Survival in vitro of motoneurons identified or purified by novel antibody-based methods is selectively enhanced by muscle-derived factors

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Summary

Motoneurons were identified in vitro by a new method using the SCI monoclonal antibody. They constituted up to 30% of total neurons in cultures of whole spinal cord from 4.5-day chicken embryos, and survived for at least 5 days in the presence of muscle extract, but not in its absence. By contrast, other neurons and floor-plate cells survived without muscle-derived factors. Motoneurons were purified to homogeneity by 'panning' on dishes coated with SCI antibody; they developed rapidly even in the absence of other spinal cells. Concentrations of muscle extract required for half-maximal motoneuron survival were indistinguishable in pure and mixed cultures, suggesting that muscle-derived factors act directly on motoneurons. Other purified growth factors tested, including ciliary neurotrophic factor, did not have the survival-promoting activity of muscle.

Key words: motoneuron growth factors, chicken spinal cord, panning, ciliary neurotrophic factor, fibroblast growth factor.

Introduction

In terms of its function in the adult animal, the spinal motoneuron is one of the best-studied of all the cells of the nervous system. Furthermore, the timing and pattern of its early development in the embryo are well described in several species. It was using this system, for example, that Hamburger and colleagues (reviewed in Hamburger, 1977) first provided evidence for the existence of muscle-derived motoneuron growth factors necessary for motoneuron survival at early stages, and probably involved in regulation of many subsequent key events in embryonic and neonatal motoneuron development (Henderson, 1988; Oppenheim and Havercamp, 1988). That these factors remain still to be identified (Barde, 1989) reflects, at least in part, the cellular complexity of the spinal cord and hence of cultures prepared from it. Until quite recently, it was not possible to be sure that a putative trophic substance actually enhanced survival or neurite outgrowth of motoneurons as opposed to other neurons in these cultures, and it remains impossible to ascertain whether such a factor affects only motoneurons.

Faced with these difficulties, investigators have adopted two complementary approaches: (i) identification of motoneurons in mixed cultures; (ii) enrichment or purification of motoneurons before culturing. For example, antibodies against choline acetyltransferase have been used to identify cholinergic neurons in cultures of spinal cord (Smith et al. 1986). However, such antibodies are difficult to use with early embryonic cells and are not completely specific, since other cholinergic neurons appear at early stages in the neural tube. The most widely used technique for identification has involved back-filling by retrograde transport of fluorescent or enzyme tracers injected in the limb bud (Eagleson and Bennett, 1983; Tanaka and Obata, 1983; Calof and Reichardt, 1984; O'Brien and Fischbach, 1986; Schaffner et al. 1987; Smith et al. 1986; Tanaka, 1987; Martinou et al. 1989, 1990). Providing that appropriate precautions are taken to prevent diffusion of tracer from the site of injection, such methods provide unambiguous identification of motoneurons projecting to the limb. However, they present several disadvantages. First, only motoneurons that are sufficiently developed to transport the tracer can be labelled. Second, not all the motoneurons in a given segment of spinal cord are labelled: unlabelled populations can thus comprise both motoneuronal and non-motoneuronal cells. Third, the tracer itself may affect development in culture, or be lost quite rapidly. Such phenomena probably help to explain the wide variation in motoneuron abundance reported by investigators using apparently very similar culture systems (for a detailed comparison, see Henderson, 1988).

The major advantage of retrograde labelling using
fluorescent tracers has been to allow the preparation of highly enriched motoneuronal populations by use of the fluorescence-activated cell sorter. Indeed, since the physical characteristics of the fluorescent cells have been well-defined, it has proved possible to sort without prior retrograde labelling (Martinou et al. 1989). This approach has provided new insights into factors directly affecting motoneuron development in vitro but is costly and labour-intensive. For this reason, other workers have routinely enriched cultures for motoneurons: (a) by dissection at early stages, at which motoneurons are relatively abundant (Masuko et al. 1979; Berg and Fischbach, 1978; Henderson et al. 1981, 1983; Longo et al. 1982); (b) by culture of the ventral regions of the neural tube (Smith et al. 1985); or (c) by use of density gradient fractions in which choline acetyltransferase activity is concentrated (Schnaar and Schäffner, 1981; Flanagan et al. 1985; Doehrmann et al. 1987; Martinou et al. 1989). It is clear, however, that such techniques cannot provide purification of motoneurons to the same extent as cell sorting.

