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Leukemia Inhibitory Factor Promotes the Neuronal Development of Spinal Cord Precursors From the Neural Tube

L.J. Richards, T.J. Kilpatrick, P.F. Bartlett, and M. Murphy
The Walter and Eliza Hall Institute of Medical Research, Victoria, Australia

Recent evidence from our laboratory has shown that leukemia inhibitory factor (LIF) can act early in peripheral nervous system development. We have investigated a potential role of LIF in the developing spinal cord. In explants and dissociated cultures of spinal cord primordium, LIF stimulated a profuse neurite outgrowth. To determine if these effects were related to neuronal differentiation, cells were plated at low cell density and stained for neurofilament. LIF stimulated an increase in the number of newly differentiated neurons, without inducing proliferation of the precursors. Given that LIF has previously reported effects as a cholinergic switching factor for sympathetic neurons, we investigated whether LIF had similar effects in these spinal cord cultures. LIF increased the number of cholinergic neurons in proportion to its overall effect on the stimulation of all neurofilament positive neurons in the culture. These data show that LIF stimulates the generation of spinal cord neurons from their precursors and further implicates a role for LIF in nervous system development. © 1992 Wiley-Liss, Inc.

Key words: spinal cord development, LIF, neurons, neuronal differentiation, growth factors

INTRODUCTION

The mammalian central nervous system (CNS) is developmentally derived from the neuroepithelial cells of the neural tube. The development of the tube segregates along its rostrocaudal axis; rostral development occurs initially with the formation of the ventricles and the cephalic compartments, which eventually give rise to the brain. Caudal development is first marked by a lengthening of the tube until the complete embryonic axis is formed, and, from this primordium, the spinal cord develops. The development of these distinct and complex structures from an apparently homogeneous population of epithelial cells within the neural tube is clearly a very complex process, dependent on many different factors.

We have been studying the early events in this process and have examined the potential role of growth factors in the early proliferative and differentiative phases of development from the neural tube. The fibroblast growth factors (FGFs) were found to be potent stimulators of proliferation of neuroepithelial cells derived from the telencephalon and mesencephalon (Murphy et al., 1990). In addition, at high concentration, the FGFs stimulated the differentiation of these cells into neurons and glia (Murphy et al., 1990). The effects of the FGFs were shown to be entirely dependent on the presence of insulin-like growth factor I (IGF-I), which alone acts as a survival factor for the neuroepithelial cells (Drago et al., 1991a). The cells synthesize IGF-I endogenously in vitro and most likely in vivo as well (Drago et al., 1991a). At least one member of the FGF family, basic FGF, is also produced at this stage of development. Thus, both of these factors are implicated in the control of the early development of the brain.

We have also begun to study the development of the primordial spinal cord from the neural tube. Although derived from an apparently similar precursor population to that giving rise to the brain, the obvious differences in development of the spinal cord which occur very early must presumably be reflected by intrinsic differences within the subpopulations of precursor cells and/or by different local environmental factors. However, this area has not been extensively studied and no purified factors have been shown to influence the differentiation of neuroepithelial cells in the spinal cord primordium. Most of the previous work on spinal cord development has been directed toward older spinal cord cultures, after neuronal differentiation has occurred, to examine factors which affect neuronal phenotype or survival (Calof and Reichardt, 1984; Giess and Weber,

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Address reprint requests to Mark Murphy, The Walter and Eliza Hall Institute of Medical Research, c/o Post Office, Royal Melbourne Hospital, 3050 Victoria, Australia.

1984; Smith et al., 1986; McManaman et al., 1990; Oppenheim et al., 1988; Houenou et al., 1991; Nawa and Patterson, 1990; Martinou et al., 1989), or neurite outgrowth around the time of synaptogenesis (Dohrmann et al., 1986; Heaton and Wayne, 1986; Heaton, 1988; Iwasaki et al., 1989a,b).

In an earlier study, it was shown that muscle-conditioned medium increases choline acetyltransferase (ChAT) activity and motoneuron survival (Martinou et al., 1989). One factor partially purified from this medium has similar properties to a cholinergic neuronal differentiation factor (CDF), which directs the choice of neurotransmitter phenotype made by cultured rat sympathetic neurons (Patterson and Chun, 1977). Sequence analysis and biological assays have, in turn, demonstrated that this protein is identical to leukemia inhibitory factor (LIF; Yamamori et al., 1989), a factor which has multiple activities outside the nervous system (Hilton et al., 1988; Gearing et al., 1987; Williams et al., 1988; Abe et al., 1986; Baumann and Wong, 1989). We have also shown that LIF can act very early in nervous system development. LIF stimulates both the generation of sensory neurons from the embryonic neural crest and the differentiation of precursor cells into sensory neurons in early dorsal root ganglia (DRG) cultures (Murphy et al., 1991). In older DRG cultures, LIF maintains the survival of differentiated neurons.

