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Leukaemia Inhibitory Factor or Related Factors Promote the Differentiation of Neuronal and Astrocytic Precursors within the Developing Murine Spinal Cord

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Abstract

Previously we have shown that leukaemia inhibitory factor (LIF) potentiates the development of murine spinal cord neurons *in vitro*, suggesting that it, or related factors, may play an important regulatory role in neuronal development. We have further investigated this role and show here that the generation of neurons in cultures of embryonic day 10 spinal cord cells is inhibited by antibodies to the β subunit of the LIF receptor. Since there are more undifferentiated precursors in antibody-treated cultures than in control and LIF-treated cultures, it is concluded that the primary action of LIF, or related molecules, is to promote neuronal differentiation, not precursor survival. In addition, the failure of LIF to support neuronal survival in the period immediately following differentiation suggests that the increased numbers of neurons generated with LIF are not attributable to its neurotrophic action. By selecting neuronal precursors on the basis of their inability to express class I major histocompatibility complex molecules, it was shown that LIF acted directly upon these cells and not via an intermediary cell. LIF also appears to be involved in regulating the differentiation of astrocytes, since it increases the number of glial fibrillary protein (GFAP)-positive cells present in the cultures and since the spontaneous production of GFAP-positive cells is blocked by antibodies to the LIF β receptor. These findings suggest that LIF or related factors promote the differentiation of neural precursors in the spinal cord, but that they are not involved in preferentially promoting precursors down a specific differentiation pathway.

Introduction

The development of the central nervous system from stem cells to mature neurons is characterized by a series of overlapping steps, the first of which is the proliferation of stem cells within the ventricular zone of the neural tube. The proliferating cells give rise to neurons which then migrate away from the ventricular zone, differentiate and form connections with target tissues. Glial cell progenitor proliferation and differentiation commences after neuronal proliferation and differentiation has occurred and, in many parts of the nervous system, astrocyte differentiation occurs after primary neuronal process outgrowth is completed. These steps appear to be at least partially controlled by cell-extrinsic factors which maintain cell survival, stimulate proliferation and regulate differentiation.

We have been studying the role that soluble growth factors play in regulating these early events in nervous system development. Previously, we showed that leukaemia inhibitory factor (LIF) stimulated the generation of neurons in cultures of both embryonic murine neural crest and spinal cord (Murphy *et al.*, 1991; Richards *et al.*, 1992). LIF belongs to a group of structurally related cytokines, which include ciliary neurotrophic factor (CNTF), oncostatin M (OsM), growth-promoting activity and interleukin 6 (Bazan, 1991). These factors also have similar signalling pathways and act through receptor complexes containing shared components (Gearing *et al.*, 1992; Ip *et al.*, 1992). The LIF receptor comprises two subunits, gp130 and LIF receptor β (LIFRβ; Gearing *et al.*, 1992; Ip *et al.*, 1992). In addition to these subunits, another receptor component, CNTFRα, is required for CNTF binding (Ip *et al.*, 1992, 1993). CNTFRα is predominantly expressed in cells of the nervous system (Ip *et al.*, 1993), and thus the actions of CNTF may be more restricted to cells of the nervous system than LIF, which has effects on many cell types within the body (Hilton, 1992). Cells containing CNTFRα, however, respond equally well to LIF and to CNTF. For example, CNTF and LIF both support the survival of motor neurons *in vitro* (Arakawa

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et al., 1990; Martinou *et al.*, 1992; Henderson *et al.*, 1993) and *in vivo* (Sendtner *et al.*, 1990; Oppenheim *et al.*, 1991; Hughes *et al.*, 1993).

These cytokines have also been implicated in glial development. CNTF, together with serum and extracellular matrix, induces the *in vitro* differentiation of type 2 astrocytes from O-2A progenitors isolated from rat optic nerve (Hughes *et al.*, 1988; Lillien *et al.*, 1990). It has also been suggested that CNTF may be produced by astrocytes; indeed, CNTF-like molecules (Hughes *et al.*, 1988) and CNTF mRNA have been detected in rat optic nerve (Stockli *et al.*, 1991). Recently both CNTF and LIF have been shown to promote the differentiation of O-2A progenitors, derived from the optic nerve, into both oligodendrocytes and astrocytes (Mayer *et al.*, 1994).

Previously we have shown that LIF stimulated a twofold increase in the generation of neurons from spinal cord precursors when placed onto monolayers of 3T3 fibroblast cells (Richards *et al.*, 1992). Here we show that neuronal differentiation of spinal cord precursors was almost totally dependent on factors acting through the LIF receptor and that the previous neuronal differentiation which occurred in the absence of exogenous LIF was probably due to the presence of endogenous LIF, or related factors. At this stage of development, LIF acts directly on neuronal precursors and is primarily a differentiation factor. Immediately after differentiation, LIF is unable to maintain neuronal survival (Richards *et al.*, 1992) in the spinal cord cultures. However, here we show that LIF is able to support the survival of mature spinal cord neurons, analogous to its ability to support the survival of mature motor neurons (Martinou *et al.*, 1992). In addition, we also find that in the presence of serum, LIF potentiates the differentiation of precursor cells into glial fibrillary acidic protein (GFAP)-positive astrocytes. Together, these results suggest that LIF or related cytokines act as differentiation agents for spinal cord precursors, regardless of their lineage.

Materials and methods

Mouse strains and dissections

The CBA mice used in these experiments were housed and bred at the Walter and Eliza Hall Institute of Medical Research (WEHI) animal house. In addition, we used a transgenic mouse line, H253, which contained an inserted *lacZ* gene under the control of the 3-hydroxy-3-methylglutaryl coenzyme A reductase promoter, as previously described (Tan and Breen, 1993). In this transgenic mouse line, the *lacZ* gene is inserted into the X-chromosome, which during development is randomly inactivated, so that cells which express the *lacZ* transgene do so independently of their cell lineage. This mouse line was housed and bred at the Department of Pathology, University of Melbourne. Using this system, all cell types generated in the culture—not only the neuronal population—could be examined by staining for β-galactosidase activity. Mice heterozygous for the transgene were mated and embryos containing the *lacZ* transgene were identified using biopsied material and assessing for the production of a blue reaction product using X-gal histochemistry (described below). The spinal cords of *lacZ*-positive embryos were then dissected.