In 1984, Tanaka and Obata described a monoclonal antibody named SCI which, in transverse sections of embryonic chicken spinal cord, labelled motoneurons, ventral epithelium (floor-plate cells) and sensory fibers in the dorsal funiculus. The labelling of motoneurons disappeared by 7 to 8 days of incubation and was highly specific to chick. We show now that this antibody can be used to provide unambiguous identification of motoneurons in vitro. Using appropriate embryos and culture conditions, motoneurons can represent up to 30% of the total population, and all of them, but not other spinal cord cells, need exogenous factors if they are to survive in culture. Furthermore, the SCI antibody can also be used to purify motoneurons by the recent technique of panning (Barres et al. 1988). Muscle extracts, but not any known growth factor tested, can keep such purified cells alive for days.

Materials and methods

Neuronal cell cultures

Spinal cords were dissected from 4.5-day Leghorn chicken embryos (Hamburger–Hamilton stage 24–25, incubation at 37.6°C), treated with trypsin and dissociated as described elsewhere (Henderson et al. 1984). They were cultured in Ham's F12 medium supplemented with penicillin (100 I.u. ml\(^{-1}\)), streptomycin (100 μg ml\(^{-1}\)), glutamine (2 mm), insulin (10 μg ml\(^{-1}\)) and glucose (10 mm) on 12-mm glass coverslips. These had been coated with polyornithine (1.5 μg ml\(^{-1}\); 30 000 M, Sigma) for 30 min at room temperature and then incubated with laminin from EHS sarcoma at 3 μg ml\(^{-1}\) in F12 medium for at least 2 h in the CO\(_2\) incubator. Supplemented, muscle extract etc. were added to each well (Costar 24-well plates; 600 μl per well) and these were then seeded with 30 000 spinal cord cells. Cultures were incubated at 37.2°C in 5% CO\(_2\)/95% air and saturating humidity. Cultures of dorsal and ventral spinal cord and of other brain regions were prepared in the same way (Taguchi et al. 1986). Basic FGF (Boehringer) and TGF-beta (R & D Systems) were used according to suppliers' instructions. Ciliary neurotrophic factor (CNTF; a generous gift of K. Wewetzer and K. Unsicker) was purified as described by Barbin et al. (1984), except that in one case the gradient centrifugation was replaced with chromatography on heparin–Sepharose. Both preparations of CNTF showed a single band after SDS-polyacrylamide gel electrophoresis.

Immunofluorescence using SCI antibody

At indicated times of culture, neurons were fixed with acetone at -20°C, either following removal of all culture medium or by adding an excess of acetone in the presence of culture medium. They were rinsed with PBS, incubated with supernatant from hybridoma SCI (dilution 1:10) containing 15% fetal calf serum for 30 min at 37°C, washed again in PBS and fixed with 10% formaldehyde for at least 10 min at room temperature. After five washes with PBS containing 2% BSA (w/v), the cells were incubated with biotinylated goat anti-mouse IgG (dilution 1:100; Amersham) for 30 min at 37°C, washed, and incubated with phycoerythrin–streptavidin complex (dilution 1:50; Amersham), or with fluorescein–streptavidin (dilution 1:100; GIBCO-BRL) following the supplier's instructions. After stabilization of the phycoerythrin, samples were mounted in either the mountant provided for phycoerythrin, or with Citifluor mountant for fluorescein. They were observed using standard fluorescence optics; no fading was observed even over long periods. No staining was observed when antibody SCI was replaced with an irrelevant mouse IgG, or when the biotinylated antibody was replaced by biotinylated goat anti-rabbit antibody (Amersham) or omitted. Similar staining patterns were obtained when SCI binding was revealed using a TRITC-coupled goat anti-mouse antibody (Cappel, not shown).

Immunofluorescence on cell suspensions

After trypsinization and dissociation, SCI supernatant was added to the suspension of total spinal cord cells at a final dilution of 1:10 and incubated at 37°C for 30 min in the CO\(_2\) incubator. Cells were then washed on a 20% metrizamide cushion in the presence of DNAase (0.1 mg ml\(^{-1}\)) by centrifugation for 15 min at 1200 revs min\(^{-1}\). The cells were then resuspended in 1 ml culture medium in the presence of biotinylated secondary antibody (diluted 100-fold) for 30 min at 37°C. After further washing, streptavidin–fluorescein (1:100) was added at 37°C for 30 min. The cells were then washed and mounted after fixation, or observed directly.

Tissue extracts and culture supplements

Extracts of innervated neonatal chick muscle were prepared as described (Henderson et al. 1983). Briefly, muscles were homogenized in the presence of a cocktail of protease inhibitors and centrifuged. Aliquots of the high-speed supernatant were stored at -20°C. Unless otherwise indicated, neonatal muscle extract was used at a protein concentration of 6 μg ml\(^{-1}\). Laminin was prepared from homogenates of the mouse EHS sarcoma (generously provided by Marc Vigny).

