Ciliary neurotrophic factor and leukemia inhibitory factor promote the generation, maturation and survival of oligodendrocytes in vitro

Margot Mayer[1], Kishore Bhakoo[2] and Mark Noble[1,3,*]

[1]Ludwig Institute for Cancer Research, 91 Riding House Street, London W1P 8BT, UK
[2]Institute for Child Health, Guilford Street, London WC1N 1EH
[3]Department of Biochemistry and Molecular Biology and Department of Anatomy and Experimental Embryology, University College London, Gower St. WC1E 6BT.

*Author for correspondence

SUMMARY

We have found that CNTF and LIF are pleiotropic modulators of development in the O-2A lineage. Both molecules enhanced the generation of oligodendrocytes in cultures of dividing O-2A progenitors. CNTF and LIF also promoted oligodendrocyte maturation, as determined by expression of myelin basic protein, and could promote oligodendrocyte survival to an extent comparable with insulin-like growth factor-1 or insulin. In addition, LIF and CNTF both promoted the differentiation of O-2A progenitors into type-2 astrocytes but only when applied in the presence of extracellular matrix (EnMx) derived from cultures of endothelial cells. The ability of CNTF and LIF to enhance differentiation of O-2A progenitors along either of the alternative pathways of oligodendrocyte and astrocyte differentiation suggests that these proteins are able to enhance the process of differentiation per se, while the actual path of differentiation promoted is determined by the presence or absence of additional molecules in the extracellular environment.

Key words: ciliary neurotrophic factor, leukemia inhibitory factor oligodendrocyte, oligodendrocyte-type-2 astrocyte progenitor, differentiation

INTRODUCTION

Ciliary neurotrophic factor (CNTF) is a molecule of increasing interest in developmental neurobiology due to its ability to function as a modulator of survival and differentiation of a variety of neurons and of glial cells of the central nervous system (Barbin et al., 1984; Hughes et al., 1988; Lillien et al., 1988, 1990; Arakawa et al., 1990; Lillien and Raff, 1990; Sendtner et al., 1990; Martinou et al., 1992; Barres et al., 1993). In respect to the modulation of glial cell differentiation, recent studies on oligodendrocyte-type-2 astrocyte (O-2A) progenitors (Raff et al., 1983b) isolated from rat optic nerves have indicated that CNTF, and a CNTF-like protein produced by type-1 astrocytes, can cause transient expression of glial fibrillary acidic protein (GFAP, a marker of astrocytic differentiation; Bignami et al., 1972) in O-2A progenitors in vitro (Hughes et al., 1988; Lillien et al., 1988). Moreover, when applied in the presence of extracellular matrix produced by cultures of endothelial or meningeal cells, CNTF induces O-2A progenitors to develop fully into type-2 astrocytes (Lillien et al., 1990).

As CNTF mRNA can first be detected in rat optic nerves beginning 1 week after birth (Stöckli et al., 1991), the time when small numbers of cells with the antigenic characteristics of type-2 astrocytes can first be identified in cell suspensions from this tissue (Raff et al., 1983a; Fulton et al., 1991), it is possible that this molecule could promote the generation of type-2 astrocytes in vivo. Unfortunately, as it has not yet been possible to identify type-2 astrocytes in vivo, despite many attempts (Barres et al., 1990; Fulton et al., 1991; Skoff and Knapp, 1991), any role CNTF might play in normal development of the O-2A lineage is not clear.

In our present studies, we show that CNTF promotes the generation, survival and maturation of oligodendrocytes in vitro, with the generation of type-2 astrocytes being promoted only if CNTF is applied in the presence of extracellular matrix of endothelial cells. As the peak period of oligodendrocyte generation in the rat optic nerve (Skoff et al., 1976a,b) occurs at about the same time that CNTF mRNA is first seen in this tissue (Stöckli et al., 1991), we suggest that if CNTF does play a role in the modulation of glial differentiation in vivo, it is more likely to be as a promoter of oligodendrocyte development than as an inducer of type-2 astrocyte generation. We have also found that leukemia inhibitory factor (LIF), but not interleukin-6 (IL-6), has similar effects to CNTF on cells of the O-2A lineage.

MATERIAL AND METHODS

Mixed optic nerve cultures

Cultures were prepared by isolation of O-2A lineage cells from optic

nerves of embryonic or 7-day-old rats as described previously (Raff et al., 1983b; Noble and Murray, 1984).

