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Survival Effect of Ciliary Neurotrophic Factor (CNTF) on Chick Embryonic Motoneurons in Culture: Comparison with Other Neurotrophic Factors and Cytokines

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In previous studies, it has been demonstrated that ciliary neurotrophic factor (CNTF) has a potent survival effect on various populations of neurons in culture, in particular, neurons isolated from chick ciliary, dorsal root sensory, and sympathetic ganglia (Barbin et al., 1984). After recent investigations demonstrated that CNTF prevents the degeneration of motoneurons in newborn rats after axonal lesion (Sendtner et al., 1990), the question arose as to whether CNTF also has a survival effect on embryonic chick motoneurons at the developmental stage where physiological cell death occurs. To study this, it was essential to develop an isolation and culture procedure for the survival of chick E6 spinal motoneurons in which non-neuronal cells were eliminated and the motoneurons were highly enriched. In these cultures, virtually all of the initially plated motoneurons survived for at least 3 d in the presence of muscle extract, which was chosen as a positive control. Retrograde labeling of the motoneurons prior to their isolation showed that there is more than an 80% enrichment for motoneurons by the method used. The retrogradely labeled neurons also fulfilled the morphological criteria (diameter of neurons, appearance of processes) to identify motoneurons independent of retrograde labeling. Under these conditions, CNTF at a concentration of 1.5 ng/ml (EC_{50}, 0.023 ng/ml) supported maximally 64% of the initially plated spinal motoneurons after 3 d and 53% after 6 d (the longest time period investigated). Other neurotrophic factors, such as NGF, brain-derived neurotrophic factor (BDNF), and neurotrophin-3, had no survival effect at all, even at concentrations up to 10 μg/ml for NGF and BDNF. However, basic fibroblast growth factor (basic FGF) supported 51% and acidic FGF (in the presence of heparin) supported 35% of the initially plated motoneurons after 3 d. The survival effect of basic FGF and CNTF was additive, resulting in a 100% survival of the initially plated motoneurons over a period of 1 week. Of other mitogens and cytokines tested, only insulinlike growth factor I and II (IGF-I, IGF-II) and insulin showed a small survival effect. The maximal survival effect of the plated motoneurons was 15%, compared to 5% survival of the motoneurons without the addition of specific survival factors. The survival effects of IGF-I and IGF-II, and of insulin in particular, were only reached at high concentrations.

The motoneuron survival during the embryological period of naturally occurring cell death has been shown to depend on the targets of these neurons, that is, the skeletal muscle cells (Hamburger, 1975; for review, see Oppenheim, 1989). Attempts to identify, purify, and characterize the responsible molecule(s) have so far been of only limited success. It could be demonstrated that neurotrophic activity present in muscle extract and myotube-conditioned medium is inactivated by heating and exposure to proteases, suggesting a proteinaceous nature of the responsible molecule(s) (Calof and Reichardt, 1984; Flanigan et al., 1985; Kaufman et al., 1985; Smith et al., 1985; Oppenheim et al., 1988; Martinou et al., 1989). The presence of neurotrophic activity in extracts of skeletal muscle leaves open the question as to whether the skeletal muscle cells, or the nerve fibers supplying them, are the source of this activity. In this context, the recent observation seems to be relevant that CNTF, which is present in high concentrations in peripheral nerves (Manthorpe et al., 1986), prevents the lesion-mediated degeneration of motoneurons in newborn rats (Sendtner et al., 1990). In this species, the period of physiological cell death is over at birth (motoneuron cell death occurs between E15 and E20; Harris and McCaig, 1984). We therefore asked whether CNTF also exhibits neurotrophic activity on motoneurons during the developmental period in which the physiological cell death occurs. It had previously been demonstrated that CNTF also exhibits survival activity *in vitro* on embryonic sympathetic and sensory dorsal root ganglion neurons in addition to ciliary neurons (Barbin et al., 1984). To obtain reliable and comprehensive information on the potential neurotrophic action of CNTF on embryonic motoneurons *in vitro*, it was essential to establish an isolation and purification procedure with conditions conducive to the survival of virtually all the initially plated motoneurons for several days after the addition of skeletal muscle extract, which served as a positive control for the neurotrophic activity on motoneurons. After having achieved the appropriate isolation

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and culture conditions, we then set out to analyze the survival activity of CNTF and to compare it with a variety of other defined neurotrophic factors and cytokines.

We report here that low concentrations of CNTF resulted in a long-term survival of more than 60% of the plated motoneurons, and basic fibroblast growth factor (basic FGF) (at approximately a 10-fold higher concentration) resulted in a 50% survival. By combining CNTF and basic FGF, a 100% survival rate of motoneurons was accomplished. A large majority of all the neurotrophic factors and cytokines studied had no survival activity at all. A small survival effect was observed with insulinlike growth factor I and II (IGF-I, IGF-II), and also with insulin, but only at high concentrations.