Preparation of panning dishes

Polyethylene Petri dishes (100 mm) were coated with 100 μg ml\(^{-1}\) of secondary antibody (affinity-purified goat anti-mouse IgG, Cappel) in 10 ml Tris buffer pH 9.5 for 12 h at 4°C. Dishes were washed 3 times with PBS and SCI hybridoma supernatant diluted 1:5 in PBS was incubated in the dishes for 1 h at room temperature. Dishes were then washed twice with PBS and incubated with BSA (0.2% w/v in F12 medium) for 20 min at room temperature in order to avoid non-specific binding.
Panning procedure
Suspensions of spinal neurons were added to the panning dish (3–5 cord equivalents per plate in 12 ml complete medium). After 1 h incubation at room temperature, each plate was washed 8 times with PBS with gentle swirling to remove non-adherent cells.

Culture of adherent cells from the panning dish
Elution of cells from the dishes was achieved by competition with an excess of SCI (2–3 ml of undiluted supernatant) and slow shaking (about 20 min). Dishes were rinsed with 2×1 ml culture medium and eluted cells combined with the first fraction. Any cells remaining attached were discarded. SCI-positive cells (approx. 20,000 per cord) were washed by centrifugation at 1000 revs min⁻¹ for 10 min, and seeded on polyornithine and laminin (PORN–laminin)-coated coverslips at a density of 25,000 per 16 mm culture well.

Elimination of floor-plate cells by subdissection
Spinal cords dissected from 4.5-day embryos were divided using micro-scissors along a line dorsolateral to the limits of the floor-plate region stained by SCI antibody. The portion containing the right-hand anterior horn still attached to the floor plate was discarded, only the left anterior horn being retained for subsequent panning.

Elimination of floor-plate cells by centrifugation
Before panning, spinal cord cells were centrifuged for 15 min at 4000 revs min⁻¹ on a 6.8% metrizamide cushion in F12 medium containing 0.1 mg ml⁻¹ DNAase. Only those cells retained by the metrizamide cushion were used in the panning procedure.

Results
Identification of motoneurons in mixed cultures using SCI antibody
We first confirmed the specificity of the SCI antibody using the phycoerythrin–streptavidin complex as fluorophore (Fig. 1). As described by Tanaka and Obata (1984), the only cells labelled within the 5-day embryonic chick spinal cord were the motoneurons and floor-plate cells. Ventral roots and dorsal root ganglia were also intensely stained; however, the latter are completely removed during dissection of the spinal cord.

Cells from different regions of the central nervous system of 4.5-day chicken embryos were cultured on glass coverslips coated with polyornithine and laminin (PORN–laminin), in serum-free F12 medium supplemented with neonatal muscle extract (see Materials and methods). After 2 days in culture, coverslips were fixed with acetone, incubated with SCI antibody and processed for indirect immunofluorescence. No labelling was observed in cultures of embryonic telencephalon or mesencephalon, or of cells from the dorsal half of the spinal cord (Table 1). In cultures of ventral or total spinal cord, however, two morphologically distinct cell types were labelled by SCI antibody: large, multipolar neurons (Fig. 2A) and flat cells with a fibroblastic morphology (Fig. 2B). The latter represented between 10% and 30% of the total SCI-labelled population in 2-day cultures of total spinal cord. When anterior horns were freed of floor plate by microdissection, this value fell to <5%. The fibroblast-like cells must therefore be floor-plate cells from the ventral epithelium.

At later stages (Tanaka et al. 1989), SCI also labels some neurons in the column of Terni. In order to exclude the possibility that the SCI-positive neurons observed in vitro were not motoneurons but preganglionic neurons that had not yet migrated, separate cultures were prepared from thoracic, and from lumbar and brachial regions of the spinal cord (not shown). The abundance of SCI-labelled neurons after culture in the presence of muscle extract was at least as great in limb-segment cultures, as expected for motoneurons (Oppenheim et al. 1989). Residual survival of SCI-labelled neurons in basal medium was identical in cultures from different segments. It remains possible that a small number of labelled cells in cultures of total cord are preganglionic neurons, but these can be

![Fig. 1. Staining pattern of 5-day embryonic chicken spinal cord using SCI monoclonal antibody. Acetone-fixed cryostat sections (15 µm) of unfixed embryos were labelled with SCI antibody using phycoerythrin as fluorochrome. Labelling was found on the motoneurons (mn) in the anterior horn, the floor plate cells in the ventral epithelium (ve) and in the dorsal root ganglion (drg) and its processes projecting to the dorsal funiculus. The cell bodies of the ganglionic neurons are completely removed during spinal cord dissection before culture.](image-url)
Table 1. In vitro labelling with SCI antibody reflects in vivo pattern but is not correlated with growth response to muscle extract

<table>
<thead>
<tr>
<th>Neuronal cultures</th>
<th>Growth response to muscle extract</th>
<th>SCI immunoreactivity in vitro</th>
</tr>
</thead>
<tbody>
<tr>
<td>E5 dorsal spinal cord</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>E5 ventral spinal cord</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>E5 telencephalon</td>
<td>+++</td>
<td>—</td>
</tr>
<tr>
<td>E5 mesencephalon</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>E8 dorsal root ganglion</td>
<td>+</td>
<td>+++</td>
</tr>
</tbody>
</table>

1 Taguchi et al. (1986). The few telencephalic neurons stained in situ by SCI (H. Tanaka, unpublished) are lost in culture.