In this paper, we have investigated a potential role for LIF in the early stages of development of the spinal cord. We find that LIF stimulates process outgrowth from explants of the spinal cord primordium isolated prior to neuronal differentiation and innervation in the mouse (embryonic day 10, E10). This correlates with increased neuronal differentiation in cultures of cells from dissociated E10 spinal cord in the presence of LIF. Thus, LIF may stimulate the development of spinal cord neurons from their precursors in the embryonic neural tube.

MATERIALS AND METHODS

Isolation of Spinal Cord Cells

Embryos were obtained from E10 CBA mice. The heads were removed and the caudal part of the neural tube, or embryonic spinal cord, which forms a closed tube by E10, was removed together with the surrounding somites (25–34 somites) from the remainder of the embryo. The section of the spinal cord used in all experiments extended from the otic vesicle to the caudal end of the cord. This tissue was subsequently incubated in Dispase II (Boehringer) in HEPES-buffered Eagle's medium (HEM) for 15 min at 4°C and for 6 min at 37°C. The tissue was then transferred to HEM containing 1.0% (w/v) fetal bovine serum (FBS) and 0.001% (w/v) DNase and the spinal cord was dissected free of the surrounding ectoderm, somites, and mesoderm, using the tissue plane created by Dispase incubation essentially as described previously for the preparation of the mesencephalic and telencephalic regions of the neural tube (Murphy et al., 1990; Drago et al., 1991b). Inspection at this stage revealed clean spinal cords free of contaminating mesoderm. These cords were plated directly onto 24 well plates (Linbro, Glasgow, Scotland) for explant cultures. For preparation of dissociated cell suspensions, the spinal cords were then incubated at 37°C in Ca^{2+}/Mg^{2+} Hank's balanced salt solution (Hank's medium) containing 0.02% (w/v) EDTA, 10 mM Hepes, 0.025% (w/v) trypsin, and 0.001% (w/v) DNase, pH = 7.6, for 12 min. The reaction was stopped by the addition of FBS, the cells were washed in Hank's medium, and single cells were prepared by gently triturating the suspension. An average of 1.5×10^5 cells were obtained from the dissection of each embryo.

Culture of Spinal Cord Explants

Following dissection, spinal cord explants were plated directly onto plastic 24 well (Linbro) plates, which had been precoated with fibronectin (50 μg/ml; Boehringer-Mannheim, Indianapolis, IN) for 30 min and then removed. Initially, the explants were plated in a minimal amount of medium to promote their adhesion to the substrate. This medium consisted of Monomed (CSL, Melbourne, Australia) with 0.05% FBS (v/v), with or without the addition LIF (murine recombinant, specific activity = 10^8 units/mg) at a concentration of 10^4 units/ml. The cultures were then incubated at 37°C with 5% carbon dioxide overnight, after which more medium was added to the wells to prevent dehydration of the explants. These cultures remained viable for at least 7 days.

Primary Culture of Dissociated Spinal Cord Cells

Spinal cord cells (5×10^4) were plated onto 96 well plates (Linbro) precoated with fibronectin in Monomed medium and 0.05% FBS ± LIF in a final volume of 100 μl. Assays were normally performed over 5 days, after which time the cultures began to deteriorate. Cell counts were performed after harvesting the cells with trypsin and trituration. Process outgrowth was quantitated at day 5 by scoring the number of processes emanating from each discrete clump of cells. Cells were also plated onto confluent, irradiated (40 Gy) monolayers of Balb/c-3T3 cells on glass 12 well slides in Monomed medium and 0.05% FBS (v/v), at a density of 1.5×10^3 cells/well. After specified times, slides were fixed in methanol at −20°C for 30 min, stained for the presence of 150 kD neurofilament (NF) as described below, and the number of positively stained cells per slide

was quantitated. Numbers in all cases are the mean and standard deviation of six determinations.

^{3}H-Thymidine Experiments

Balb/c-3T3 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS (v/v) until a confluent healthy monolayer developed. Confluent monolayers of cells were irradiated (40 Gy) and then washed in Monomed with 0.1% FBS, just prior to plating the spinal cord cells. Spinal cord cells were plated onto the monolayers at a cell density of 6×10^3 cells/well in plastic 8 well slides (Lab-Tek, Naperville, IL) in Monomed with 0.1% FBS. Half an hour after plating, ^{3}H-thymidine was added to each well at a concentration of 0.03 μCi/ml and, after 3 days, the cultures were fixed and stained for NF, before being dipped in NTB-2 emulsion for autoradiography. The irradiated 3T3 monolayer took up ^{3}H-thymidine because the cells were still dividing at the start of the experimental period. Following 2 weeks of autoradiography, the slides were developed and examined microscopically for the presence of silver grains over the NF positive (NF^+) cells. The percent of neurons which had incorporated ^{3}H-thymidine was determined by counting at least 250 NF^+ cells for each condition.