Experiments were performed using dissociated spinal cord cells from mouse embryos at day 10 (E10, where E0 was the day a vaginal plug was detected) or E15, or dorsal root ganglion cells from mice at postnatal day 2. Staging of the mice according to Theiler (1972) indicated that, for the spinal cord dissections, the CBA mice were close to E10.0 and the *lacZ* mice were close to E10.5. The dissection procedure has been previously described for spinal cord (Drago *et al.*, 1991; Richards *et al.*, 1992) and dorsal root ganglion cells (Murphy *et al.*, 1993).

Preparation of purified neuronal precursor cells by MHC class I-negative selection

Neuronal precursors can be isolated from E10 spinal cords since they differ from other neural cells by not expressing class I major histocompatibility complex (MHC) molecules in response to interferon-γ stimulation (Bartlett *et al.*, 1989; Bailey *et al.*, 1994). Class I-negative cells were selected by removing the class I-positive population using magnetic bead sorting. Spinal cords from E10 embryos were dissected and dissociated, and 10^6 cells were incubated for 24 h in Dulbecco's modified Eagle medium (DMEM) with 10% (vol/vol) fetal bovine serum (FBS) and 100 units of interferon-γ per well, in plastic 24 well plates. After 24 h, the cells were sorted by incubating with a mouse anti-class I MHC antibody (a gift from Dr G. Morahan, WEHI) for 30 min, washing three times, and incubating for a further 30 min with magnetic Dynabeads coated with sheep anti-mouse immunoglobulin antibody (Dynal, Skøyen, Norway). The coated dynabeads were prewashed three times in DMEM, 10% (vol/vol) FBS; all steps were conducted at 4°C, and during incubation the cells and beads were continuously rotated to ensure thorough mixing. After 30 min, a sample of cells was removed and examined microscopically to ensure that some of the cells and magnetic beads had formed rosettes. The cells and beads were then placed next to a magnet in a dynabead cell sorter (Dynal) and the supernatant, which contained cells that had not bound to beads, was carefully removed and resorted.

Approximately 3×10^5 cells from the starting population of 10^6 cells were unbound. To determine the percentage of class I-negative cells in this sorted population, an aliquot of the sorted cells was incubated for 15 min on ice with a sheep anti-mouse fluorescein-conjugated antibody (Silenus, Melbourne, Australia). These cells were washed three times in phosphate-buffered saline (PBS), mounted, and viewed using fluorescence microscopy. The percentage of class I-negative cells obtained through this sorting procedure was >99%.

Preparation of BALB/c 3T3 monolayer, fibronectin and poly-DL-ornithine/laminin-coated substrates

To obtain a confluent 3T3 monolayer, 5×10^3 BALB/c 3T3 cells per well were grown in DMEM with 10% (vol/vol) FBS in Permanox 8 chamber slides (Lab-Tek, Naperville, IL) and cultured for 3 days. The 3T3 monolayers were irradiated (40 Gy) and washed three times in Monomed medium (CSL, Melbourne, Australia) prior to plating the spinal cord cells.

Fibronectin-coated substrates were prepared by coating either 13 mm glass coverslips (Deckglasser, Germany) or 96 well plates (Linbro) with a solution of 50 μg/ml fibronectin (Boehringer Mannheim) diluted in PBS for at least 30 min at room temperature. To ensure that the wells did not dry out, the fibronectin was removed just prior to plating the cells.

Poly-DL-ornithine/laminin-coated 60 well HL-A plates (Nunc, Naperville, IL) were prepared over 2 days, prior to plating the cells (this protocol was provided by Dr A. Davies). Poly-DL-ornithine (Sigma) was used at a concentration of 0.5 mg/ml, dissolved in 0.15 M borate acid buffered to pH 8.56 with NaOH. The poly-DL-ornithine was sterile-filtered and plated onto the wells overnight at room temperature. The following day the wells were washed three times in HEPES-buffered Eagle medium (HEM) and allowed to air-dry in a sterile laminar flow cabinet. Laminin (Collaborative Research, Bedford, MA) was used at a concentration of 20 μg/ml and plated on top of the poly-DL-ornithine coated substrate for 4 h in a humidified incubator at 37°C. Just prior to plating the cells, the laminin was aspirated off and the wells were washed once in HEM for the purified neuronal precursor experiments and in HEM with 10% (vol/vol) FBS for all other assays.

Cytokines and inhibitory antibodies

LIF was obtained from AMRAD (Melbourne, Australia) and was used at a concentration of 10^4 units/ml (specific activity 10^8 units/mg). To determine LIF activity produced by 3T3 cells, these cells were cultured for 3 days at confluence in DMEM containing 10% FBS, conditioned medium was harvested, concentrated 10-fold and applied to an S-Sepharose fast-flow column, equilibrated in 50 mM phosphate buffer, 0.02% Tween-20, pH 7.0. The column was washed in this buffer, a gradient of 0–1 M NaCl was applied, and fractions containing protein were pooled and concentrated, and LIF bioactivity was determined as previously described (Gearing *et al.*, 1987). The rabbit anti-LIF receptor antibody was provided by Immunex Corporation (Seattle, WA); the antigen was a fusion protein of the extracellular portion (soluble form) of the murine LIFRβ (Gearing *et al.*, 1991) and the FLAG octapeptide (IBI, Newhaven, CT), expressed in CHO cells and purified by affinity chromatography (IBI). The antigen was injected in complete Freund's adjuvant. The resulting polyclonal antiserum detects LIFRβ on Western blots and immunoprecipitates LIFRβ (J. Peschon, personal communication).

Cell culture

To assess the degree of neuronal differentiation, E10 spinal cord cells were plated onto irradiated 3T3 monolayers in Permanox 8 chamber slides (Lab-Tek) at a density of 6×10^3 cells per well in Monomed with 0.1% (vol/vol) FBS in the presence or absence of LIF. Class I-negative neuronal precursors were plated onto polyornithine/laminin-coated wells of HL-A plates at 1.6×10^3 cells per well in Monomed medium. The cells were cultured in a humidified incubator at 37°C and 5% CO_2 in air. After specified times, cells were fixed and stained for neurofilament, as described below. To assess the effects of the anti-LIF receptor antibody on sensory neuron survival, dorsal root ganglion cells were cultured as previously described (Murphy *et al.*, 1993). All experiments were performed at least three times.