Materials and Methods

Materials. NGF, purified from the mouse submandibular gland, and brain-derived neurotrophic factor (BDNF), purified from the porcine brain, were provided by Dr. H. Rohrer and Dr. Y.-A. Barde from our laboratory. COS-cell-conditioned medium containing recombinant mouse neurotrophin-3 (NT-3) was provided by Y.-A. Barde (Hohn et al., 1990). The following factors were the generous gifts of the following laboratories or scientists: recombinant human acidic (acidic FGF) and basic (basic FGF) fibroblast growth factor from Dr. W. Knoerzer, Progen Biotechnik, Heidelberg, FRG; recombinant human transforming growth factor α (TGFα) from Dr. R. Derynck, Genentech, San Francisco, CA; recombinant human insulinlike growth factors I and II (IGF-I, IGF-II) from Dr. A. Skottner, KabiVitrum AB, Stockholm, Sweden; recombinant human interleukin-1β (IL-1β) from Dr. A. Gronenborn, Max-Planck-Institute for Biochemistry, Martinsried, FRG; and recombinant rat CNTF from Dr. Nikos Panayotatos, Regeneron Pharmaceuticals, Tarrytown, NY. Other proteins and reagents were purchased from the following companies: platelet-derived growth factor (PDGF), purified from human serum, and recombinant porcine transforming growth factor β1 (TGFβ1), British Bio-Technology, Oxford, UK; mouse epidermal growth factor (EGF), bovine insulin, human transferrin, soybean trypsin inhibitor, poly-DL-ornithine hydrobromide, rhodamine B isothiocyanate, and penicillin G, Sigma, Munich, FRG; recombinant mouse interleukin-3 (IL-3) and interleukin-6 (IL-6), Genzyme, Boston, MA; metrizamide, Serva, Heidelberg, FRG; laminin and Leibovitz's L-15 medium, Gibco, Eggenstein, FRG.

Primary culture of chick motoneurons. In principle, we followed the procedure of Dohrmann et al. (1986, 1987). However, because a series of detailed changes were made that proved to be crucial for the improvement of the method, the entire procedure will be sequentially presented to ensure that the experiments can be reproduced.

Lateral motoneuron columns were carefully dissected from the lumbar part (1.5 mm length) of the spinal cord of 6 chick embryos (E6) as described by Masuko et al. (1979), with the exception of using tungsten forceps for the isolation of the ventrolateral part of the spinal cords instead of razor blades. The dissected tissues were stored in cold calcium- and magnesium-free glucose- (4 gm/l) supplemented Hanks' balanced salt solution (HBSS). After washing with HBSS, the tissues were treated with 1 ml 0.03% trypsin-HBSS in a 50-ml Falcon tube at 37°C in a shaking water bath (about 30 cycles/min) for 20 min. They were then rinsed with cold HBSS, transferred into 1 ml cold HBSS containing 0.1% soybean trypsin inhibitor, and triturated mildly with a fire-polished siliconized Pasteur pipette by 8 strokes. After removal of the cell suspension, 1 ml cold HBSS was added to the remaining fragments, and the procedure was repeated twice. The resultant cell suspension (3 ml) was filtered through a 50-μm nylon mesh and layered onto 4 ml cold 6.8% metrizamide in HBSS with 25 mM HEPES (pH, 7.4) in a 12-ml siliconized conical glass tube. After centrifugation at 400 × *g* for 15 min at 4°C, the intermediate layer (0.4 ml) was collected and transferred into another siliconized tube containing 6.5 ml cold culture medium. The culture medium consisted of 75 ml Leibovitz's L-15 medium supplemented with glucose (4 gm/l), 15 ml 0.15 M sodium bicarbonate, 10 ml heat-inactivated and filtered horse serum, and 0.1 ml 10^5 U/ml penicillin G. The medium was buffered with 5% CO_2 and used within 1 month. After centrifugation at 100 × *g* for 7 min at 4°C, the supernatant was removed, and the cells were gently resuspended in culture medium, then plated in Greiner 4-well culture dishes (well diameter, 10 mm; C. A. Greiner und Söhne GmbH, Nürtingen, FRG). Cell densities were 1000–2000 cells per well. The dishes used were precoated with poly-DL-ornithine [0.5 mg/ml in 0.15 M sodium borate buffer (pH, 8.3)] overnight at 4°C, rinsed twice with PBS, and subsequently incubated for 5–6 hr with laminin (10 μg/ml in serum-free culture medium) in a 5%-CO_2 incubator. The solution was removed just before cell plating. The cell preparation procedure was accomplished within 2.5 hr. Cells were incubated at 37°C in a humidified incubator supplied with 5% CO_2. Tissue extracts or factors to be tested were added 1 hr after plating. The culture medium was changed after 24 and 72 hr. Cells were counted in predetermined areas corresponding to 8% (see Table 1, Figs. 3, 4) or 23% (see Table 2, Fig. 5) of each well bottom. Initial cell numbers were determined 3 hr after plating. Only large phase-bright cells (≥14 μm in diameter) were included in the evaluation of the extent of motoneuron survival.

Retrograde labeling of motoneurons. In some experiments, motoneurons were retrogradely labeled *in vivo* by rhodamine B isothiocyanate in accordance with Dohrmann et al. (1986). Particular care was taken to insert rhodamine B isothiocyanate crystals into as many places of the hind limb as possible. After 24 hr, some of the operated embryos were processed for 10-μm frozen sections after formaldehyde fixation. The other embryos were used for motoneuron preparations following the procedures described above. After 5 hr in culture, cells were rinsed with HBSS, fixed with 4% formaldehyde in PBS at room temperature for 20 min, rinsed with PBS, then mounted in glycerol-PBS (1:1).

Preparation of skeletal muscle extract. For the dissection of embryonic chick (E20) leg muscles, particular care was taken to remove the skin and sciatic nerve trunk as completely as possible. Homogenization was performed with an Ultra-Turrax (Jahnke and Kunkel, Staufen, FRG) in 4 vol PBS containing phenylmethylsulfonyl fluoride (0.1 mM), EDTA (1 mM), *N*-ethylmaleimide (1 mM), and benzamidine (1 mM) on ice. The homogenate was centrifuged at 100,000 × *g* at 4°C for 1 hr. After storing overnight at −70°C and thawing, the supernatant was concentrated by ultrafiltration (Centricon-10, Amicon; cutoff, 10,000), at 4°C. The residual extract was rediluted to the original volume with cold PBS and stored in 70-μl aliquots at −70°C until use. The protein concentration was determined by the method of Bradford (1976) using BSA as a standard.