Immunohistochemistry

Neurofilament staining. For staining of processes emanating from the cell clusters, cells were plated onto fibronectin-coated 60 well HLA plates (Lux, IL) in Monomed medium and 0.05% FBS at a concentration of 5×10^3 cells/well ± LIF at 10^4 units/ml. At day 5 the cultures were fixed in methanol at −20°C for 30 min and washed three times in phosphate-buffered saline (PBS) with 1% FBS (v/v). They were then incubated for 30 min with a rabbit anti-150 kD NF antibody (Chemicon, CA) diluted 1:400 in PBS with 1% FBS (v/v) and washed as above. For fluorescence microscopy, the cells were incubated with a fluorescein-conjugated sheep anti-rabbit antibody (Silenus, Australia) diluted 1:100 for 30 min and washed a further three times. For peroxidase staining of cells on 3T3 monolayers, the cells were incubated as above with anti-150 kD NF antibody and were then incubated with a biotinylated goat anti-rabbit antibody (1:200 dilution; Vector, Burlingame, CA) for 30 min. An avidin/biotin complex (1:100 dilution each; Vector) was then added for 30 min followed by a solution containing 10 ml of MTPBS, 10 mg 3,3′-diaminobenzidine (Dakopatts, CA), and 15 μl H_2O_2 for 6 min and then washed a further three times. The slides were then mounted in 1,4-diazabicyclo(2.2.2)-octane (DABCO; Merck, Munich) in glycerol (see Murphy et al., 1990) for fluorescence microscopy or Aquamount mountant (BDH, England) for peroxidase staining and coverslipped. NF^+ cells and processes were detected by fluorescence microscopy and peroxidase-stained cultures were examined by brightfield microscopy. Background staining controls substituted normal rabbit serum for the anti-150 kD NF antibody and showed no staining.

ChAT staining. E10 spinal cord cells were plated onto plastic 8 chamber slides (Lab-Tek) at either 100,000 cells/well on fibronectin or 6,000 or 12,000 cells/well on irradiated 3T3 monolayers. Cells were cultured in Monomed with 10% FBS (v/v) ± LIF at 10^4 units/ml. After 4 days in culture, cells were fixed in Zamboni's fixative (Zamboni and De Martino, 1967) for 1 hr at 4°C, then washed three times in PBS with 1% FBS (v/v). (PBS with 1% FBS was used as the diluent in all solutions.) Normal mouse serum (1:200) was then applied as a blocking solution for 30 min followed by normal sheep serum (1:200). The cells were then washed three times in PBS with 1% FBS (v/v) and the primary ChAT antibody (raised in rabbit; a gift from Dr. Miles Epstein, University of Wisconsin) was applied at a 1:5,000 final dilution overnight at 4°C. For negative control wells, normal rabbit serum (1:200 dilution) was applied instead of the ChAT antibody. The cells were then washed three times in PBS with 1% FBS (v/v) and incubated with a rhodamine isothiocyanate-conjugated sheep anti-rabbit IgG at 1:100 dilution for 30 min (Vector). For double-labeling experiments of ChAT and NF, the cells were first stained for ChAT immunoreactivity as above and were then refixed in methanol and stained for NF using a fluorescein-conjugated sheep anti-rabbit (1:100; Silenus) second layer. The cells were then washed three times in PBS with 1% FBS (v/v), then twice in distilled water and mounted. Since both primary antibodies were rabbit derived, some cross-reactivity may have occurred; however, 90% of the neurons which stained for NF were ChAT negative, and all of the $ChAT^+$ cell bodies had NF^+ neurites, confirming their neuronal identity.

RESULTS

LIF Stimulates Process Outgrowth From the Neural Tube

We recently showed that LIF stimulates the development of sensory neurons in cultures of neural crest obtained from E9 murine embryos (Murphy et al., 1991). In these experiments, lumbar to cervical regions of the neural tube are explanted into culture and the neural crest cells migrate out from the explants. LIF was noted also to influence the appearance of explants, increasing their apparent viability and process outgrowth (data not shown). We repeated these experiments on explants of E10 neural tubes caudal to the otic vesicle, corresponding to the spinal cord primordia, when most

