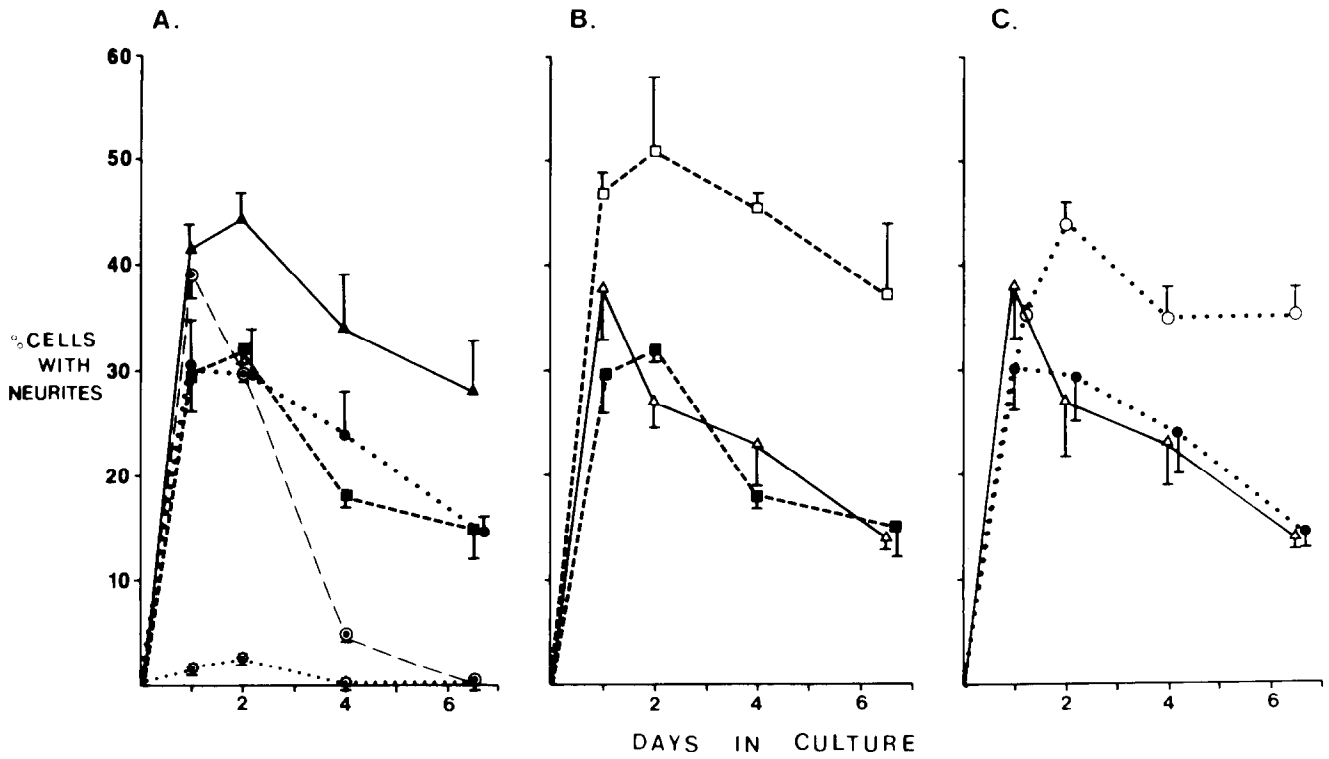

FIG. 13. Effect of different conditioned media on motoneuron survival. Motoneurons were grown under the following conditions and fixed and evaluated at the indicated times: (A) (⊙) untreated polylysine substratum, unsupplemented serum-free growth medium. (⊙) NOPA-treated substratum, unsupplemented serum-free growth medium. (■) NOPA-treated substratum, serum-free growth medium supplemented with 50% $SCCM_{SF}$. (●) NOPA-treated substratum, serum-free growth medium supplemented with 50% FCM_{SF}. (▲) NOPA-treated substratum, serum-free growth medium supplemented with 50% MCM_{SF}. (B) (△) NOPA-treated substratum, serum-free growth medium supplemented with 20% MCM_{SF}. (■) NOPA-treated substratum, serum-free growth medium supplemented with 50% $SCCM_{SF}$. (□) NOPA-treated substratum, serum-free growth medium supplemented with 50% $SCCM_{SF}$ plus 20% MCM_{SF}. (C) (△) NOPA-treated substratum, serum-free growth medium supplemented weith 20% MCM_{SF}. (●) NOPA-treated substratum, serum-free growth medium supplemented with 50% FCM_{SF}. (○) NOPA-treated substratum, serum-free growth medium supplemented with 50% FCM_{SF} plus 20% MCM_{SF}.

component of myotube conditioned medium can promote motoneuron neurite outgrowth has been confirmed in this study. Only one peak of neurite outgrowth-promoting activity has been seen during the fractionation procedures employed in the present study, and this material behaved in a manner that establishes its similarity to the BCE factor purified by Lander and colleagues (1982, 1983a).

In addition to its neurite outgrowth-promoting activity, MCM in the culture medium of motoneurons enhances their survival over periods of greater than 2 days in culture. This is consistent with the results of Bennett *et al.* (1980) who found that motoneurons—labeled *in ovo* with horseradish peroxidase and later identified in the cultures—needed myotube-conditioned medium to survive for 48 hr in mixed spinal cord cultures. There are several possible explanations of this apparent trophic activity, and two were examined in different tests.