Results

Evaluation of the purity of motoneuron cultures

In the retrograde-labeling experiments, the fluorescence was confined to the lateral motoneuron column. Outside the spinal cord, cells of lumbar dorsal root ganglia were also labeled (Fig. 1*A,B*). After careful dissection of the area of motoneuron columns and dissociation of the tissues, cells were cultured on polyornithine-laminin substrate for 5 hr and fixed for phase-contrast and fluorescence microscopy (Fig. 1). Without metrizamide fractionation, the cells proved to be heterogeneous (Fig. 1*C*). In contrast to small cells, which were not labeled, a high proportion of the large neurons were fluorescence labeled, identifying them as motoneurons. Some cells of intermediate size were also labeled (data not shown), which possibly represent younger motoneurons.

Metrizamide fractionation markedly augmented the percentage of labeled motoneurons (Fig. 1*E,F*); that is, 83% of the total cells were found to be labeled after 5 hr in culture. Most of the labeled neurons were large. The rest consisted of intermediate-sized cells, putatively young motoneurons. A small percentage (<10%) of the cells could not be identified. Nevertheless, they seemed to represent neurons as they showed neurite outgrowth after a few days in culture. There was no evidence for the presence of non-neuronal cells after metrizamide fractionation.

Modification of the isolation and culture procedures necessary for long-term survival of motoneurons

To obtain high rates of long-term survival, the following methodological criteria proved to be essential: (1) Because motoneurons are very fragile, the enzymatic digestion and the trituration