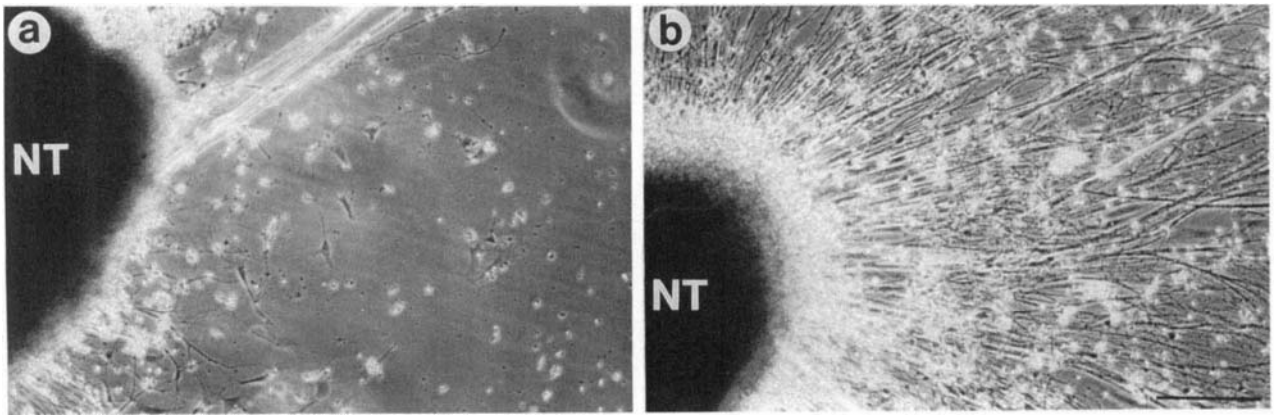


Fig. 1. Stimulation of process outgrowth from neural tube explants by LIF. Photomicrographs are of explants of E10 spinal cords cultured in the presence or absence of LIF in 24 well plates at day 7 in vitro to display process outgrowth. Shown are **(a)** control cultures and **(b)** explants cultured in the presence of LIF. The stacked and fasciculated arrays of axon bundles made quantitation of process outgrowth from the explants impossible. NT = neural tube. Scale bar = 200 μm.

of the neural crest has already migrated, but little neuronal differentiation has yet occurred (Cochard and Paulin, 1984; see also Lance-Jones, 1982). In order to see if LIF might be acting on neurons or their precursors in the spinal cord, we reduced the serum concentration in the assays to 0.05% to reduce proliferation of non-neuronal cells, without necessarily affecting neuronal differentiation (Ziller et al., 1983). In these cultures, there was little cell migration away from the explants and the substratum remained essentially free of cell bodies. Under these conditions, LIF stimulated profuse outgrowth of neuritic processes from the explants (Fig. 1b) compared with control cultures (Fig. 1a). The LIF-treated explants extended processes straight out onto the fibronectin-coated substratum, some as single processes and some in fasciculated bundles, occasionally many layers deep, making the number of processes per explant impossible to count. The stimulation of process outgrowth first became apparent at day 3 and increased up to a maximum at day 7, after which the cultures began to deteriorate.

LIF Stimulates Process Outgrowth From Dissociated Neuroepithelial Cultures

To further investigate the stimulation of process outgrowth described above, single cell suspensions were made (see Materials and Methods) of the spinal cord primordium and plated onto 96 well plates in the presence and absence of LIF. At less than 2.5×10^4 cells plated/well and in 0.05% serum, there was poor survival of the cultures and little process outgrowth, but at higher cell densities (5×10^4 cells/well) the cells formed aggregates over the first 2 days of culture. Soon after they formed, these aggregates began to put out processes, and in the LIF-treated cultures (Fig. 2b,c) significantly more processes arose than in control cultures (Fig. 2a). The number of processes in the cultures reached a maximum after 5 days, at which time the average number of processes in the LIF-treated cultures was approximately 4 times that in the controls (2,600 ± 500 processes/well in the presence of LIF compared to 680 ± 60 processes/well in control cultures, n = 6; $P < 0.001$, t-test). The processes in these cultures stained positively with the 150 kD anti-NF antibody (Fig. 2c), establishing their neuritic identity.

There was no significant difference in total number of viable cells in the LIF-treated cultures compared to controls after 5 days (LIF-treated cultures, $2.0 \pm 0.38 \times 10^5$ cells/well; control cultures, $2.2 \pm 0.34 \times 10^5$ cells/well; n = 6). This suggests that the increase in process outgrowth by LIF was not due to survival effects of LIF on the bulk population of neuroepithelial cells.

In addition, the effect was dependent on the concentration of LIF, with 50% maximal stimulation of process outgrowth at approximately 50 units/ml of LIF and maximal activity achieved at over 100 units/ml (Fig. 3). This concentration range is similar to that reported for other effects of LIF, such as those on the differentiation of the leukemic cell line M1 (Hilton et al., 1988) and on the survival of sensory neurons (Murphy et al., 1991).

LIF Stimulates an Increase in Differentiated Neurons in Neural Tube Cultures

It was possible that the stimulation of process outgrowth by LIF in the neuroepithelial cultures was due to an increase in neuron number. To study neuronal differentiation, it was necessary to culture the precursor cells at a lower cell density in order to identify and stain