For embryonic cultures, 8,000 cells were cultured on poly-L-lysine glass coverslips (PLL; Sigma; M_r 175,000; 20 µg/ml) in 0.3 ml Dulbecco's modified Eagle's medium (DMEM) supplemented with 25 µg/ml gentamicin, 2 mM glutamine, 1 µg/ml bovine pancreas insulin (Sigma), 100 µg/ml human transferrin (Sigma), 0.0286% (v/v) BSA pathocyte (Miles Laboratories, Inc), 0.2 µM progesterone (Sigma), 0.10 µM putrescine (Sigma), 0.45 µM L-thyroxine (Sigma), 0.224 µM selenium (Sigma) and 0.49 µM 3,3′,5-triiodo-L-thyroxine (Sigma) (DMEM-BS; Bottenstein and Sato, 1979). Embryonic cultures also received 10 ng/ml of PDGF ± 10 ng/ml of CNTF, LIF or IL-6 daily, as detailed in the text.

Purification of O-2A progenitor cells

Purified O-2A progenitors derived from 7-day-old rats were prepared by using a specific antibody-capture assay (Wysocki and Sato, 1978) adapted to the O-2A lineage by Barres et al. (1992). Negative selection with the Ran-2 antibody (Bartlett et al., 1981) was used to eliminate type-1 astrocytes, followed by anti-galactocerebroside (GalC) antibody (Ranscht et al., 1982) panning to remove oligodendrocytes. The antigenic phenotype of O-2A progenitors (A2B5$^+$/GalC$^-$) allows purification of these cells from the remaining cell suspension by capture on a tissue culture dish coated with A2B5 antibody (Eisenbarth et al., 1979). After attachment, unbound cells were removed and the plate was washed with DMEM-BS. The bound cells were then trypsinized and plated on PLL-coated coverslips in a 24-well plate in DMEM-BS at densities indicated in the Results and in following experimental descriptions. After the cells were allowed to adhere for 1 hour, 300 µl of DMEM-BS was added. This procedure yielded 2×10^5 O-2A progenitor cells from an initial 2×10^6 mixed cells from rat optic nerve. In the final culture, A2B5$^-$ cells (type-1 astrocytes and oligodendrocytes) represented <0.5% of the total cells while >99.5% of cells were O-2A progenitors. Antibodies (as serum-free hybridoma supernatants) for coating the plates were applied at: anti-Ran-2 [2.5 µg/ml], anti-GalC [2.5 µg/ml], A2B5 antibody [5 µg/ml]. Cells were allowed to bind to the specific plates for 20-30 minutes in a 37°C incubator. Cells were not exposed to fetal bovine serum at any time during the immunopanning procedure, in contrast with the protocol of Barres et al. (1992, 1993).

Platelet-derived growth factor (PDGF$_{AA}$) and basic fibroblast growth factor (bFGF), kind gifts from C. George-Nascimento and L. Coussens (Chiron Corporation, Emeryville, CA) were added daily at a concentration of 10 ng/ml, while CNTF, LIF or IL-6 were added daily at concentrations of 0.2-10 ng/ml as described for each experiment. Recombinant human IGF-I (a kind gift of Genentech) was applied at a concentration of 100 ng/ml and insulin at 1 µg/ml. Native CNTF (Barbin et al., 1984) was a kind gift from Michael Sendtner and recombinant human CNTF (Lin et al., 1989) from Frank Collins (Synergen, CO). Experiments were initially carried out with native CNTF and confirmed with recombinant CNTF. No difference was seen between the native and recombinant material. Recombinant human LIF was a kind gift from John Heath. Recombinant human IL-6 was obtained from Promega.

Immunocytochemistry

All antibodies were diluted in Hank's Balanced Salts solution (Imperial Laboratories) containing 0.05% w/v sodium azide (BDH-Merck), 5% heat-inactivated donor calf serum (DCS; Imperial Laboratories) and buffered to pH 7.4 with Hepes (Sigma) prior to use, and applied to cells for between 30 and 45 minutes. Antibodies to cell surface antigens were applied directly to living cells. To visualise the cytoplasmic antigens, GFAP and myelin basic protein (MBP) cells were permeabilised at −20°C for 15 minutes with methanol that had previously been cooled to −70°C. After antibody staining, coverslips were washed in distilled water and mounted cell side down in a drop of 2.5% w/v solution of 1,4 diazobicyclo-2.2.2 octane in glycerol to

retard fading of fluorescein (Johnson et al., 1982) and sealed with nail varnish. Specimens were viewed on a Zeiss Axiophot microscope equipped with phase- and interference-contrast optics, epifluorescent illumination and selective filters for rhodamine, fluorescein and coumarin.