Cell counting and subculture to assess astrocyte differentiation

To assess glial differentiation, E10 spinal cord cells were cultured at 1.5×10^4 cells per well on fibronectin-coated wells of 96 well plates in Monomed, 10% (vol/vol) FBS. These cells grew to a high cell density during the course of the culture period, making it impossible to directly assess the percentage of GFAP-positive cells. In order to assess this, the cells were dissociated after 9 days in culture with trypsin–versene (CSL) for 10 min on a rocking table at 37°C. FBS was then added to inhibit the enzyme activity and the cells were gently passed through a glass pipette several times to form a single-cell suspension. The cells were either immediately cytospun onto glass microscope slides with Shandon filter cards (Astmoor, UK; 450 g/m^2) using a Shandon Southern cytospin apparatus, or replated onto fibronectin-coated (50 μg/ml), Permanox 8 chamber slides (Lab-Tek) in Monomed with 10% (vol/vol) FBS. These cells were cultured for an additional 2 days without the further addition of LIF or the anti-LIF receptor antibody. Both the replated cells and the cytospun cells were then fixed and stained for the presence of GFAP (see below). The percentage of GFAP-positive cells was then determined by counting at least 500 randomly selected cells per cytospin.

Assessment of LIF mRNA in BALB/c 3T3 cells

3T3 cells were cultured in 25 cm^2 tissue culture flasks (Falcon) in 15 ml DMEM with 10% (vol/vol) FBS until they reached confluence, irradiated (40 Gy), washed three times in Monomed and then cultured in Monomed with 0.1% (vol/vol) FBS, in the presence or absence of LIF, for a further 3 days. The 3T3 cells were then trypsinized and RNA was isolated from the cells using a guanidinium extraction procedure (Chomczynski and Sacchi, 1987). Poly(A)$^+$ RNA was extracted from the cells using a proteinase K/sodium dodecyl sulphate/oligo(dT) procedure (Gonda *et al.*, 1982). First-strand cDNA was synthesized as previously described (Allan *et al.*, 1990) from at least 100 ng of RNA and used as a template for a polymerase chain reaction (PCR). PCR reactions contained 200 mM dNTP, 1 mM primers, standard PCR buffer (Perkin-Elmer, Melbourne, Australia) with Mg^{2+} at 2.5 mM, and 1.25 units of *Taq* polymerase (Perkin-Elmer). Cycle conditions were 94°C for 1.5 min, 60°C for 2 min and 72°C for 3 min through 25 cycles. Primers for LIF and the glycolytic enzyme GAP-DH, which was used as a control for RNA quality and quantity, were as previously described (Allan *et al.*, 1990). PCR reaction products were analysed by electrophoresis through 1.2% agarose gels and then transferred to a Zeta probe membrane (Bio-Rad, Hercules, NY) in 0.4 M NaOH (Reed and Mann, 1985) for 2–6 h for Southern analysis. Filters were prehybridized and hybridized in 50% formamide, 5 × standard saline citrate (SSC), 5% sodium dodecyl sulphate, 10 mM sodium phosphate, and 1% skim milk powder at 42°C. DNA fragments (Allan *et al.*, 1990) were radiolabelled with [^{32}P]dATP (Bresatec, Adelaide, Australia) by random priming. Following overnight hybridization, filters were washed to a final stringency of 0.2 × SSC at 65°C and autoradiographed (Kodak XAR5 film) with two intensifying screens at –70°C.

Immunohistochemistry

For neurofilament, MAP2 and GFAP staining, cultures were first fixed in methanol at –20°C for 30 min and then washed three times in PBS with 1% (vol/vol) FBS. The wells were then incubated with either a rabbit anti-150 kDa neurofilament antibody (used at a dilution of 1:400; Chemicon, Temecula, CA), mouse anti-MAP2 (used at a dilution of 1:400; Sigma, USA) or a rabbit anti-GFAP antibody (used at a dilution of 1:50; Dako) for 30 min and washed a further three times in PBS, 1% (vol/vol) FBS. After the initial incubation with the primary antibody, cells were then peroxidase-stained with a Vectastain kit (Vector, Burlingame, CA) for GFAP and neurofilament antibodies or an Elite Vectastain kit (Vector) for MAP2 staining. Cells were washed three times in pure water and mounted in Aquamount (BDH, Poole, UK).

Detection of the lacZ reporter gene using the Xgal reaction

To detect the expression of β-galactosidase by the *lacZ* transgene, histochemical staining was performed by incubating the cells overnight at 37°C with 0.1% X-Gal (Diagnostic Chemicals, Freehold, NJ), 2 mM $MgCl_2$, 5 mM EGTA, 0.01% (wt/vol) sodium desoxycholate, 0.02% (wt/vol) Nonidet P-40 (BDH, Poole, UK), 5 mM $K_3Fe(CN)_6$ and 5 mM $K_4Fe(CN)_6.6H_2O$. The cultures had been previously fixed in a freshly prepared solution of 4% (wt/vol) paraformaldehyde and 0.5% (vol/vol) glutaraldehyde in PBS. These cultures could then be refixed in methanol and stained for neurofilament, as described above.

Statistical analysis

Where numbers of cells are expressed as total cells per culture, the numbers are expressed as mean ± SD, and Student's *t*-test was used to determine the degree of difference between conditions (Zar, 1984). Where data are expressed as percentages of cells per condition, the percentages were arcsin-transformed and statistical analyses were performed. Data were then back-transformed and numbers are expressed as percentage ± SD. In these experiments, levels of significance were determined using confidence intervals (Zar, 1984).