Excluded by using only limb segment cultures (Oppenheim et al. 1989). We therefore consider that SCI antibody unambiguously identifies motoneurons in culture.

Motoneuron survival in mixed cultures is selectively enhanced by muscle-derived factors

The effects of muscle-derived factors on identified motoneuron survival were apparent when total spinal neurons were cultured for 2 days on PORN-laminin in the presence and in the absence of neonatal muscle extract (Fig. 3). In its presence, labelled motoneurons were abundant and well-developed (Fig. 3B). In the absence of muscle-derived factors, whereas other spinal cells survived normally and were visible by their background fluorescence (Fig. 3A), only rare motoneurons were present, together with SCI-positive cell debris. Many floor-plate cells survived in the absence of muscle extract (not shown). In certain experiments, survival even of other cells was enhanced by muscle extract; we attributed this to small variations in basal conditions and not, since this requirement was inconsistently expressed, to a physiologically significant mechanism. Since the mounting medium used for preservation of phycocerythrin fluorescence did not allow cells to be visualised using Nomarski optics, all motoneuron counts presented here are expressed relative to total cells estimated by background labelling. Any cell showing non-neuronal morphology was excluded from counts of either fluorescent or total cells: floor-plate cells were thus not taken into account.

Cultures were fixed after different times in culture, in the presence or absence of muscle extract (Fig. 4). In the initial cell suspension, labelled cells were only weakly fluorescent, as a result of degradation of SCI antigen by trypsin during cell dissociation. Intense SCI labelling was initially seen in intracellular perinuclear vesicles, presumably corresponding to the Golgi apparatus (not shown), and was subsequently expressed at the cell surface on cell body and neurites. When muscle extract was present, full expression of SCI antigen was obtained after 2 to 3 days in vitro on a maximum of 25–30% of total neurons (Fig. 4). This percentage varied from culture to culture in the range 5–30% (5–50% in cultures of ventral spinal cord), perhaps as a function of the exact developmental stage of the embryos, but more likely because of slight variations in the conditions of mechanical dissociation, to which motoneurons are extremely sensitive. From 3 days in culture onwards, the SCI labelling became rapidly more diffuse on individual cells (which were thus impossible to quantify precisely) and was barely detectable after 5 days in culture, although many neurons survived (not shown). This disappearance of labelling, corresponding to a theoretical age of 7.5 to 9.5 days in ovo, most probably reflects the developmentally regulated pattern of expression already reported in vivo (Tanaka and Obata, 1984).

In the absence of muscle extract, only low levels (<5% of total cells) of SCI-positive cells were observed, at all stages of culture (Fig. 4). This difference could be explained by a survival-promoting effect of muscle extract, but equally well if muscle-derived factors were simply acting to increase expression of SCI antigen on cells whose survival is muscle-independent. In order to differentiate between these possibilities, parallel cultures were maintained for 1 day with or without muscle extract. At this point, all cultures were changed to medium containing muscle extract. High percentages of SCI-positive cells were

Fig. 2. Morphology of SCI-labelled cells in cultures of total and ventral spinal cord. Cells from 4.5-day chick embryonic spinal cord were cultured for 2.5 days in the presence of muscle extract and stained by indirect immunofluorescence using SCI monoclonal antibody, as described in Materials and methods. (A) Large multipolar neuron (motoneuron); (B) fibroblast-like cells (floor-plate). Scale bar=50 μm.
Muscle enhances motoneuron survival

Fig. 3. Motoneuron survival in culture is muscle-dependent. Parallel cultures of total 4.5-day spinal cord were seeded in the absence (A) and the presence (B) of neonatal muscle extract at a final protein concentration of 5 μg ml⁻¹. They were processed for SC1 immunofluorescence after 2 days in culture. (A) In the absence of muscle extract, most cells, visible by their background fluorescence, survived after 2 days in serum-free medium, but only fluorescent debris remained of the motoneurons. (B) Several healthy motoneurons after 2 days in the presence of muscle extract. Scale bar=50 μm.

once again only seen in those cultures maintained continuously in the presence of muscle extract (Fig. 5), demonstrating that deprivation of muscle-derived factors for 24 h was sufficient to initiate cell death in the motoneuron population.