The following antibodies were used: Mouse IgM monoclonal antibody A2B5 (Eisenbarth et al., 1979; hybridoma supernatant, 1:3) was used in identification of O-2A progenitors and type-2 astrocytes (Raff et al., 1983a,b). Mouse IgG$_3$ monoclonal anti-GalC antibody (Ranscht et al., 1982; hybridoma supernatant, 1:3) was used as a specific label of oligodendrocytes (Raff et al., 1978). Mouse IgG$_1$ monoclonal anti-MBP antibodies (Groome, 1980; dilution 1:500) were a kind gift of Dr Nigel Groome. Rabbit antiserum to bovine GFAP (Dakopatts, 1:500) was used to identify astrocytes (Bignami et al., 1972). All fluorescein- and rhodamine-conjugated secondary layer antibodies (1:100) were from Southern Biotechnology Associates, USA.

BrdU incorporation assay

To examine DNA synthesis, cultures were incubated with 10 µM 5-bromodeoxyuridine (BrdU, Sigma) for 4 hours. BrdU incorporation into nuclei of cells synthesizing DNA was visualized using anti-BrdU antibody (Gratzner, 1982; Becton Dickinson). Prior to antibody application, cells were fixed with methanol (10-20 minutes, −20°C) and then exposed to 0.02% paraformaldehyde in HBBS+5%DCS for 60 seconds followed by 0.07 M NaOH for 7-10 minutes. Coverslips were rinsed several times in HBSS+5% DCS and incubated with anti-BrdU antibodies for 30 minutes. After several washings, cells were then incubated with rhodamine-conjugated goat anti-mouse-IgG$_1$ for 30 minutes and washed, mounted and examined as described before.

Lucifer Yellow pulse-labelling

Purified O-2A progenitor cells were plated on a coverslip and incubated with bFGF +/− CNTF, LIF or IL-6 for 48 hours. Cells were plated at 5000 cells/coverslip to allow for loss of cells during the extensive prelabelling procedure and incubated with anti-GalC antibody under sterile conditions for 30 minutes. As anti-GalC antibodies are rapidly internalised by oligodendrocytes (Dyer and Benjamins, 1988), application of only this single layer of antibody did not allow cells to be followed in culture, in contrast with previous pulse-labelling strategies that we have utilised (e.g., Raff et al., 1983b; Noble and Murray, 1984). We could, however, identify the prelabelled oligodendrocytes for extended periods in vitro with the following method: following labelling with anti-GalC antibody, cells were extensively washed with sterile DMEM and incubated with a biotinylated goat anti-mouse IgG$_3$ antibody for 30 minutes. Coverslips were again washed and incubated with Lucifer Yellow-coupled streptavidin (Molecular Probes) for a further 30 minutes. The coverslips were washed and the labelled cells were grown in culture for an additional 4 days in their original culture medium, supplemented daily with 10 ng/ml bFGF ± 2 ng/ml CNTF or 4 ng/ml LIF. With this procedure, 100% of the GalC$^+$ cells (i.e., all oligodendrocytes) present at the time of labelling were stained and cells retained the labelling up to 8 days, even following antibody internalisation (unpublished observations). After 4 days the prelabelled cells were fixed with 4% para-formaldehyde and stained with A2B5 and anti-GalC antibodies for 20 minutes. A2B5 antibody was visualised with a biotinylated goat-anti mouse IgM antibody and streptavidin-coumarin. The freshly applied anti-GalC antibody was visualised with rhodamine-conjugated goat anti-mouse IgG$_3$. All oligodendrocytes present at the time of initial labelling were double labelled with Lucifer Yellow and rhodamine. All newly generated oligodendrocytes were only labelled with rhodamine. A2B5$^+$ cells were coumarin$^+$.