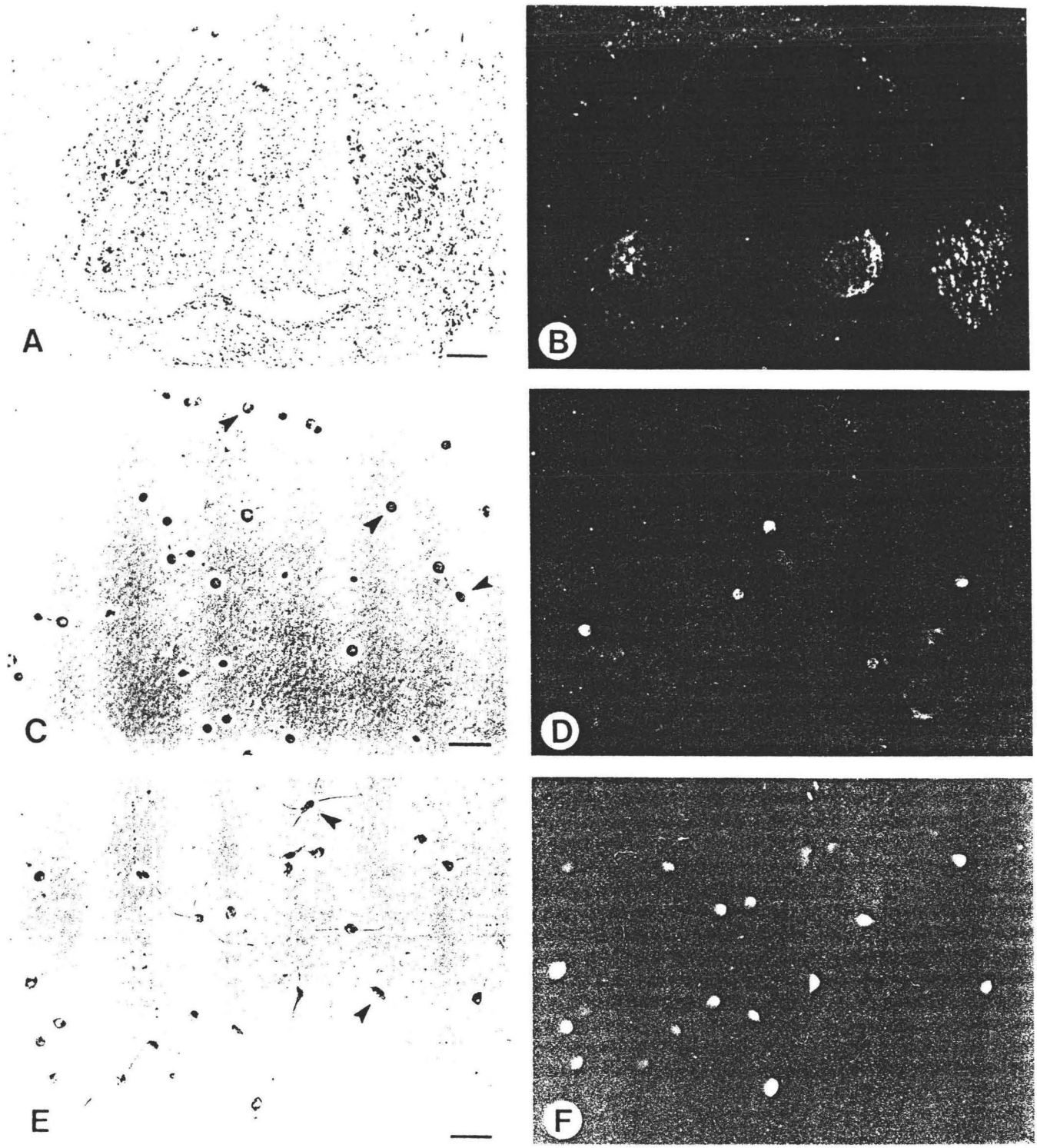

Figure 1. Retrograde labeling of spinal motoneurons. Motoneurons were labeled for 24 hr *in ovo* by inserting rhodamine B isothiocyanate crystals into 2 or 3 sites of each hind limb of E5 chick embryos. *A* and *B*, Phase-contrast (*A*) and fluorescence (*B*) pictures of a frozen section of the lumbar spinal cord of an operated embryo. Scale bar, 100 μm. Note that only motoneuron columns were labeled within the spinal cord, and that cells of a dorsal root ganglion were also labeled. *C–F*, Phase-contrast (*C, E*) and fluorescence (*D, F*) pictures of cells before (*C, D*) and after (*E, F*) motoneuron enrichment. Cells were fixed after a 5-hr incubation and mounted in glycerol-PBS (1:1). Scale bars, 50 μm. *Arrowheads* in *C* and *E* show the large cells, which are not labeled.

procedures had to be evaluated and then standardized (see Materials and Methods). Moreover, as soon as the neurons were in culture, the mechanical disturbances (e.g., during medium change) had to be kept to a minimum. (2) During the preparation of the cells, the temperature had to be kept low in order to prevent degenerative changes from occurring at a higher temperature. (3) Ca^{2+}-free medium had to be used, buffered with bicarbonate rather than with phosphate during cell preparation.

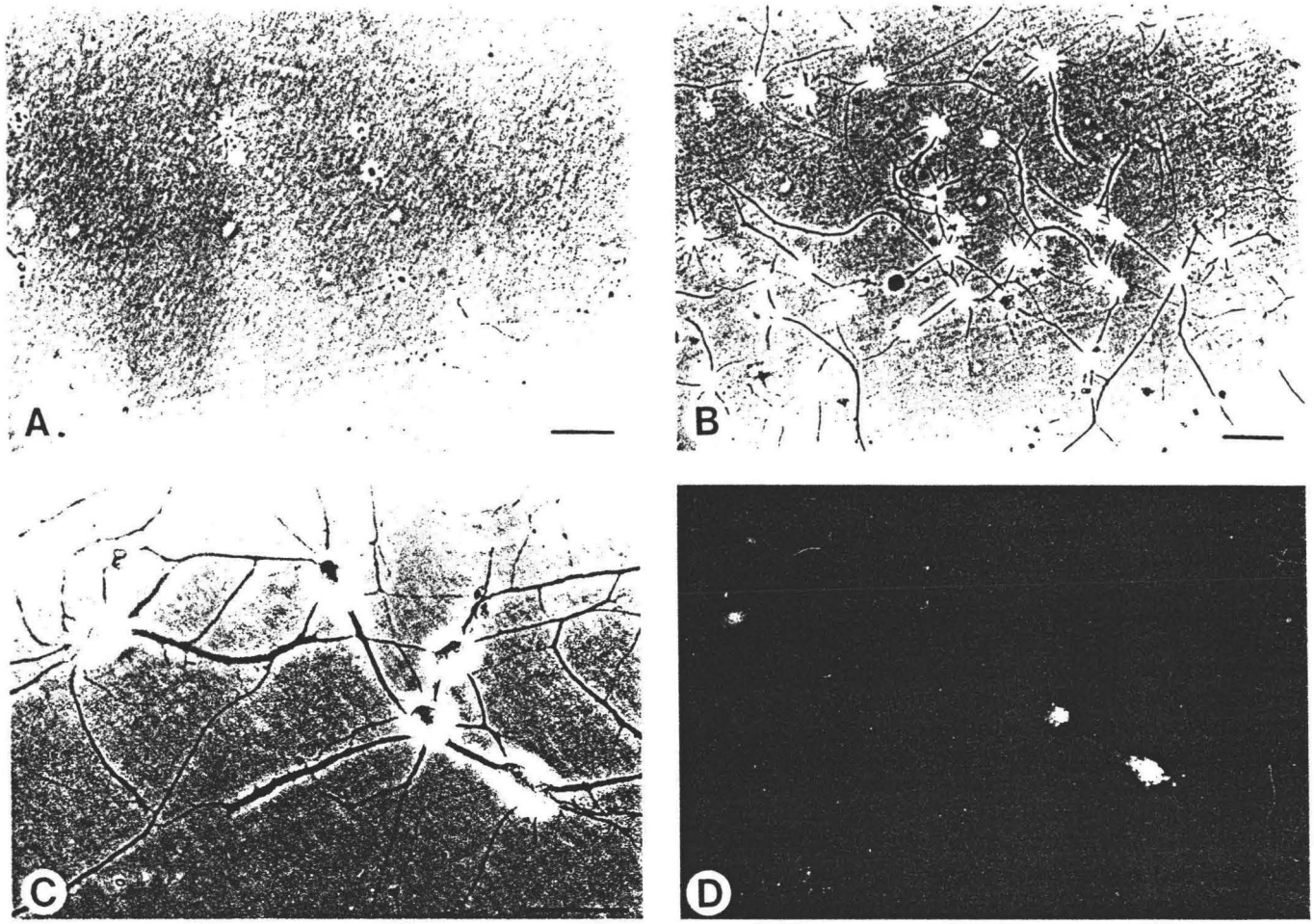

Figure 2. Photomicrographs of motoneurons cultured for 3 d in absence (*A*) or in presence (*B–D*) of skeletal muscle extract (100 μg/ml). A phase-contrast (*C*) and a fluorescence (*D*) picture of the same field at a larger magnification show that some of the retrogradely labeled motoneurons still maintain a fluorescent dye. Scale bar, 100 μm in *A* and *B*; 50 μm in *C*.