The survival-promoting effect of muscle extract on identified motoneurons in mixed cultures on PORN–laminin was dose-dependent (Fig. 6). The concentration of protein required for half-maximal survival in the experiment shown was 4.7 μg ml⁻¹.

Purification of motoneurons by panning on SC1 antibody

Although high levels of SC1 expression were only detected after 2 days in culture (Fig. 4), the use of biotinylated secondary antibody and streptavidin–
fluorescein allowed us to detect relatively faint SC1 staining on the membrane of living freshly dissociated spinal cord cells. Immediately after treatment with trypsin, cells were dissociated and incubated with SC1 in suspension. After washing by centrifugation, 30% of cells present showed SC1 immunofluorescence on at least part of their membrane. We used these SC1-binding sites to develop a method of motoneuron purification based on the panning procedure recently applied to retinal ganglion cells by Barres et al. (1988).

Polystyrene Petri dishes were coated with affinity-purified goat anti-mouse IgG antibodies at pH 9.5 (see Materials and methods), and then incubated with SC1 hybridoma supernatant. After saturation of non-specific protein-binding sites with BSA, suspensions of spinal cord cells were incubated on the panning plate. One hour later, non-adherent cells were removed by washing and then tightly bound cells (Fig. 7B) were eluted with an excess of SC1 supernatant. The panned cells were plated on coverslips coated with PORN-laminin and cultured for 1 day or more in the presence of muscle extract, before being fixed and stained using SC1. All (>99%) of the cells were SC1-positive, and most of them rapidly developed quite complex neuronal morphology (Fig. 8A,B,C,E).

The adhesion of cells to the panning dish was the result of a specific interaction of SC1 antibody with the SC1 antigen. When SC1 was omitted, or replaced by hybridoma supernatant containing anti-myosin antibodies, no cells attached. Similarly negative results were obtained when neurons from the dorsal part of the spinal cord were incubated on dishes coated with SC1.

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**Fig. 4.** Time course of development of intense SC1 immunoreactivity in culture, and dependence upon muscle-derived factors. At different times of culture in the presence (H) or the absence (+) of muscle extract, total spinal cord cells were fixed and stained with SC1 antibody. Strongly labelled cells of neuronal morphology were counted as a percentage of total cells, estimated from their background fluorescence. The absence of intensely labelled motoneurons immediately after trypsin treatment was confirmed by immunofluorescence on spinal cord dissociates (see text for description of weakly labelled cells). Indicated values are mean ± range of duplicate determinations.

**Fig. 5.** Identified motoneurons in mixed cultures are committed to death within 24 h in the absence of muscle extract. Cultures of total 4.5-day spinal cord were seeded in the presence (H) or the absence (+) of muscle extract. After 24 h (arrow), all media were changed for medium containing muscle extract. At indicated times, sample cultures were fixed and processed for SC1 immunofluorescence. Indicated values are mean ± range of duplicate dishes (for 2 points, duplicate was lost).

**Fig. 6.** Dose-dependence of the survival-promoting effect of muscle extract on motoneurons is unaffected by the presence of other spinal cord cells. Cultures of ventral spinal cord (C) or motoneurons purified by panning (●) were grown in the presence of the indicated concentrations of neonatal muscle extract. Two days later, the number of SC1-positive neurons per field (arbitrary units) was determined by immunofluorescence (ventral spinal cord) or phase-contrast microscopy (panned motoneurons). In each case, higher concentrations of muscle extract than those shown caused aggregation and cell death. Calculated concentrations of total muscle protein for half-maximal survival are 4.7 μg/ml (ventral spinal cord) and 3.2 μg/ml (panned motoneurons). Values shown are mean ± range for duplicate dishes.
The SC1 staining seen on panned cells was not due to non-specific binding of the high concentration of antibody used to elute the cells; when SC1-negative cells from the dorsal spinal cord were taken through the elution procedure, they showed only background levels of fluorescence (Fig. 8D). Further evidence for the specificity of interaction was the observation that motoneurons often developed neurites within 3 h when cultured on the polystyrene panning dish, especially when SC1 alone was used for coating (not shown). Their survival in these conditions was muscle-dependent but quantitatively variable, making it unreliable to perform bioassays using motoneurons directly attached to the dish.

Elution from the panning dish was therefore performed using SC1 supernatant to compete for binding sites on the cells and on the dish. When serum-containing hybridoma medium was used instead of SC1, approximately 2-fold less cells were eluted, all of which, however, were SC1-positive. Both trypsin and Ca$^{2+}$ and Mg$^{2+}$-free phosphate-buffered saline removed all cells from the dish, but viability of these cells in culture was extremely poor. Even using SC1 supernatant, approximately 5% of attached cells remained bound to the dish. When these were detached by a more vigorous stream of medium, they too were found to contain >95% SC1-positive cells. It thus seems that the major determinant of specificity in the panning procedure is the binding step. However, elution with specific antibody provides the best compromise between efficiency of elution and preservation of motoneuron viability.