Motoneurons might take up and use up the substratum-binding factor during the course of long-term cultures, and therefore might require a continuous supply of the factor to maintain their neurites and remain healthy. To rule out this possibility, the substratum-binding factor was partially purified from MCM and added back to the medium of motoneuron cultures every 2 to 3 days for 6 1/2 days. Cultures supplemented in this way fared no better than cultures grown in medium without supplement.

It is also possible that the survival-enhancing effect of MCM added to motoneuron culture medium is due to a general medium conditioning effect. Such an effect could be due to the secretion by other cultured cells of nutritive substances which the motoneurons do not themselves make in culture, or which they make in insufficient quantities for their own utilization. This possibility is certainly consistent with the results reported here, although it is not the only possible explanation of them. A finding that medium conditioned by myotubes was the only type of conditioned medium capable of supporting motoneuron survival would have suggested that a target-specific trophic molecule was involved. A finding that any type of conditioned medium was as effective as myotube conditioned medium would

have argued in favor of a general medium-conditioning effect. In fact, the experimental data lie somewhere between these two possibilities. MCM_{SF}, $SCCM_{SF}$, and FCM_{SF} all enhanced motoneuron survival, but more motoneurons survived for extended periods in MCM_{SF} than in FCM_{SF} or $SCCM_{SF}$. The results of these tests of different conditioned media are complicated, and must be interpreted with caution. Despite their ability to enhance motoneuron survival over the course of 1 week, FCM_{SF} and $SCCM_{SF}$ appeared to depress initial neurite outgrowth (24 hr) compared to controls (serum-free growth medium alone). This indicates that substances which interfere with initial cell attachment, survival, or neurite outgrowth might be present in FCM_{SF} and $SCCM_{SF}$. Considering that the effects of such substances would be superimposed on any survival-enhancing effects, it is not possible—on the basis of the data presented—to determine accurately the relative quantities of survival-enhancing substance(s) in the three conditioned media.