Results

LIF is required for neuronal development on 3T3 monolayers

Previously, we showed that LIF increased the number of neurons on 3T3 monolayers; however, the observed enhancement by LIF was only two-fold that of control cultures. This small increase in neuronal differentiation did not account for the large amount of stimulation of neurite outgrowth previously observed with LIF from spinal cord explant cultures grown on a fibronectin-coated substrate (Richards *et al.*, 1992). It was possible that the neuronal differentiation of spinal cord precursors observed on the 3T3 monolayers was partly due to the endogenous production of LIF by the 3T3 cells. To investigate this possibility, we performed reverse transcriptase PCR on RNA isolated from 3T3 cells using oligonucleotide primers designed to anneal specifically to LIF cDNA. Using this protocol, and by performing Southern blot analysis and hybridization with a radioactive LIF probe, it was found that the cells did indeed contain mRNA for LIF (Fig. 1). In addition, conditioned medium from the 3T3 cells was found to have significant LIF bioactivity (equivalent to 0.36 ng LIF/ml of medium). Thus, 3T3 cells synthesize LIF mRNA and produce LIF bioactivity.

In order to inhibit the action of endogenous LIF, we added an anti-LIFRβ antibody to the cultures. This antibody inhibits LIF mediated survival of dorsal root ganglion sensory neurons *in vitro* (Fig. 2), the effects of which could also be reversed by the addition of excess LIF, indicating that cell death did not result from direct antibody-induced toxicity. The specificity of the antibody for LIFRβ was also shown from these experiments, where at low concentrations of antibody, levels of LIF as low as 10 units/ml (100 pg/ml) could abrogate inhibitory effects of the antibody (Fig. 2). Further, the LIFRβ antibody did not interfere with the survival response of the dorsal root ganglion neurons to nerve growth factor. At 48 h of culture, no significant difference ($P>0.1$) was seen between cultures treated with nerve growth factor (81 ± 8%) and those treated with nerve growth factor and LIFRβ antibody (83 ± 8%). These data further confirm the specificity of the LIFRβ antibody for LIF or related factors. Corroborative support for the specificity of this antibody comes from the finding that its effects on dorsal root ganglion neurons match exactly the responses of dorsal root ganglion neurons from mice disrupted in the gene for LIFRβ (Ware *et al.*, 1995).

When the spinal cord cells were plated onto 3T3 monolayers in the presence of the LIFRβ antibody, very few neurons arose (Fig. 3). In the first 24 h of culture, the number of neurons in antibody-treated cultures was approximately 15% of the number which arose in the LIF-treated cultures. There was no subsequent generation of neurons at later time points and, in fact, the number of neurons decreased (Fig. 3). Thus, the generation of neurons which occurred on the 3T3 monolayers in the absence of added LIF was dependent upon endogenously produced factor(s), acting through the LIFRβ.

LIF promotes neuronal differentiation in the developing spinal cord cultures

To investigate the mechanism by which LIF stimulated neuronal development within the spinal cord cultures, we attempted to determine whether LIF was directly stimulating neuronal differentiation or whether it was promoting the survival of the neuronal precursor which subsequently differentiated into a mature neuron. It was therefore necessary to be able to identify all the spinal cord precursor cells plated onto the 3T3 monolayer, so that we could observe their subsequent development. To do this, we used spinal cord cells derived from transgenic mice which constitutively expressed the *lacZ* gene,

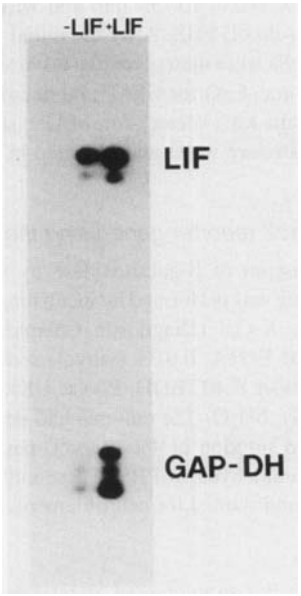

FIG. 1. 3T3 cells express LIF mRNA. 3T3 cells were cultured in the presence and absence of LIF, cDNA was prepared, PCR products were generated using primers for both LIF and GAP-DH genes, and the products were separated on agarose gels as described in Materials and methods. Shown is an autoradiograph of the Southern blot of the PCR products, hybridized with both ^{32}P-labelled LIF and GAP-DH probes (specific hybridization products are indicated at the side of the autoradiograph).

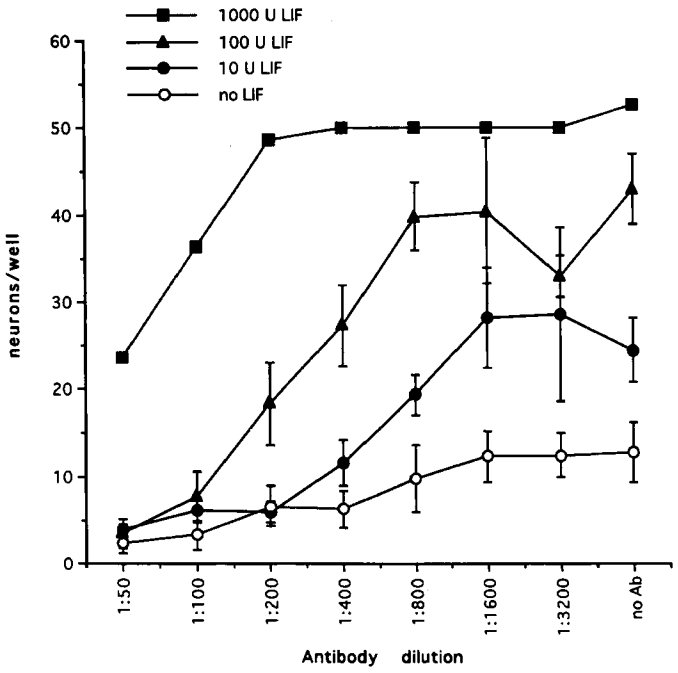

FIG. 2. Effect of anti-LIF receptor antibody on sensory neuron survival. Dorsal root ganglion cells from postnatal day 2 mice were prepared and plated at 100 cells per well in wells of HL-A plates in the presence of different dilutions of LIF and anti-LIF receptor antibody. After 2 days, numbers of live neurons were determined and are expressed as mean ± SD (n = 6), with the exception of assays conducted at 1000 U/ml, where numbers are the average of two determinations.

allowing all spinal cord-derived cells in the culture to be identified by β-galactosidase staining. These cells were plated onto 3T3 monolayers, fixed after 24 h and stained for the presence of β-galactosidase. In the LIF-treated cultures, 85% of the total β-galactosidase-positive cells also stained positively for neurofilament. In comparison, <40% of the β-galactosidase-positive cells had differentiated into neurofilament-positive neurons in cultures in which the action of LIF had been prevented by treatment with the anti-LIF receptor antibody (Fig. 4). At this time, there was no significant difference between the number of β-galactosidase-positive cells in the LIF-treated cultures (1120 ± 200) compared to cultures treated with the anti-LIF receptor antibody (960 ± 430, n = 8). These results show that in the first 24 h of culture, more overt neuronal differentiation of precursor cells occurred in the presence of LIF than in the presence of the anti-LIFRβ antibody, while at the same time there was little influence of LIF on the survival of the cells.