One surprising observation was that only about 2% of total spinal cord cells (i.e. approximately 7–15% of SC1-positive cells) adhered to the panning plate. Many SC1-positive neurons remained in suspension and when cultured on PORN-laminin proved to be perfectly viable, and indistinguishable on morphological grounds from those that bound to the dish. These unbound motoneurons represented ca. 10% of total unbound neurons. This was unlikely to reflect a limited number of binding sites on the dish, as using different cell densities for panning (from 2×10^5 to 10^6 cells per dish), the percentage of cells bound was approximately constant. Furthermore, when non-adherent cells were incubated on a fresh panning dish, none attached. Since further increasing the cell density resulted in clumping of cells, routinely 3 spinal cord equivalents were panned per 100 mm polystyrene dish, with a yield of approximately 10^5 cells per dish. Similar results were obtained when panning was performed on limb segment dissociates (not shown).

In different experiments, populations of cells panned on SC1 contained variable proportions (from 10% to 50%) of cells with non-neuronal morphology, corresponding to SC1-positive floor-plate cells. Although these did not hinder counting of motoneurons, confirmation of the direct neurotrophic effect of muscle extract in this preparation made it necessary to develop a system in which reproducibly only motoneurons were present. No cell-surface marker for chicken floor-plate cells is known; we therefore subdissected anterior horns before panning. More than 98% of the panned cells showed neuronal morphology in culture. After 1.5 days, almost no cells survived in basal medium whereas 90% of those initially attached survived in the presence of muscle extract (Fig. 9). In an alternative approach, more suitable to large-scale preparations, we took advantage of the observation that on continuous 3–20% metrizamide density gradients, SC1-positive cells of non-neuronal morphology were found near the bottom of
Fig. 8. Morphology and SCI expression of motoneurons purified from total spinal cord by panning on SCI antibody.
(A) Phase-contrast micrograph of panned motoneurons after 2 days of culture on PORN-laminin in the presence of neonatal muscle extract. (B) Low-power view of cells from the first panning step cultured on PORN-laminin for 2 days in the presence of neonatal muscle extract and subsequently stained for SCI immunofluorescence. All cells are SCI-positive, and most show clear neuronal morphology. (C) High-power view of an SCI-stained motoneuron. (D) SCI immunofluorescence of panned neurons to which dorsal neurons were added just before elution from the panning dish by an excess of SCI supernatant. Dorsal neurons survive (arrows) but remain clearly unreactive for SCI. (E) Detail of motoneuron growth-cone morphology after 2 days in culture. Presumptive axon shown arises from a motoneuron out of field on bottom left. Scale bar 50 μm (shown in E for A,C,D,E).
Muscle enhances motoneuron survival

Factors promoting survival of purified motoneurons

The survival of the purified motoneurons, counted using phase-contrast optics, was enhanced by neonatal muscle extract in a dose-dependent manner (Fig. 6). This did not result from differential adhesion, as 3 h after seeding in a typical experiment, 12.7 and 15.3 viable cells per field were counted in the presence and absence of muscle extract, respectively, representing a plating efficiency of 40–50%. Approximately 3.2 μg ml⁻¹ of muscle protein were required for half-maximal survival after 2 days on PORN-laminin, a value indistinguishable from that required for half-maximal motoneuron survival in mixed cultures using the same extract (Fig. 6).

We tested the ability of some other known growth factors to reproduce this survival-promoting effect. Murine nerve growth factor (NGF), recombinant human basic fibroblast growth factor (bFGF), ciliary neurotrophic factor (CNTF) from chicken embryo eye extract and human transforming growth factor-beta (TGFβ₁) had no significant effect on motoneuron survival (Table 2; Fig. 10), although each sample used was shown in parallel to be biologically active in other
Table 2. Effect of known growth factors on survival of identified and purified motoneurons

<table>
<thead>
<tr>
<th>Growth factor</th>
<th>Source</th>
<th>Range of concentrations tested</th>
<th>Survival effect on motoneurons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscle extract</td>
<td>P6 chick leg</td>
<td>0.1–20 μg ml⁻¹</td>
<td>++</td>
</tr>
<tr>
<td>NGF</td>
<td>Mouse</td>
<td>1–10 ng ml⁻¹</td>
<td>-</td>
</tr>
<tr>
<td>bFGF</td>
<td>Human</td>
<td>0.5–4 ng ml⁻¹</td>
<td>-</td>
</tr>
<tr>
<td>CNTF</td>
<td>Chicken eye</td>
<td>1.5–45 TU ml⁻¹</td>
<td>-</td>
</tr>
<tr>
<td>TGFβ1</td>
<td>Human</td>
<td>0.5–10 ng ml⁻¹</td>
<td>-</td>
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</tbody>
</table>

Abbreviations: nerve growth factor (NGF), basic fibroblast growth (bFGF), ciliary neurotrophic factor (CNTF), transforming growth factor-beta (TGFβ1), trophic units for chicken ciliary neurons (TU).