Survival assay

To examine the survival of oligodendrocytes in the presence of various factors, purified O-2A progenitor cells from 7-day-old rat

pups were cultured at a density of 3000 cells/coverslip for 48 hours in DMEM/BS. After confirming on parallel coverslips that 100% of the cells differentiated into GalC[+] oligodendrocytes, the cells were washed 3 times in DMEM and replaced in new culture wells containing either DMEM alone or in combination with insulin, IGF-1, CNTF, LIF or IL-6 at appropriate concentrations. Factors were added daily and cells were stained after a total of 4 days in culture.

MTT assay

The assay was performed as described by Mosmann (1983) and Barres et al. (1992) and additionally combined with immunofluorescence. MTT (3-[4,5-dimethythiaziazol-2yl]-2,5-diphenyl tetrazolium bromide, Sigma) was dissolved in PBS at 5 mg/ml. The stock solution was filtered through a Millipore filter (0.22 µm) and added to the culture medium at a dilution 1:10. The plates were incubated at 37°C for 2 hours. In live cells, the tetrazolium ring is cleaved into a visible dark blue formazan reaction product. Cells were then fixed with 4% paraformaldehyde and stained with anti-GalC antibodies as described. Live cells appear with dark blue cytoplasm in bright-field phase microscopy.

Preparation of endothelial cell matrix (EnMx)

Cultures of bovine aortic endothelial cells (kindly provided by J. Folkman, Harvard Medical School) were cultured after three passages on PLL-coated coverslips in DMEM/10% foetal calf serum. Cells were grown for 3 days to confluency with one medium change. The culture medium was then aspirated and the cells were treated with 20 mM ammonia for 15-20 minutes at room temperature (Lillien et al., 1990). The remaining EnMx was washed 5 times with DMEM and coverslips, kept at 37°C, were used within 2 hours.

RESULTS

CNTF promotes the generation and survival of oligodendrocytes derived from purified O-2A progenitors

To determine whether CNTF could promote the generation of oligodendrocytes, we initially examined the effects of this factor on populations of purified O-2A progenitor cells. As division of O-2A progenitor cells is required to prevent their premature differentiation into oligodendrocytes (Raff et al., 1983a; Noble and Murray, 1984; Raff et al., 1984, Noble et. al., 1988; Raff et al., 1988), we first examined the effects of CNTF on cells grown in the presence of basic fibroblast growth factor (bFGF), a mitogen for both O-2A progenitors and oligodendrocytes (Eccleston and Silberberg, 1985; Saneto and deVellis, 1985; Bögler et al., 1990; McKinnon et al., 1990; Mayer et al., 1993). In these conditions, differentiation of O-2A progenitors is inhibited so long as they are first purified away from the other cells of the optic nerve (McKinnon et al., 1990; Mayer et al., 1993). Moreover, the ability of bFGF to promote oligodendrocyte division means that the generation of oligodendrocytes in the presence of bFGF is not necessarily associated with a cessation of cell division.

The daily addition of bFGF + 2 or 4 ng/ml of CNTF for 6 days (Fig. 1C,D), but not for 3 days (Fig. 1A,B), was associated with an increase in the proportion of O-2A lineage cells that were oligodendrocytes (Fig. 1C), as compared with cultures exposed to bFGF alone. CNTF application was also associated with a reduction in the total numbers of O-2A progenitors present in the cultures at this time (Fig. 1D). Effects on cell number appeared to be distinct from effects on differentiation, as all concentrations of CNTF tested were associated

with a significant fall in the total number of progenitor cells even though the lower concentrations of CNTF applied did not promote increases in oligodendrocyte number. CNTF application did not induce differentiation of O-2A progenitors into type-2 astrocytes, as no GFAP[+] type-2 astrocytes were observed in these cultures at any time point examined.

As the increased numbers of oligodendrocytes seen in O-2A progenitor cultures exposed to 2 or 4 ng/ml of CNTF for 6 days could have arisen from an increased generation of oligodendrocytes and/or from enhanced division or survival of these cells after they had been generated, we separately examined each of these possibilities.

Increases in oligodendrocyte number did not appear to result from enhanced oligodendrocyte division, as judged by analysis of cultures grown in the presence of bFGF ± CNTF and labeled with BrdU for 4 hours. We found that 9±3% of GalC[+] oligodendrocytes had BrdU-labelled nuclei in cultures exposed to bFGF alone, as compared with figures of 6±3, 4±1, 2±1 and 4±2% for cells exposed to bFGF + 0.2, 0.5, 2 and 4 ng/ml of CNTF, respectively.