(4) In contrast to previous procedures (Dohrmann et al., 1986), the number of embryos used for individual experiments had to be kept to a minimum in order to reduce the time necessary for cell preparation. In any case, the preparation time did not exceed 2.5 hr. (5) For the metrizamide gradient centrifugation, the number of cells layered on the gradient had to be reduced, resulting in a higher enrichment of motoneurons in the interface compared to previous experiments (Dohrmann et al., 1986).

The effect of embryonic muscle extract on motoneuron survival

In the presence of muscle extract, nearly 100% of the motoneurons survived and showed extensive neurite outgrowth after 3 d in culture (Fig. 2*B*). In the absence of extract, most of the cells died (Fig. 2*A*). When retrogradely labeled motoneurons were cultured with muscle extract, the percentage and absolute number of fluorescent cells (Fig. 2*C,D*) decreased much faster than did the number of large neurons representing motoneurons. Similar results were obtained using 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindo-carbocyanine perchlorate (DiI) (Honig and Hume, 1986) as a retrograde marker (data not shown). This indicates that the reduction of the number of fluorescent cells was due to the fading of the fluorescence rather than to cell death (O'Brien and Fischbach, 1986). Therefore, motoneuron survival was determined by counting large neurons (Figs. 1*E,F*; 3). Although there were also some intermediate-sized neurons that were retrogradely labeled, these cells were not counted as motoneurons in order to achieve a higher reliability. Under these conditions, motoneuron survival reached about 100% after 3 d in culture, but declined to about 60% after 6 d, even if the medium (controls and muscle extract) was renewed on the 3rd and 5th d (Fig. 3). In the absence of muscle extract, about 70% of the initially plated motoneurons survived after 24 hr, but after 3 d, only a small number of neurons survived (Fig. 3). Therefore, in routine assays, the extent of the survival of motoneurons was determined after 3 d in culture.

Survival effects of specific neurotrophic molecules and cytokines

Of all the molecules tested, CNTF and basic FGF proved to be the most potent molecules (Table 1). The survival activity of acidic FGF could be increased when the cultures were supplemented with heparin, which prevents inactivation of acidic FGF (Gospodarowicz and Cheng, 1986). IGF-I, IGF-II, and insulin showed minor effects. The concentration–response curves for these active molecules (Fig. 4) showed that the highest survival effects could be obtained by: (1) CNTF, 64% survival at 1.5 ng/ml; (2) basic FGF, 51% at 30 ng/ml; (3) acidic FGF, 18% at 300 ng/ml; (4) acidic FGF in the presence of 1 μg/ml heparin, 35% at 100 ng/ml; (5) IGF-I, 15% at 100 ng/ml; (6) IGF-II, 15% at 300 ng/ml; and (7) insulin, 16% at 25 μg/ml. The EC_{50} values

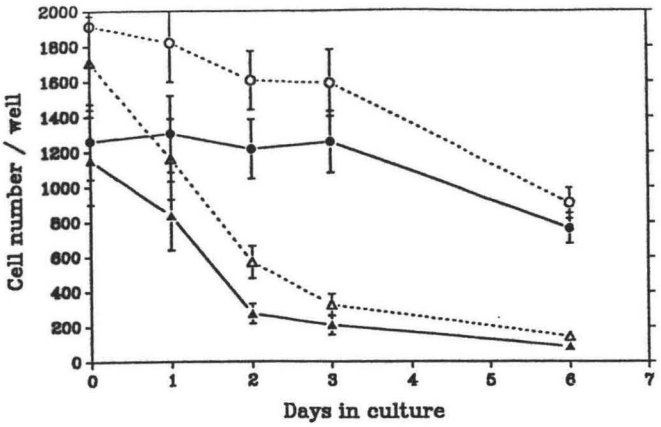

Figure 3. Survival of total cells (*open symbols*) and large neurons (*solid symbols*) in the presence (*circles*) or absence (*triangles*) of skeletal muscle extract (100 μg protein/ml). Values are mean ± SEM ($n = 6$).

were 0.023 ng/ml for CNTF and 0.28 ng/ml for basic FGF. For IGF-I, IGF-II, and insulin, exact EC_{50} values could not be determined, as the maximal effects were very small compared to the controls.

The concentration of heparin was critical for the improvement of the activity of acidic FGF. The concentrations used in the present experiments (1 μg/ml) did not seem to be maximal for the survival activity of acidic FGF. However, higher concentrations of heparin resulted in a detachment of the neurons from the culture dishes. Even with 1 μg/ml heparin, neuron detachment started after 3 d of incubation (data not shown). NGF, BDNF, PDGF, EGF, TGFα, TGFβ1, IL-1β, IL-3, and IL-6 had no discernable effect even when supramaximal concentrations

Table 1. Motoneuron survival activities of known molecules

Molecule	Concentration	Survival[a]
Control		–
NGF (mouse)	10 μg/ml	–
BDNF (porcine)	10 μg/ml	–
NT-3 (mouse, rec.)[b]	1:50-dilution[b]	–
CNTF (rat, rec.)	500 pg/ml	+++
Basic FGF (human, rec.)	10 ng/ml	++
Acidic FGF (human, rec.)	300 ng/ml	+
Acidic FGF + Heparin	100 ng/ml + 1 μg/ml	++
PDGF (human)	5 ng/ml	–
EGF (mouse)	10 ng/ml	–
TGFα (human, rec.)	10 ng/ml	–
TGFβ1 (porcine, rec.)	5 ng/ml	–
IL-1β (human, rec.)	100 units/ml	–
IL-3 (mouse, rec.)	100 units/ml	–
IL-6 (mouse, rec.)	50 units/ml	–
IGF-I (human, rec.)	100 ng/ml	+
IGF-II (human, rec.)	300 ng/ml	+
Insulin (bovine)	25 μg/ml	+
Transferrin (human)	100 μg/ml	–

rec.: recombinant.