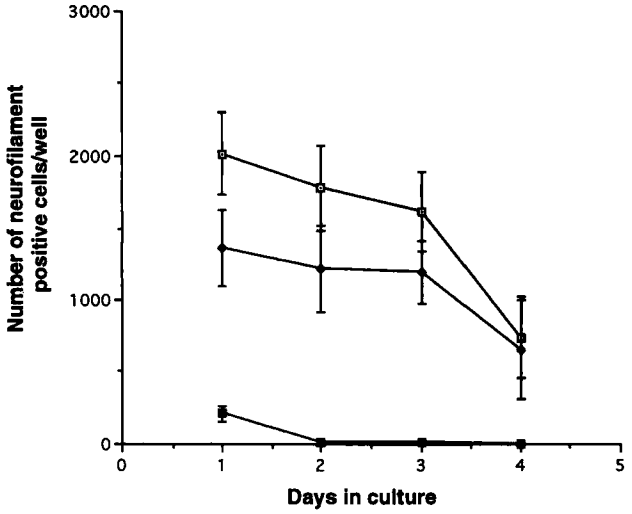

FIG. 3. LIF or a related molecule is necessary for the generation of neurons from spinal cord precursors. E10 spinal cord cells were plated at 6000 cells per well on 3T3 cells in the presence of LIF (□), control (◆) or anti-LIF receptor antibody (□), and at daily intervals cultures were fixed and assessed for the total number of neurofilament-positive cells per well. Shown are the mean ± SD of eight wells.

The time course of the appearance of differentiated neurons (β-galactosidase-positive, neurofilament-positive cells) and loss of undifferentiated precursor cells (β-galactosidase-positive, neurofilament negative cells), using cells from these *lacZ* transgenic mice, shows that in the first 24 h of culture most of the precursor cells differentiate into neurons (Fig. 5) in the presence of LIF. Later in the culture period, there is little additional neuronal differentiation and the remaining precursor cells are lost (Fig. 5). Thus, LIF stimulates the neuronal differentiation of precursor cells, and those cells which have not differentiated by 24–36 h probably die.

LIF acts directly on the neuronal precursor and not via accessory cells in the culture

The above experiments suggest that LIF could induce neuronal differentiation without promoting neuronal precursor cell survival. Since these experiments were performed on whole spinal cord cultures, we wanted to investigate whether LIF could act directly on neuronal precursor cells to induce neuronal differentiation in the absence of other cell types within the culture. We therefore isolated a population of neuronal precursor cells by sorting the neuroepithelial cells on the basis of whether they could be induced to express MHC class I (Bartlett *et al.*, 1989; Bailey *et al.*, 1994). It was also important to remove 3T3 cells from these cultures, as they also clearly influenced the development of the spinal cord cells. Thus, the purified neuronal precursors were plated onto poly-ornithine/laminin-coated substrates and incubated in the presence and absence of LIF. In the presence of LIF, significantly more neurons differentiated than in medium alone

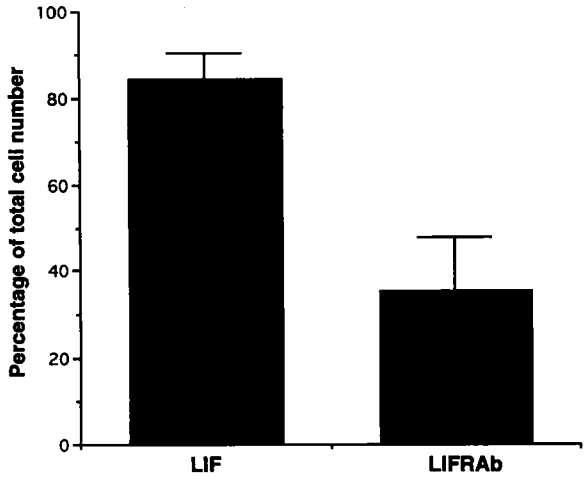

FIG. 4. LIF receptor antibody blocks neuronal differentiation of spinal cord precursors. E10 spinal cord cells were isolated and dissociated from *lacZ* transgenic mice, cultured on 3T3 monolayers in the presence of LIF or the anti-LIFRβ antibody (LIFRAb) for 24 h and then stained for the presence of β-galactosidase and neurofilament. This graph shows the mean percentage ± SD (n = 8) of neurofilament-positive cells as a proportion of total β-galactosidase-positive cells, and indicates that a significantly greater percentage (P < 0.005) of the precursor cell population had differentiated into neurons in the presence of LIF.

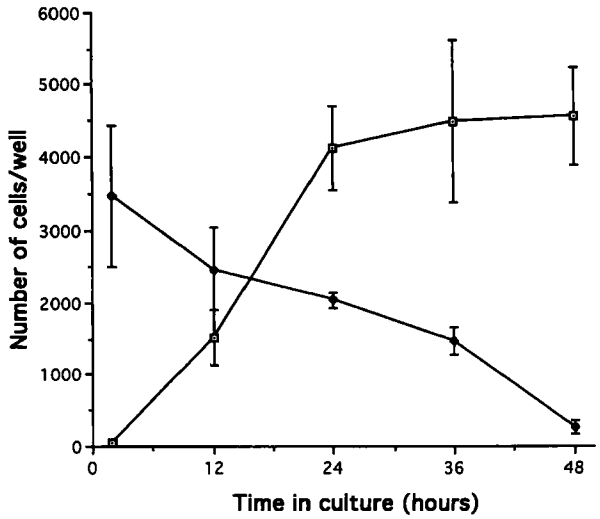

FIG. 5. Time-course of the differentiation of spinal cord precursors on 3T3 monolayers into neurofilament-positive neurons, in the presence of exogenous LIF. The number of β-galactosidase-positive, neurofilament-negative (◆) and neurofilament-positive cells (□) was determined over a 48 h period. Shown are the mean ± SD of counts from eight wells per time-point.