To determine whether the generation of new oligodendrocytes in cultures exposed to CNTF was sufficient to explain the differences between these cultures and O-2A progenitor cultures exposed to bFGF alone, we developed a novel pulse-labelling strategy that enabled us to identify newly generated oligodendrocytes in our cultures (see Materials and Methods and Fig. 2). Briefly, cultures grown for 48 hours in the presence of bFGF + CNTF were sequentially prelabelled, in sterile conditions, with monoclonal anti-GalC antibody, biotin-conjugated anti-IgG3 antibodies (which bound to the anti-GalC antibody) and streptavidin-conjugated Lucifer Yellow (which bound to the biotin-conjugated second antibody). Prelabelled cells were then grown for a further 4 days in the presence of bFGF ± CNTF. Cultures were then labelled with fresh anti-GalC antibody, followed by rhodamine-conjugated anti-IgG₃ antibodies. With this method, newly generated oligodendrocytes were rhodamine[+]Lucifer Yellow[−], while oligodendrocytes present at the time of prelabelling were rhodamine[+]Lucifer Yellow[+] (Fig. 2). CNTF was applied at 2 ng/ml, this being the optimal effective concentration for this factor in the experiments of Fig. 1. The procedure of prelabelling did not appear to alter the behaviour of the cultures, as prelabelled and parallel control cultures contained the same distribution of cell types and comparable cell numbers in parallel experiments (data not shown).

The proportion of newly generated oligodendrocytes in cultures grown in the presence of bFGF + CNTF was 4-fold greater than the proportion of newly generated oligodendrocytes seen in cultures exposed only to bFGF (Fig. 3). The total number of oligodendrocytes present at the time of prelabelling (i.e., 48 hours) was similar in cultures exposed to bFGF or to CNTF + bFGF (52±21 versus 51±10 cells/coverslip, respectively). Cultures exposed to bFGF alone for a further 4 days after Lucifer Yellow labelling contained 44±14 new oligodendrocytes/ coverslip, as compared with 161±12 new oligodendrocytes for cultures exposed to CNTF + bFGF ($P<0.001$). Application of CNTF was also associated with a reduction in the numbers of O-2A progenitors present, from 426±72 in cultures exposed to bFGF to 248 ±26 in cultures exposed to CNTF + bFGF ($P<0.001$). The difference in oligodendrocyte numbers between cultures grown in the presence or absence of