[a] Survival activities were assayed after 3 d in culture and estimated with: +, 15–20% (statistically significant from each blank control, $p < 0.01$) survival; ++, 35–55% survival; +++, 55–75% survival; –, not significantly different from control (survival, 8.3 ± 3.8%, mean ± SD; $n = 18$). The results were combined from different experiments.

[b] Conditioned medium of NT-3 cDNA-transfected COS-cells.

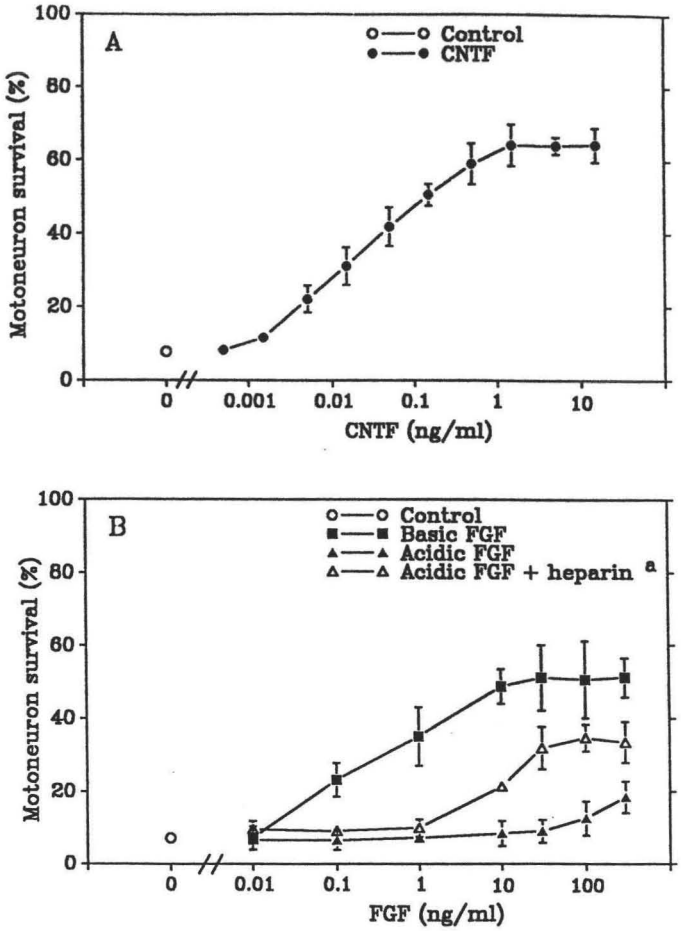

Figure 4. Concentration–response curves for CNTF (*A*) and acidic and basic FGF (*B*). Survival activities were assayed after 3 d in culture. In *B*[a], heparin (1 μg/ml) was added to the culture only for the first 24 hr to avoid cell detachment induced by excess heparin, while acidic FGF was present for 3 d.

(with respect to biological effects on other cell types) were used. Also, NT-3, a new neurotrophic molecule of the NGF–BDNF gene family (Leibrock et al., 1989; Hohn et al., 1990), was used in these experiments. Concentrations of NT-3 protein produced by transfected COS-cells, which supported the survival of chick embryonic nodose ganglion neurons or trigeminal mesencephalic neurons in culture (Hohn et al., 1990), had no survival effect on motoneurons.

For NGF and BDNF, the absence of survival activity on chick motoneurons seems to be conclusive insofar as these 2 neurotrophic molecules showed survival activity on other populations of chick embryonic neurons in culture (see Barde, 1989; Hohn et al., 1990), and the negative results cannot be explained by the possible species specificity of these 2 molecules. Unfortunately, no comparable information is available on PDGF, TGFα, TGFβ, EGF and all the interleukins tested. Therefore, it must be taken into consideration when looking at these negative results that there are possible differences in the species specificities of these molecules.

During the preparation of this manuscript, Martinou et al. (1990) reported on a survival effect of TGFβ in rat motoneurons cultured on a layer of astrocytes. As rat astrocytes have been shown to produce CNTF (Lillien et al., 1988; Stöckli et al., 1989), it cannot be decided whether the observed survival effect

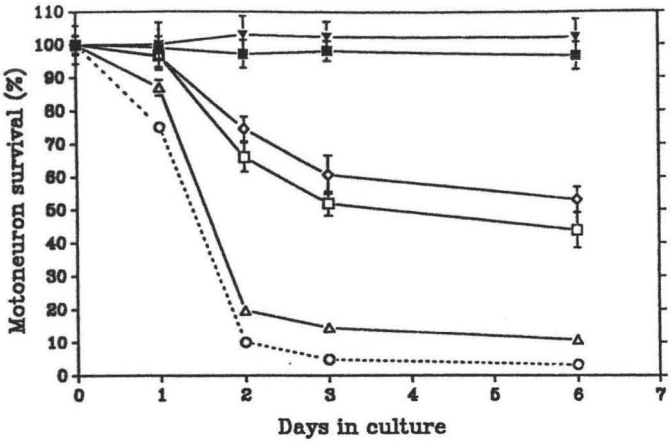

Figure 5. Survival of motoneurons in presence of CNTF, basic FGF, and IGF-I. *Open circles* and *dashed curve,* control; *open diamonds,* CNTF (1.5 ng/ml); *open squares,* basic FGF (30 ng/ml); *open triangles,* IGF-I (1 μg/ml); *solid squares,* CNTF with basic FGF; *solid triangles,* CNTF with basic FGF and IGF-I.

was a direct or an indirect one. Furthermore, the number of surviving neurons after 4 d in culture on lysed astrocytes was so small (about 5% of the initially plated neurons) that their results cannot be compared with those presented here.