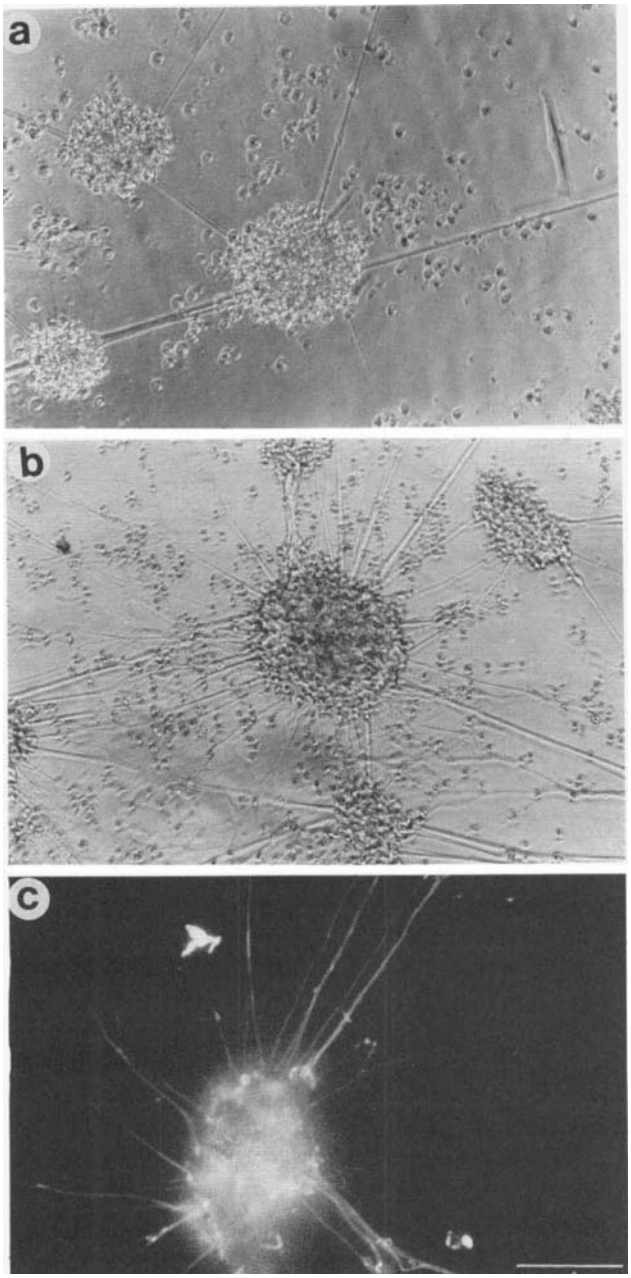


Fig. 2. LIF increases neurite outgrowth in dissociated neural tube cultures. Dissociated cultures of E10 neural tubes (5×10^4 cells) were incubated in the presence or absence of LIF in 96 well plates for 5 days, as described in Materials and Methods. Shown are phase-contrast photographs of **(a)** control cultures and **(b)** cultures incubated with LIF. Also shown is a similar culture **(c)** from spinal cord precursors, incubated with LIF and stained for NF antibody demonstrating NF^+ staining of the processes. Scale bar = 100 μm.

individual neurons, without the cells aggregating into large clumps. Thus, cells were plated at low density (1,500 cells/well of 8 mm diameter on glass slides) onto

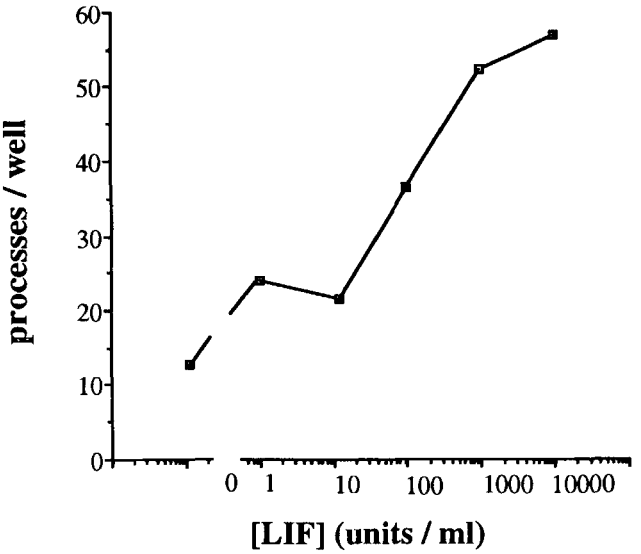
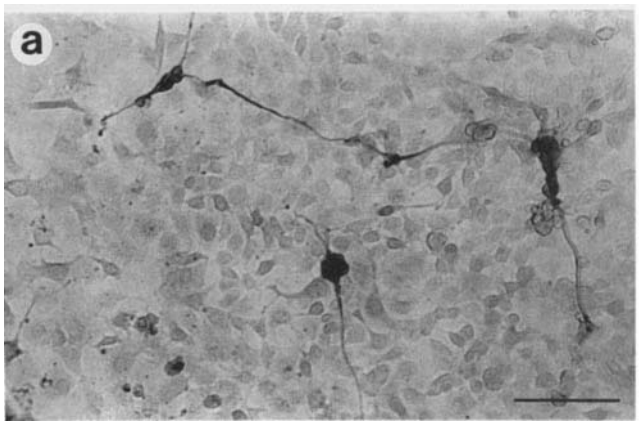