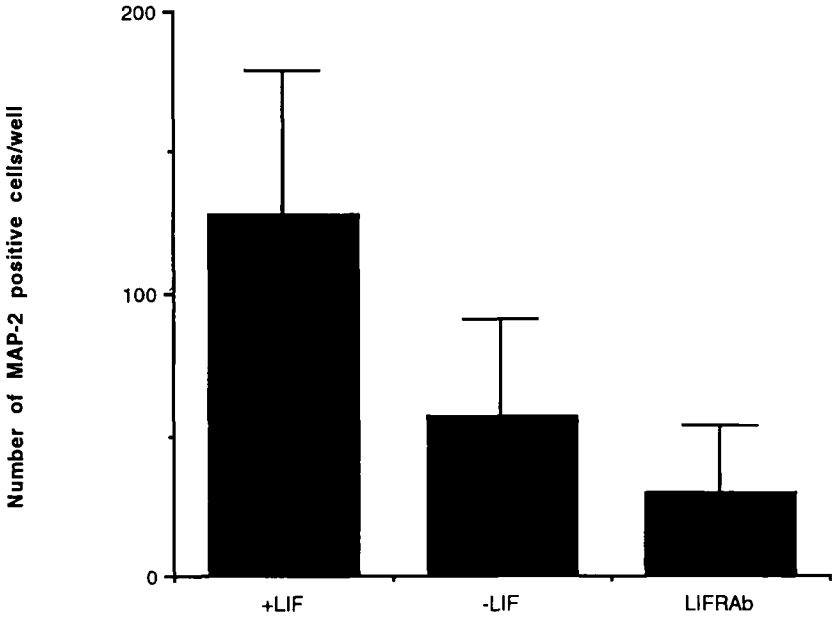

FIG. 6. LIF acts directly on the neuronal precursor cell. Purified neuronal precursors were plated at 1.6×10^3 cells per well in medium alone, in the presence of LIF, or LIFRβ antiserum. Mean ± SD of between 8 and 11 wells per condition is presented. LIF significantly increased the number of neurons ($P < 0.005$) over control and LIFRβ antibody cultures.

(Fig. 6), establishing that LIF could act directly on the neuronal precursors to stimulate differentiation. Further, addition of LIFRβ antiserum to these cultures resulted in a further decrease in the number of neurons arising compared to medium alone (Fig. 6), and this level was ~20% of the differentiation which occurred in LIF-treated cultures, indicating that most of the differentiation which occurred was dependent on LIF or related factors.

LIF promotes the survival of fully differentiated neurons

Previously, LIF has been reported to support the survival of sensory and motor neurons *in vitro* (Murphy *et al.*, 1991, 1993; Martinou *et al.*, 1992). Our previous studies, however, showed that in cultures of spinal cord cells from E10, the neurons generated in response to LIF did not survive in the presence of LIF. To determine whether this was a transient failure of LIF to support newly differentiated neurons, neurons from the spinal cord of E15 mice were isolated and plated at 6×10^3cells per well onto irradiated 3T3 monolayers. It was found that there was a significant increase in the number of neurons that survived for 3 days in the presence of exogenous LIF, compared to control cultures or those cultured with the anti-LIF receptor antibody (Fig. 7).

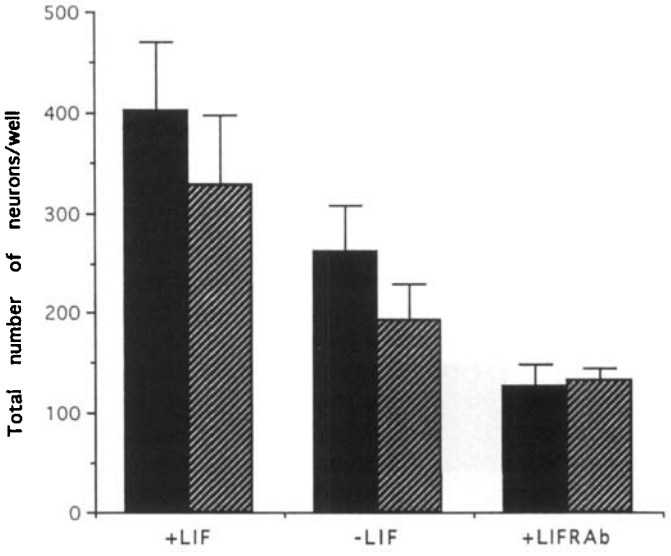

FIG. 7. LIF potentiates the survival of mature neurons isolated from E15 spinal cords. E15 spinal cord cells were plated onto 3T3 monolayers and cultured for 1 day (solid columns) or 3 days (hatched columns). LIF significantly increased the number of neurons present (+LIF) over control (–LIF; $P < 0.01$) and anti-LIF receptor antibody (+LIFRAb) cultures ($P < 0.001$). In each condition, the number of neurons surviving in each of eight wells was assessed, and the mean ± SD is shown.

LIF promotes the differentiation of astrocytes

In preliminary experiments, we had noted that if spinal cord cells were incubated in the presence of high serum (10% FBS), LIF appeared to stimulate an increase a subpopulation of non-neuronal adherent cells which may have been glia. To further investigate this possibility, E10 spinal cord cells were cultured on a fibronectin-coated substrate, at a density of 1.5×10^4 cells/well in 96 well plates and, at various times after plating, stained for the presence of GFAP (Antanitus *et al.*, 1975). Previous experiments have shown that GFAP does not arise *in vitro* until the equivalent of E17 (Abney *et al.*, 1981), and thus E10 cultures were maintained for up to 11 days after plating. After this time the cells in the cultures were flat and adherent and often formed multilayered aggregates, making the enumeration of GFAP-positive cells difficult. Therefore, to determine the total number of cells in the cultures and the proportion which were GFAP-positive, cultures were trypsinized and the cells were either replated at a lower cell density, or immediately cytospun onto glass microscope slides. The total cell number of cells per well after 9 days was slightly