Finally, it is possible that the survival-enhancing activity of myotube-conditioned medium is due to the presence in it of a "motoneuron trophic factor." However, survival of all motoneurons over the course of 1 week in culture did not occur, even under the optimum conditions tested in these experiments (motoneuron growth medium supplemented with 50% MCM_{SF}). This suggests that motoneurons may require further trophic support than that provided by MCM_{SF}. It may be that myotubes in culture do not secrete an appropriate "motoneuron trophic factor." Alternatively, myotube conditioned medium may contain too little of such a trophic factor, or the factor may be present in too degraded a state, to be completely effective. If such a factor can be identified in MCM_{SF}, it will be interesting to see whether production of it *in vivo* is regulated during embryogenesis in a way consistent with the known schedule of motoneuron development. The limited production by muscle of such a factor, or limitations in the access of motoneurons to it, may be influential in naturally occurring motoneuron death. If no trophic factor is present in MCM_{SF}, i.e., if the survival-promoting activity of MCM_{SF} is entirely due to nutritive substances, it will be important to determine whether or not a motoneuron trophic factor exists, and can be isolated from some other source.

It is noteworthy that, for the first 2 days in culture, motoneurons plated on an appropriate substratum survive well without any added conditioned medium, raising the possibility that motoneurons may not absolutely require any survival factor, in the way that some neurons require nerve growth factor. However, other explanations are consistent with these findings. The motoneurons were taken from the embryo just prior to the period of naturally occurring cell death. At this point, they may not have required any trophic factors for their survival, but they may have developed this requirement with time in culture. An alternative explanation is that the motoneurons required a trophic factor at the time of plating, but could survive a few days in its absence because the culture substratum was optimal. This phenomenon has been observed in other systems. For example, rat sympathetic neurons—which require nerve growth factor for long-term survival *in vivo* and *in vitro*—can survive and extend neurites for at least 2 days in the absence of NGF, provided that they have plated onto extracellular matrix produced *in vitro* (Lander *et al.*, 1982). Chick parasympathetic neurons, when cultured on an appropriate substratum, can be maintained 6 days without any survival factor (Nishi and Berg, 1981); on untreated polylysine or collagen substrata, they survive for only 24 hr unless an appropriate trophic factor is supplied to them (Varon *et al.*, 1979).

In summary, the requirements of spinal motoneurons in culture are beginning to be elucidated. Neurite outgrowth appears to be dependent on the presence on the culture substratum of a factor present in MCM_{SF} and other conditioned media. Long-term survival of motoneurons can be enhanced by the addition of conditioned media to the growth medium. This may reflect the presence in conditioned media of a trophic molecule, nutritive substances, or both.

Note added in proof. Our recent results indicate that the major protein component of the substratum-binding, neurite outgrowth-promoting activity in MCM is laminin. Affinity-purified antibodies directed against laminin can immunoprecipitate all neurite outgrowth-promoting activity (NOPA) from MCM. SDS-polyacrylamide gels of the immunoprecipitated NOPA show a single protein band that comigrates with authentic laminin. Upon reduction it is converted to two bands that comigrate with the heavy and light chains of laminin (M_r's of $\sim 0.4 \times 10^6$ and $\sim 0.2 \times 10^6$). When MCM is fractionated by ammonium sulfate precipitation, ion exchange chromatography, and gel filtration, the peak of NOPA is associated with a peak of laminin immunoreactivity. This purified NOPA also contains a protein that comigrates with authentic laminin in nonreducing SDS-polyacrylamide gels. Results of electrophoresis under reducing conditions suggest that the heavy chain of MCM "laminin" may undergo proteolytic degradation during purification. Laminin, in association with laminin-binding molecules, appears to comprise the substratum-binding, neurite outgrowth-promoting factor found in conditioned media (CM's) from a variety of other cell types (A. D. Lander *et al.*, *Soc. Neurosci. Abstr.* **10**, 40). The fact that antibodies to purified laminin fail to block the activity of substrata treated with CMs, including MCM (Fig. 9), may reflect structural differences between CM-derived laminin and laminin derived from other cell types or species, or it may reflect the fact that molecules bound to laminin in CMs prevent antibodies from gaining access to important laminin epitopes (A. D. Lander, personal communication).

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