Fig. 3. The effect of LIF on process number is dependent on the concentration of LIF in the assay. Cultures from spinal cord primordium were plated as described in Figure 2, and the number of processes per culture was determined after 5 days of incubation. The results are the mean numbers of six determinations for each concentration and are of one representative experiment from a series of five experiments.

irradiated Balb/c-3T3 monolayers, which enhances the survival of the neuroepithelial cells at low cell density (see Murphy et al., 1990). As shown in Figure 4b, 4% of the cells initially plated were NF^+ neurons (Fig. 4a shows the neuronal morphology of these cells with large round cell bodies and exhibiting process extension) and in control cultures, the number of these cells increased by a little over 3-fold to approximately 200 neurons/well. In the LIF-treated cultures, the rate of increase in neuronal numbers was twice that of controls and the total number of neurons in these cultures reached approximately 400 neurons/well after 3 days (or 26% of the number of cells originally plated; Fig. 4b). Therefore, LIF significantly increased the total number of differentiated neurons in these cultures. After 3 days, the number of NF^+ cells declined at the same rate in LIF-treated and control cultures, indicating that LIF did not provide a survival advantage for the newly differentiated neurons after 3 days under these culture conditions.

To determine whether the increase in neuronal number in the LIF-treated cultures was due to a stimulation of precursor proliferation, ^{3}H-thymidine was added to the cultures at the time of plating. Dissociated E10 spinal cord primordial cells were plated onto 3T3 monolayers and cultured for 3 days in the presence or absence of LIF. The cultures were then fixed and stained for NF and processed for autoradiography (Fig. 5). There



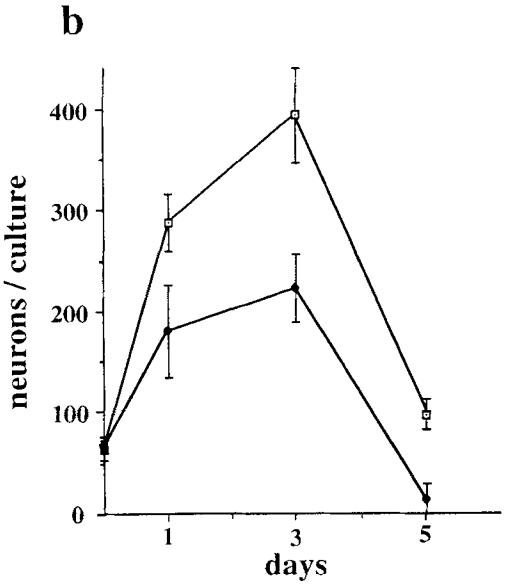


Fig. 4. Effect of LIF on neuronal differentiation of E10 neural tube cells. E10 neuroepithelial cells were plated at low cell density (1.5×10^3 cells/well) on irradiated (40 Gy) confluent monolayers of 3T3 cells, on glass 12 well slides, as described in Materials and Methods. At specified times after plating (4 hr, then 1, 3, and 5 days), the cultures were fixed and stained for 150 kD NF. NF^+ neurons on top of the 3T3 monolayer are shown in **a.** The number of neurons per culture was determined for six cultures at each of the specified time points, and the mean and standard deviations are shown in **b.** Scale bar = 100 μm.

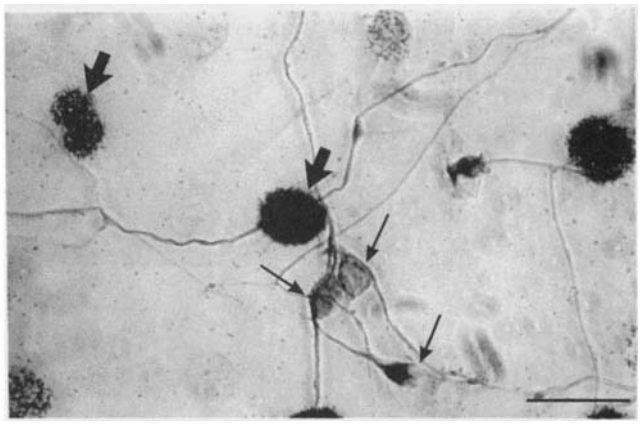


Fig. 5. LIF has no effect on precursor proliferation. To determine whether the increase in neuron number may be due to neuronal proliferation, we incubated neuroepithelial cells on 3T3 monolayers with ^{3}H-thymidine (see Materials and Methods). LIF did not increase the percentage of NF^+ neurons which incorporated ^{3}H-thymidine in these cultures. This photomicrograph shows several neurons (indicated by small arrows) stained for NF, which have not incorporated ^{3}H-thymidine, in contrast to the densely labeled cell bodies of the irradiated 3T3 cells (indicated with large arrows; see Materials and Methods). Scale bar = 50 μm.

was no significant difference between LIF-treated and control cultures in the percentage of the NF^+ cells which had incorporated ^{3}H-thymidine (13.1 ± 5.18% of the cells in LIF-treated cultures compared to 12.2 ± 2.75% in control cultures, n = 4). Further, most of the neurons which were labeled with ^{3}H-thymidine were only lightly labeled in comparison to the irradiated 3T3 monolayer cells. The irradiated 3T3 monolayer took up ^{3}H-thymidine because they were still dividing at the start of the culture period (see Materials and Methods). This suggests that, since the ^{3}H-thymidine was present during the entire experimental period, those neurons which were labeled had originated from precursors which synthesized only low amounts of DNA, only during the early culture period, and may not have been actively dividing for the remainder of the 3 days. Thus, the increase in neuron number in the presence of LIF is not due to the stimulation of neuronal precursor proliferation.