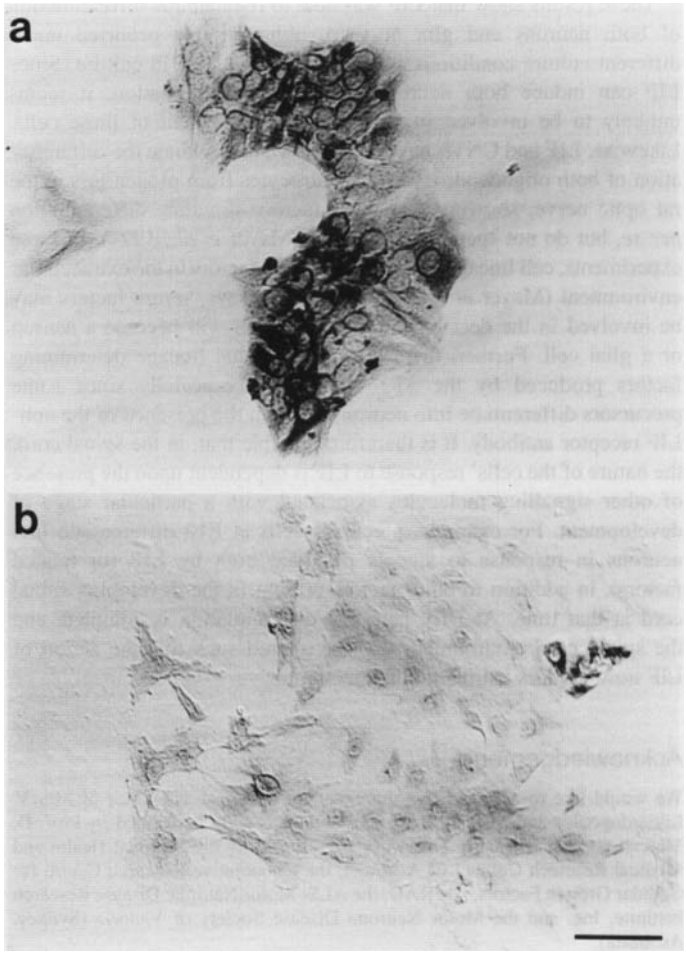

FIG. 8. Photomicrographs demonstrating that LIF promotes the expression of GFAP in long-term cultures of E10 spinal cord precursors. After 9 days in culture, cells grown in the presence (A) or absence (B) of LIF were dissociated, replated and cultured for a further 2 days, and stained for the presence of GFAP. Bar = 100 μm.

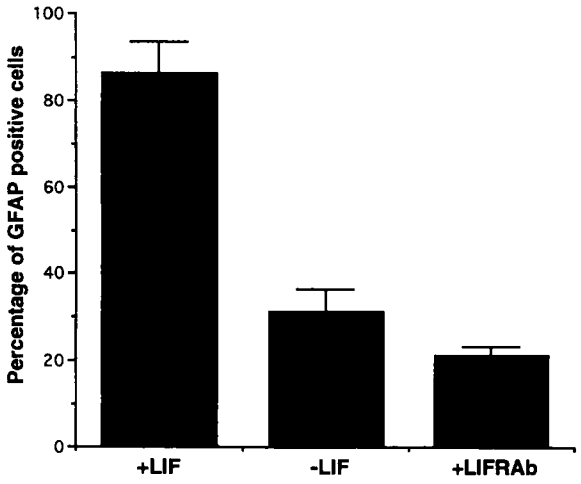

FIG. 9. LIF increases the percentage of GFAP-positive cells in long-term spinal cord cultures ($P < 0.005$). Spinal cord cells were cultured for 9 days in the presence (+LIF) or absence of LIF (–LIF) or in the presence of the anti-LIF receptor antibody (+LIFRAb), and then dissociated and subcultured at low cell density for 2 days. The cultures were then stained for GFAP and the percentage of GFAP-positive cells was determined by assessing 500 randomly selected cells, in eight separate wells for each condition.

increased in the presence of LIF ($1.5 \times 10^5 \pm 0.27 \times 10^5$ cells) compared to the non-treated cultures ($0.95 \times 10^5 \pm 0.42 \times 10^5$ cells). However, when the replated cells were stained for the expression of GFAP (Fig. 8a, b), the percentage of GFAP-positive astrocytes was significantly increased in the LIF-treated cultures compared to controls and cultures treated with the anti-LIF receptor antibody (Fig. 9). In addition cells were also harvested after 9 days *in vitro*, directly cytospun and stained for GFAP. A greater percentage of these cells were also GFAP-positive in the presence of LIF: 46% compared to 9.4% in cultures treated with the anti-LIF receptor antibody.

Discussion

These results have shown that LIF has prominent effects on the development of cells of the murine spinal cord *in vitro*. LIF not only increased the number of neurons generated within these cultures (Richards *et al.*, 1992) but the production of neurons was critically dependent upon the presence of LIF. This was best shown where a specific anti-LIF receptor antibody was added to the cultures and the number of neurons generated was significantly reduced. LIF appeared to directly stimulate neuronal differentiation, as shown by experiments in which the proportion of cells which had differentiated into neurons could be definitively determined. By using *lacZ* transgenic mice and by following the fate of *lacZ*-positive cells, it was shown that LIF directed a clear shift in the percentage of neurons generated, in the absence of any significant difference in total cell number. Thus, this increase was not attributable to a selective survival of precursors in the presence of LIF. Neither could the increase be attributed to the selective proliferation of a subset of neuronal precursors, as the [^{3}H]thymidine labelling index was unaffected by exogenous LIF (Richards *et al.*, 1992). The precursor cells which did not differentiate into neurons eventually died, regardless of whether LIF was present in the culture medium, indicating that LIF was not acting as a survival agent for these cells. The finding that there were significant numbers of neurons in the presence of the LIFRβ antibody in experiments conducted with *lacZ* mice was inconsistent with our findings that only a few cells underwent neuronal differentiation in the presence of this antibody. This may have been because embryos taken from *lacZ* mice at E10 always appeared considerably more developed (to at least E10.5 according to Theiler) than their CBA counterparts (E10.0). This difference may be important since it occurs at a critical time in the development of spinal cord neurons, which are undergoing neuronal differentiation from as early as E10.0.