Fig. 10. Survival of panned motoneurons in serum-free medium on PORN-laminin in the presence of different growth factors. Panned cells (25,000 per 16 mm well) were seeded in wells containing neonatal muscle extract (●), bFGF (■), CNTF (▲), NGF (□) and TGFβ1 (◇), and with no supplement (▲). Cell survival was counted in the phase-contrast microscope at indicated times. Very similar results were obtained in two separate experiments. Although the zero time point was not measured in the experiment shown, it was confirmed independently that none of the factors tested affected cell attachment after 3 h. Concentrations of factors used were those determined to be ‘optimal’ in parallel dose–response experiments (Table 2). They were, respectively, 6 μg ml⁻¹ muscle protein (neonatal extract), 4 ng ml⁻¹ (FGF), 45 trophic units for ciliary neurons ml⁻¹ (CNTF), 1 ng ml⁻¹ (NGF), and 5 ng ml⁻¹ (TGFβ).

cell culture systems, involving chicken neurons in all cases except for TGFβ (Unsicker et al. 1987; Dreyer et al. 1989; B. Buisson and A. Dupuy-Dangeac, unpublished). Chicken TGFβ1 shows 100% sequence identity to the human factor (Jacakow et al. 1988). For each growth factor, two samples from different preparations or suppliers were tested. Parallel experiments were conducted using the same growth factors in cultures of total spinal cord. Concentrations in the ranges indicated in Table 2 were tested for their ability to increase motoneuron survival above basal levels in the absence of muscle extract, or to enhance the survival-promoting activity of a suboptimal concentration of muscle extract. Counts of SCI-positive neurons in the fluorescence microscope at 1, 3 and 4 days of culture showed that none of these factors had motoneuron survival-promoting activity, either in the presence of muscle extract or in its absence (not shown).

Discussion

The results reported here demonstrate that the SCI monoclonal antibody can be used to identify spinal motoneurons in culture. Several arguments support the hypothesis that the in vitro labelling pattern faithfully reflects the in vivo specificity. First, these large multipolar neurons closely resembled both motoneurons silver-stained in situ and motoneurons purified by cell sorting and cultured in similar conditions. Second, SCI labelling in the central nervous system was limited to cultures of the ventral region of the spinal cord and SCI-positive cells were abundant in lumbar and brachial segments, from which preganglionic neurons are virtually absent. Third, SCI labelling grew weaker with time in culture, with a time course closely resembling that reported for motoneurons (but not other SCI-positive structures) in vivo (Tanaka and Obata, 1984). As expected for motoneurons, the survival of the SCI-positive population was critically dependent on muscle-derived factors in conditions in which other spinal cord cells seemed not to share this requirement.

One major advantage of SCI over other means of identifying motoneurons in culture, apart from convenience, is that it probably labels all motoneurons present rather than a selected subpopulation. The reasons to believe this are, first, that SCI in vivo labels apparently all cells in the anterior horns at all rostrocaudal levels tested, in contrast for instance with antibodies to calcitonin gene-related peptide, which label only approximately half of the motoneurons present (Fontaine et al. 1986; New and Mudge, 1986). Second, the maximal abundance (nearly 30%) of SCI-positive neurons as a percentage of total spinal cord cells seems unlikely to be an underestimate. Indeed, this value is considerably greater than any other previously reported using retrograde labelling (see Henderson, 1988), the closest being the estimate (14%) of Schaffner et al. (1987) using 13-day mouse embryos. It is striking that the figure of 30% putative motoneurons corresponds exactly to the fraction of spinal neurons that we previously reported to put out neurites in the presence of muscle-derived fractions when cultured on tissue-culture plastic (e.g. Henderson et al. 1984).