Combination of CNTF, basic FGF, and IGF-I

The combination of CNTF and basic FGF at optimal concentrations resulted in a 100% survival of the motoneurons over a period of 1 week (Table 2, Fig. 5). The effect of IGF-I was small by itself, but became more evident when it was combined with either CNTF or basic FGF (Table 2). Even though the addition of muscle extract during the first 3 d resulted in a 100% survival of motoneurons, there was nevertheless an unavoidable decay of the number of motoneurons after 6 d; this was possibly due to neurotoxic compounds present in the muscle extract (Fig. 3).

Discussion

After previous experiments had demonstrated that CNTF prevents the degeneration of motoneurons caused by nerve lesion in newborn rats (Sendtner et al., 1990), the present investigations showed that CNTF also exhibits a marked (>60%) survival effect on chick motoneurons isolated from embryos at the beginning of the period of naturally occurring motoneuron cell death. Of a great variety of neurotrophic factors and cytokines tested, only basic FGF had a long-term survival effect comparable to that of CNTF, albeit at a 10-fold higher concentration. The combination of CNTF and basic FGF resulted in virtually 100% survival. IGF-I, IGF-II, and insulin showed small but consistent effects, though at high concentrations. Particularly high concentrations were necessary for insulin (25 μg/ml), suggesting that it most probably acted via the IGF-I receptor (Zapf et al., 1981).

To obtain reliable information on the survival effects of the different factors on motoneurons *in vitro,* it was essential to aim at isolation and culture procedures that fulfilled the following criteria: (1) A high yield of the neurons under investigation has to be ensured during the isolation procedure, to avoid a bias resulting from an under- or overrepresentation of subpopulations of neurons. (2) Non-neuronal cells have to be removed to eliminate the possibility of the observed neurotrophic effects being a result of indirect actions via non-neuronal cells. (3) The

Table 2. Additional effects of CNTF, basic FGF, and IGF-I

Factors	Motoneuron survival (%)[a]
Control	4.8 ± 1.0
IGF-I (1 μg/ml)	14.5 ± 0.5**
Basic FGF (30 ng/ml)	51.9 ± 3.6
CNTF (1.5 ng/ml)	60.7 ± 5.8
Basic FGF + IGF-I	76.1 ± 4.1**
CNTF + IGF-I	87.0 ± 4.5*
Basic FGF + CNTF	98.2 ± 3.0
Basic FGF + CNTF + IGF-I	102.5 ± 5.3 (NS)

*, $p < 0.05$.
**, $p < 0.01$.
NS, Not significant.
[a] Motoneuron survival was assayed after 3 d in culture. Values are mean ± SEM ($n = 4$). The results of the t test were indicated only for the comparisons between the values with or without IGF-I because the effect of IGF-I is relatively small.

isolation and purification procedures should be, with the addition of the appropriate survival factors, conducive to the survival of all the initially plated neurons. For peripheral sympathetic, sensory, and parasympathetic neurons, isolation and culture conditions were established that almost ideally meet these criteria (Barde et al., 1982). For neurons isolated from the CNS, the isolation procedures were generally more difficult and demanding because the neurons had to be isolated from a more complex environment. Therefore, the yield, purity, and extent of survival of the initially plated neurons was not as satisfactory as that of the peripheral neurons. This was also true for the culture conditions of chick embryonic motoneurons available so far (Dohrmann et al., 1987). Although conceptually new approaches were not used, the modification of single steps of previous procedures (as detailed in Material and Methods) resulted in a marked improvement of the method. It was possible to perform long-term survival analysis with a highly enriched population of motoneurons at low density in the absence of non-neuronal cells. Thus, the survival analysis permitted the direct counting of motoneurons and did not depend on the measurement of ChAT activity as in several previous studies (Flanigan et al., 1985; Kaufmann et al., 1985; Smith et al., 1985; McManaman et al., 1989). Determination of ChAT activity as a measure for motoneuron survival is unsatisfactory as it does not distinguish between an increased survival of motoneurons and an increase in ChAT activity in a constant or even reduced number of motoneurons. The situation becomes even more complex if non-neuronal cells are present and if the measured ChAT activity results exclusively from, or is influenced by, indirect effects via non-neuronal cells. Indeed, astrocytes do exhibit neurotrophic activity on motoneurons in culture (Eagleson et al., 1985).

So far, the evaluation of motoneuron survival *in vitro* was confined to the analysis of the effects of tissue extracts or conditioned media. In particular, skeletal muscle extract and myotube- or astrocyte-conditioned media were used (Calof and Reichardt, 1984; Eagleson and Bennett, 1983, 1986; O'Brien and Fischbach, 1986; Dohrmann et al., 1987; Schaffner et al., 1987; Martinou et al., 1989). In the present experiments, we have demonstrated that recombinant CNTF and recombinant basic FGF, and, to a much smaller extent, IGF-I, IGF-II, and insulin, result in a long-term survival of purified motoneurons. CNTF, which acts at very low concentrations, achieved a more than

60% long-term survival of motoneurons. Basic FGF, at about a 10-fold higher concentration than that of CNTF, achieved a maximal survival of about 50% of the initially plated motoneurons (Table 2). IGF-I, IGF-II, and insulin had consistent, but small, effects on motoneuron survival. Moreover, these small effects were observed only at high concentrations. The combination of CNTF and basic FGF resulted in a virtually 100% survival.