Using purified neuronal precursors, it was also shown that LIF could act directly on these cells to stimulate their differentiation. Thus, the effects of LIF in stimulating differentiation were not mediated through the stimulation of other lineages, for instance any effects that it may have on glial differentiation, which it was important to exclude since LIF does influence glial development in the spinal cord (as discussed below). The observation that neuronal differentiation was further reduced in the presence of LIFRβ antibody suggests that LIF or related factors are being produced by these cells themselves.

The anti-LIFRβ antibody experiments showed that this receptor was critically involved in the differentiation process. It has been shown that a number of ligands may be acting through this receptor, including LIF, CNTF, OsM and growth promoting activity (Gearing

and Bruce, 1992; Ip *et al.*, 1992), and preliminary data suggest that both CNTF and OsM are also able to promote the generation of neurons within the E10 spinal cord cultures (L. Richards, unpublished observations). Thus, it is possible that these factors, in addition to LIF, are produced by the 3T3 monolayer and may also contribute to neuronal differentiation.

This raises the question of which of these factors might be acting *in vivo* during spinal cord development. We have found detectable levels of LIF mRNA in the spinal cord from as early as E12 (Murphy *et al.*, 1993), consistent with a role for LIF in spinal cord development. In addition, CNTFRα is expressed at high levels throughout the neuroepithelium from at least E11 (Ip *et al.*, 1993), implying that this component has a role in the signalling process and that the entire LIF receptor complex is present at this stage of development. However, CNTF lacks a conventional secretory mechanism (Sendtner *et al.*, 1990) and is only expressed at very low levels during development (Ip *et al.*, 1993), suggesting that CNTF is unlikely to be involved in the neuronal differentiation process. The role of OsM in neuronal development is yet to be determined. So far, mice deleted for either the *LIF* (Stewart *et al.*, 1992) or the *CNTF* gene (Masu *et al.*, 1993) have revealed no defects in the development of spinal cord neurons. However, recent observations reveal developmental defects in the spinal cord neurons of mice deleted for LIFRβ (Ware *et al.*, 1995; C. Ware, unpublished observations). These findings suggest that factors apart from LIF or CNTF, but which signal through LIFRβ, are involved in neuronal development in the spinal cord.

The analysis of CNTF- and LIF-deleted animals does, however, indicate that these factors are important for the maintenance of certain spinal cord neurons. Mice with a mutant CNTF gene display subtle changes in locomotor behaviour and muscle strength associated with some motor neuron cell death (Masu *et al.*, 1993). In addition, CNTF/LIF double knockout mice show a more rapid degeneration of motor neurons compared to mice deleted only for CNTF (M. Sendtner, personal communication).

Whereas LIF can actively stimulate precursor differentiation, it is unable to support the survival of newly differentiated neurons (Richards *et al.*, 1992). Later in spinal cord development, LIF can support the survival of spinal cord neurons, as well as purified motor neurons (Martinou *et al.*, 1992). This situation also pertains in sensory development (Murphy *et al.*, 1993) and may be related to discrete activities of this family of factors, first in differentiation and second in neuronal maintenance. The survival of the newly differentiated sensory and motor neurons is probably regulated by one or more neurotrophins (Davies, 1992; Snider, 1994).

In this study we also showed that LIF promoted the expression of GFAP when precursors were cultured in the presence of 10% FBS. This finding correlates with the recent findings from LIFRβ knockout mice, in which GFAP expression is missing in the CNS of late term embryos (Ware *et al.*, 1995). It also appears that the action of LIF is predominantly on glial precursors, as mature astrocytes did not up-regulate GFAP when treated with exogenous LIF in the culture (L. Richards and T. Kilpatrick, unpublished observation). These results could reflect a pivotal role for LIF in promoting the differentiation of astrocytes; alternatively the action of LIF could be confined to stimulating the expression of GFAP. While the significance of GFAP expression is uncertain, it may be important in the acquisition of a mature astrocytic phenotype, namely the facilitation of the formation of stable astrocytic processes in response to the presence of neurons (Weinstein *et al.*, 1991). Clearly, however, further studies are required to delineate the precise role which LIF and/or related molecules play in astrocytic differentiation.

These results show that LIF was able to regulate the differentiation of both neurons and glia *in vitro*, although this occurred under different culture conditions and with varying times in culture. Since LIF can induce both neuronal and glial differentiation, it seems unlikely to be involved in the lineage commitment of these cells. Likewise, LIF and CNTF have been shown to stimulate the differentiation of both oligodendrocytes and astrocytes from progenitors in the rat optic nerve, showing that these factors stimulate differentiation *per se*, but do not specify cell lineage (Mayer *et al.*, 1994). In these experiments, cell lineage was determined by factors in the extracellular environment (Mayer *et al.*, 1994). In our assays, serum factors may be involved in the decision of whether a cell will become a neuron or a glial cell. Further, there may be neuronal lineage determining factors produced by the 3T3 monolayers, especially since some precursors differentiate into neurons, even in the presence of the anti-LIF receptor antibody. It is therefore possible that, in the spinal cord, the nature of the cells' response to LIF is dependent upon the presence of other signalling molecules associated with a particular stage of development. For example, precursor cells at E10 differentiate into neurons in response to signals provided both by LIF (or related factors), in addition to other factors present in the developing spinal cord at that time. At E16, neuronal differentiation is complete and the spinal cord environment may be altered such that the action of LIF now induces astrocytic differentiation.

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Abbreviations

CNTF	ciliary neurotrophic factor
CNTFRα	ciliary neurotrophic factor receptor α
DMEM	Dulbecco's modified Eagle medium
E	embryonic day
EGTA	ethylene glycol-bis(β-aminoethyl ether)*N,N,N′,N′*-(2-ethanesulphonic acid)
FBS	fetal bovine serum
GFAP	glial fibrillary acidic protein
HEM	HEPES-buffered Eagles medium
HEPES	*N*-(2-hydroxyethyl)piperazine-*N′*-(2-ethanesulphonic acid)
LIF	leukaemia inhibitory factor
LIFRβ	leukaemia inhibitory factor receptor β
MAP2	microtubule-associated protein 2
MHC	major histocompatibility complex
O-2A	oligodendrocyte type 2 astrocyte
OsM	oncostatin M
P	postnatal day
PBS	phosphate-buffered saline

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