This approach, which labels all motoneurons, permits a distinction to be made between factors affecting motoneurons as well as other cells, and those affecting only motoneurons. In appropriate conditions, neurons from the ventral but not the dorsal part of the spinal cord, when cultured on tissue-culture plastic, will put out neurites in response to muscle-derived factors,
posteriori. Especially when phenomena such as short-

gradients or cell sorting have frequently reported that it 
even in mixed cultures, muscle effects on motoneuron 
by panning grew as well, and often better, than those in 
spinal cord.
survival are direct, not mediated by other cells of the 
muscle extract (Fig. 7). It is probable therefore that, 
dependence of their survival response to neonatal 
terms of their morphology and in the concentration-
were collagenase, elastase and dispase at standard 
enzymatic treatment that will dissociate the spinal cord 
bling those of the total motoneuron population, both in 
the panned motoneurons had properties closely resem-
antigen, these conditions are only met in a statistically 
both labelling is lost in a developmentally regulated 
fashion after 4–5 days of culture, making motoneurons 
identifiable at later stages, and that motoneurons are 
not separated from other cell types in the culture, which 
might therefore themselves produce neurotrophic ac-
activity under the influence of muscle extract. In order to 
overcome these problems, we developed a panning 
method for isolation of motoneurons. As presented 
here, this method provides a reliable bench-top method 
for production of reasonable quantities (5×10^5) of pure 
motoneurons. The low percentage of total spinal cord 
cells that adhered to the panning dish was unexpected. 
Our results suggest that only a subpopulation of SCI-
positive cells in spinal cord dissociates expressed the 
antigen in an appropriate manner for interaction with 
the dish to occur. It is possible that the non-adherent 
SCI-positive cells were too far degraded by the trypsin 
used in dissociation; we have not yet found an 
enzymatic treatment that will dissociate the spinal cord 
without degrading the SCI antigen (other enzymes tried 
were collagenase, elastase and dispase at standard 
concentrations). Another possibility is that there are 
steric constraints for the interaction of immobilized SCI 
with the cell surface, and that, at low overall levels of 
antigen, these conditions are only met in a statistically 
infrquent subpopulation. Whatever the explanation, 
the panned motoneurons had properties closely resem-
ling those of the total motoneuron population, both in 
terms of their morphology and in the concentration-
dependence of their survival response to neonatal 
muscle extract (Fig. 7). It is probable therefore that, 
even in mixed cultures, muscle effects on motoneuron 
survival are direct, not mediated by other cells of the 
spinal cord.

Motoneurons separated from other spinal cord cells 
by panning grew as well, and often better, than those in 
mixed cultures. In contrast, others using density 
gradients or cell sorting have frequently reported that it 
was necessary to use an astrocyte feeder layer for 
survival of enriched fractions (Schaffner et al. 1987; 
Martinou et al. 1989; H. El M’hamdi and C. E. 
Henderson, unpublished data) or that development of 
purified motoneurons was less complete than in the 
presence of interneurons (O’Brien and Fischbach, 
1986). Immature motoneurons are probably not 
selected by these methods, owing to their smaller size 
and potentially lower capacity to transport tracers from 
the limb. SCI antigen, on the other hand, is present 
from 3 days in ovo (Tanaka and Obata, 1984). It is 
possible therefore that in addition to being more gentle 
than other methods, panning allows purification of 
immature motoneurons, better adapted to survival in 
vitro, and more appropriate for studies of factors 
involved in early neuromuscular contact formation. In 
support of this hypothesis, the cells bound to the 
PORN-laminin alone. 

Two purified polypeptides have been reported to 
prevent or reduce motoneuron death when adminis-
tered in vivo, although neither of them has yet been 
demonstrated to affect survival of isolated motoneu-
rons. Ciliary neurotrophic factor (CNTF) protected 
motoneurons in newborn rat facial nuclei against 
axotomy-induced degeneration, suggesting that the 
early postnatal period of sensitivity to axotomy can be 
correlated with the low levels of CNTF expressed in the 
sciatric nerve at this stage compared to the adult 
(Sendtner et al. 1990). Our finding that otherwise 
biologically active preparations of CNTF from chick 
eye do not affect survival of identified embryonic 
motoneurons (Figs 9, 10) suggests either that the in vivo 
effect of CNTF is mediated by a cell type or a co-factor 
not present in our cultures, or that sensitivity to CNTF 
is only acquired at later embryonic stages. However, it 
is also possible that the ‘CNTF’ molecules purified from 
sciatric nerve and eye may have different biological 
properties. It was recently shown that treatment of 
chick embryos with purified cholinergic development 
factor (CDF), a 22×10^3 M\textsubscript{r} polypeptide (pI4.8) of 
unknown sequence, rescued about 30% of the spinal 
motoneurons that would normally have been lost 
during the period of naturally occurring cell death 
(McManaman et al. 1990). However, CDF did not 
afffect total cell survival in cultures of motoneurons that 
were enriched but not purified, raising the possibility 
that its action might be indirect. Motoneuron survival 
quantified using SCI should provide an appropriate 
parameter by which to evaluate the neurotrophic action 
of this and other known molecules and on which to base 
a strategy for identification of the active molecule in 
muscle extracts.

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References


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