By far, the largest quantities of CNTF protein and CNTF mRNA are present in the sciatic nerve of adult rats (Manthorpe et al., 1986; Stöckli et al., 1989). At birth, when the period of normal motoneuron cell death is over, the levels of CNTF and its mRNA are just at the detection limit. They then rapidly increase during the first postnatal week, reaching adult levels by the end of the second postnatal week (Stöckli et al., 1989). The large quantities of CNTF present in the sciatic nerve of adult rats suggests the possibility of its function as a 'lesion factor' preventing motoneuron degeneration after nerve lesion. This suggestion is supported by the following observations: transection of the peripheral facial nerve in adult rats, which contains virtually exclusively axons of motoneurons, results in chromatolysis in the motoneuron cell bodies and in reactive gliosis in their vicinity. However, no degeneration of facial motoneurons occurs within 1 week (Tetzlaff et al., 1988). In contrast, transection of the facial nerve in newborn animals results in a degeneration of virtually all the neurons (Sendtner et al., 1990). The extent of degeneration decreases relatively rapidly in the postnatal period (Snider and Thanedar, 1989), paralleling the increase in the levels of CNTF in the peripheral nerves (Stöckli et al., 1989). The assumption of a causal relationship between the extent of degeneration and the levels of CNTF in the lesioned nerves is supported by the fact that the local administration of CNTF in newborn animals can almost completely prevent the degeneration of the corresponding motoneuron cell bodies (Sendtner et al., 1990). Therefore, in spite of the strong survival activity of CNTF on embryonic motoneurons in culture, it seems to be rather unlikely that CNTF is the molecule responsible for the regulation of motoneuron survival in rat embryos between E15 and E20. It still remains to be established whether a CNTF-related molecule is expressed at an earlier developmental stage, or whether an unrelated molecule of a yet unidentified nature is responsible for the developmental regulation of motoneuron survival.

FGF-like activity has been identified at very early stages (Munaim et al., 1988; Seed et al., 1988) of embryonic development and is responsible, together with TGFβ, for mesodermal induction (Slack et al., 1987; Kimmelman and Kirschner, 1987; Kimmelman et al., 1988). Both acidic and basic FGF were found in chick embryonic brains (Risau et al., 1988); however, the determined levels were generally lower than those found in adult brains (Gospodarowicz et al., 1984; Caday et al., 1990). A staining of neurons with antibodies directed against basic FGF was reported in rodents (Pettmann et al., 1986; Finklestein et al., 1988). H. Schnürch and W. Risau (personal communication) demonstrated in chicks by *in situ* hybridization that mRNA for chick basic FGF was expressed in ependyme; most of the neurons of the brain, including cell bodies of the spinal cord and autonomic and sensory ganglia, expressed acidic FGF. CNTF and both basic and acidic FGF have the structural features of cytosolic proteins. In particular, they do not have a leader sequence (Abraham et al., 1986; Lin et al., 1989; Stöckli et al., 1989), and wherever a possible secretion of FGF was analyzed in cultured cells, the results were negative (for review, see Klagsbrun, 1989). In view of the fact that both basic and acidic FGF are not secreted, at least not by the classical secretory pathway, the physiological relevance, in particular to the survival activity on central (Walicke et al., 1986) and peripheral neurons *in vitro*, is elusive.

The ChAT-inducing activity of basic FGF in heterogeneous spinal cord cell cultures (McManaman et al., 1989) does not permit a conclusion as to whether this is the result of a direct effect or the result of an indirect effect via astrocytes, which themselves produce CNTF and basic FGF *in vitro* (Ferrara et al., 1988; Hatten et al., 1988; Lillien et al., 1988). In this context, it is worth mentioning that Interferonγ increases ChAT activity in mixed cultures of human spinal cord, but this effect seems to be an indirect one mediated by non-neuronal cells (Erkman et al., 1989).

The extremely high concentrations of insulin necessary to induce a borderline survival effect on embryonic chick motoneurons suggest that insulin acts via IGF-I receptors (Zapf et al., 1981). The fact that IGF-I and IGF-II are synthesized by a great variety of tissues, including the liver, pituitary, and nervous system, and the fact that they are present in relatively high concentrations in serum and cerebral spinal fluid (Zapf et al., 1981; Haselbacher and Humbel, 1982; Haselbacher et al., 1985; Hansson et al., 1987), makes it very unlikely that they play an essential, specific role in the regulation of the embryonic survival of motoneurons. It is possible that they may act as 'cofactors' in regulating the synthesis of other neurotrophic molecules, in enhancing the effects of other neurotrophic factors, and/or, as has been demonstrated in the present experiments, in making their neurotrophic activity more apparent in the presence of FGF or CNTF. In line with these considerations is the observation by Kanje et al. (1989) that IGF-I slightly enhances regeneration of sensory nerve fibers in the lesioned sciatic nerve of the rat.

In conclusion, though CNTF and both basic and acidic FGF have a marked survival activity on purified embryonic chick motoneurons in culture, the developmental expression, at least of CNTF, and the cellular localization (CNTF, basic and acidic FGF are cytosolic molecules) make it unlikely that they represent the agents responsible for the regulation of target-dependent embryonic motoneuron survival. However, the survival activity of CNTF, and to a smaller extent of basic FGF, together with the previous observation that CNTF prevents lesion-mediated degeneration of motoneurons in newborn rats, makes these 2 molecules, particularly CNTF, promising candidates for the treatment of degenerative disorders of motoneurons.

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