Effect of LIF on Cholinergic Differentiation

LIF acts as a cholinergic differentiating factor for sympathetic neurons (Patterson and Chun, 1977; Yamamori et al., 1989). Thus, we investigated whether LIF had an effect on the number of cholinergic neurons arising in spinal cord primordial cultures when isolated prior to innervation. Cells were plated as above onto 3T3 monolayers and stained for the presence of ChAT after 3 days (Fig. 6). There was a twofold increase in the number of ChAT positive ($ChAT^+$) cells in the LIF-treated cultures. Double-labeling experiments using anti-NF and anti-ChAT antibodies on the same culture showed that all the $ChAT^+$ neurons were also NF^+ (Fig. 6). However, the proportion of NF^+ neurons that were $ChAT^+$ was approximately the same in both LIF cultures (9.75 ± 1.2%, n = 4) and in controls (9.3 ± 2.9%, n = 4), indicating that LIF did increase the number of $ChAT^+$

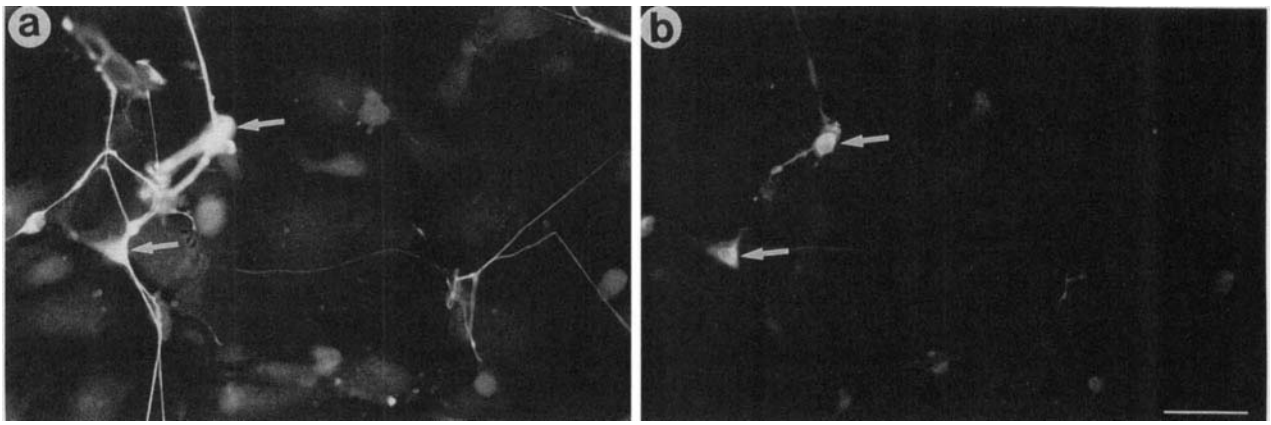


Fig. 6. Doubel-labeling of neurons with anti-ChAT and anti-NF antibodies. Cells were plated at 6,000 cells/well on plastic 8-chamber culture slides for 4 days and then stained, as described in Materials and Methods. Shown are fluorescence photomicrographs of the same neurons stained for NF **(a)** and ChAT **(b)**. Two neuronal cell bodies have been double-labeled (indicated by arrows), and their processes have been labeled with NF. Scale bar = 50 μm.

neurons in culture, but that this neuronal increase was not restricted to $ChAT^+$ neurons.

DISCUSSION

The results we present here clearly show that LIF stimulates neuronal process outgrowth from embryonic neural tube explants and in dissociated cultures of neural tube cells. The embryonic age at which these experiments were undertaken, E10, is a time when most of the cells in the neural tube are still dividing and have not differentiated (Nornes and Carry, 1978). The peak period of final division of motoneuron precursors in the spinal cord is E10–E11 and of most other spinal cord neuron precursors is E11–E14 (Nornes and Carry, 1978). The observation that there is significant neuronal differentiation in our cultures is thus quite consistent with the in vivo sequence of events and the effects of LIF that we have observed are probably upon this developmental process.

Our observations have found an increase in the number of neurites in aggregated cultures and an increase in the number of neurons in the cultures on the 3T3 monolayers with LIF. The simplest interpretation of these findings is that LIF has stimulated the generation of neurons in both cultures and this could have been accounted for by an increase in neuronal precursor proliferation, differentiation, or survival. In the 3T3-treated cultures LIF may have acted specifically on the neuronal precursors or there may have been an additional indirect effect mediated by the action of LIF upon 3T3 cells. However, we find no binding of ^{125}I-LIF to 3T3 cells (N. Nicola, unpublished observations), suggesting that there are no LIF receptors on these cells. Thus, it is unlikely that any of the effects we see are mediated through an activity of LIF on the monolayer. Clearly, the 3T3 monolayer does provide either cell-surface or other factor(s) which support the survival of the spinal cord cells at low cell density. There was also significant differentiation of the cells into neurons on the 3T3 monolayers in the absence of LIF. We have recently found that the 3T3 cells contain significant levels of LIF mRNA (L. Richards, unpublished observations), so it is possible that the observed differentiation of neurons on monolayers without exogenous LIF may have been dependent on this endogenously produced LIF.

How is LIF acting on the precursor cells? The experiments on the 3T3 monolayers with ^{3}H-thymidine incorporation show that LIF stimulates the rate of increase of neurons arising from their precursors, without influencing their proliferation. The observation that most (87%) of the neurons which arose had not incorporated ^{3}H-thymidine indicates that LIF acts upon nondividing precursor cells. LIF does not act as a survival factor for the majority of the neuroepithelial cells, but it may still act as a specific survival factor for the subpopulation of neuronal precursors; the loss of these precursors in control cultures may not be detected in total cell counts. Thus by promoting survival, LIF may be providing a permissive environment for differentiation to occur. Alternatively, its principal effect may be to directly stimulate the differentiation of the precursors. It may be possible to distinguish between these alternatives if the neuronal precursors could be identified, but at present such identification is difficult.

Studies of lineage in the chick spinal cord show that there are multipotential progenitors which give rise to a range of different neuronal types including interneu-

rons, autonomic preganglionic neurons, motoneurons, and glial cells (Leber et al., 1990) and which are present shortly before motoneurons are born. It may be that LIF is acting on these progenitors to stimulate their differentiation. Alternatively, LIF may be acting on a more restricted lineage but given that at least 26% of the plated cells differentiate into neurons in the presence of LIF, it seems more likely that LIF is stimulating the differentiation of more than one class of neurons. This is supported by the finding that only a subpopulation of the neurons are $ChAT^+$, which demonstrates the phenotypic heterogeneity of the neurons generated.

The differentiation of neurons in the spinal cord has also been shown to be influenced by epigenetic signals derived from the notochord and/or floor plate in the developing chick (Yamada et al., 1991). These experiments have shown that motoneurons can be induced to develop even in the dorsal aspects of the developing chick spinal cord, by grafting an extra notochord or floor plate section in that area. These results suggest a malleability of the spinal cord in terms of the differentiation of specific neuronal cell types that may develop under the influence of epigenetic factors, which, given our results, may include LIF.

We also have preliminary data to suggest that the effect of LIF in the CNS may be regionally specific at E10, with no response demonstrable in embryonic brain (L. Richards, unpublished observations). This would imply that at this stage of embryonic development, there are already significant differences in the neuroepithelial cells of the brain and of the spinal cord primordium, at least in the response to LIF. If LIF is influencing the precursor cells in the spinal cord primordium, this finding would mean that the precursor cells in the brain and spinal cord are already regionally committed before they have undergone differentiation. This kind of precommitment of precursor cells has been reflected in other studies of different cells of the CNS. Previously, we showed that the neuroepithelial cells of the mesencephalon and telencephalon of E10 mice respond to the FGFs but not to EGF or a range of other growth factors (Murphy et al., 1990), whereas Anchan et al. (1991) showed that neuroepithelial cells of the developing rat retina respond to EGF and TGF-α.

During the preparation of this paper, Martinou et al. (1992) found that LIF increases the survival of purified subpopulations of spinal cord motoneurons isolated postinnervation and therefore concluded that LIF must be acting directly on the neurons in these cultures. This shows that LIF can influence the survival of motoneurons postdifferentiation. In analogous studies of the development of the peripheral nervous system, we found that LIF acts to stimulate the differentiation of sensory neurons from their precursors and is also a survival factor for these neurons postinnervation (Murphy et al., 1991; unpublished observations). A similar relationship may apply for LIF in the differentiation and the subsequent survival of spinal cord neurons.

We have detected LIF mRNA in the developing spinal cord as early as E12 and up until birth by polymerase chain reaction (Murphy et al., manuscript submitted). Therefore LIF is present in vivo at a time analogous to when it is able to promote neuronal development in our in vitro assay. This is before innervation has occurred in vivo and suggests that it may act initially by mechanisms other than as a target-derived neurotrophic factor. The results of Martinou et al. (1992) suggest that LIF can also act as a survival factor for motoneurons after precursor differentiation has occurred. It is thus possible that LIF may play a role at several stages in the development of the mammalian spinal cord.

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