Day 3   **Day 6**

% of cells
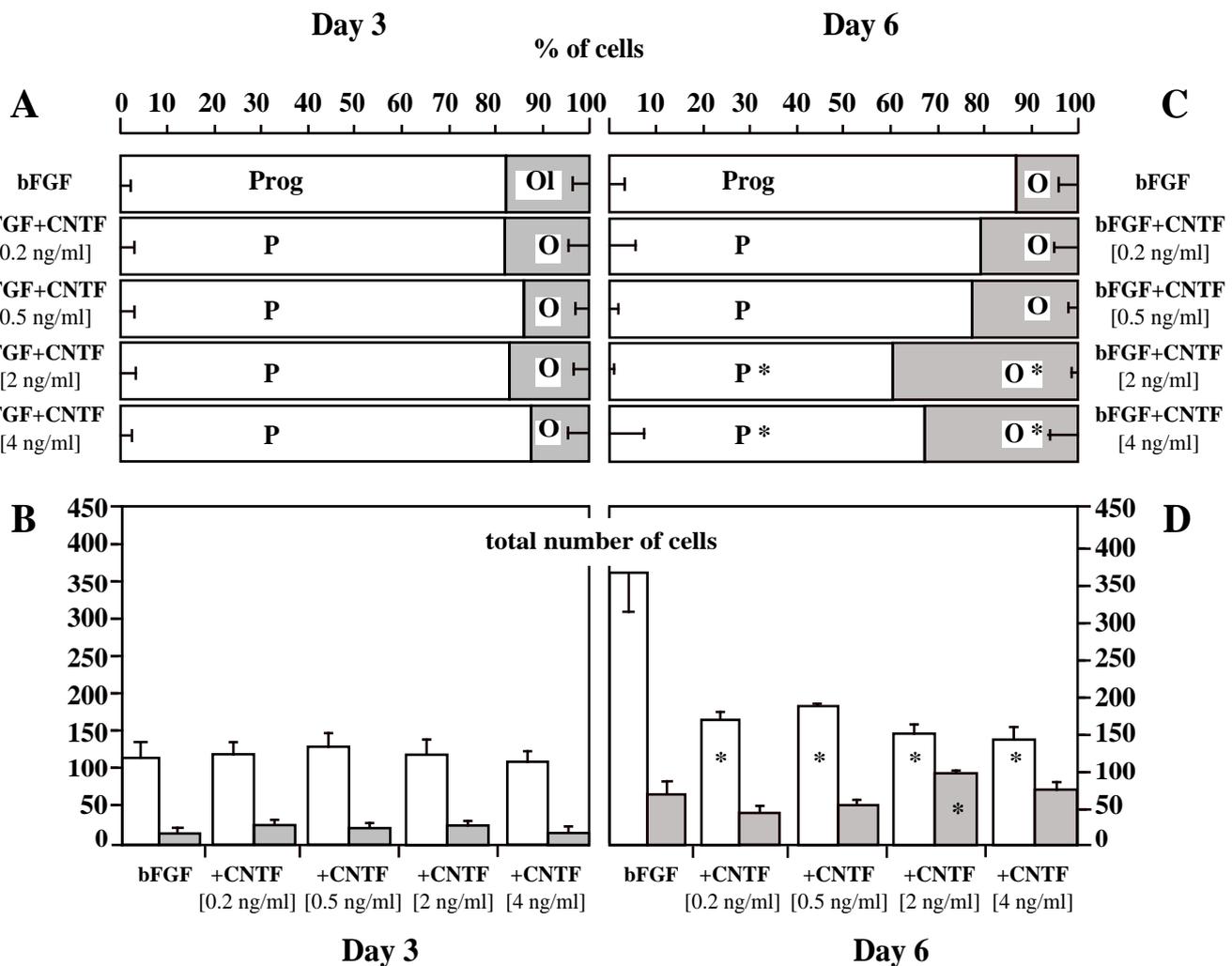
Fig. 1. CNTF enhances the generation of oligodendrocytes in cultures of purified O-2A progenitors grown for 6 days in the presence of bFGF. Purified O-2A progenitors were plated at a density of 3000 cells/coverslip and grown for 3 or 6 days in the presence of bFGF alone or bFGF ± CNTF. bFGF was added daily at 10 ng/ml; CNTF was added daily at concentrations of 0.5-4 ng/ml. The values are the mean ± s.e.m. from 4 separate isolations containing sets of 4-6 coverslips; * indicates values significantly different from control values. Application of CNTF for 3 days did not alter the proportion of cells that were oligodendrocytes (A), while application for 6 days (C) of 2 ng/ml or 4 ng/ml of CNTF was associated with a significant increase ($P<0.001$ and $P<0.005$, respectively) in representation of oligodendrocytes in these cultures. Shaded bars represent GalC$^+$ oligodendrocytes, while open bars represent cells with the A2B5$^+$GalC$^-$ phenotype of O-2A progenitors. Oligodendrocytes and O-2A progenitors were the only cell types present in these cultures. Determination of cell numbers revealed that all doses of CNTF examined were associated with reductions in progenitor number after 6 days (D), but only with increases in oligodendrocyte number at doses of 2 ng/ml or 4 ng/ml (Shaded bars represent GalC$^+$ oligodendrocytes, while open bars represent A2B5$^+$GalC$^-$ O-2A progenitors.). No significant differences between different conditions were seen on day 3 (B). The values are the mean ± s.e.m. from 4 separate isolations containing sets of 4-6 coverslips.

CNTF therefore appeared to be explicable solely on the basis of the generation of newly formed oligodendrocytes (compare Fig. 3 with Fig. 1).

We further examined the ability of CNTF to promote survival of oligodendrocytes. Cultures of purified O-2A progenitors were grown in DMEM-BS for 2 days to induce differentiation of all cells into oligodendrocytes. Cultures were then switched to DMEM (lacking additional supplements) ± CNTF (2 ng/ml) and incubated for a further 2 days, with fresh CNTF added daily. The extent of oligodendrocyte survival at this time point was defined as cells that took up and cleaved MTT to yield a visible reaction product (Mosman, 1983). For comparison, cultures receiving IGF-1 or insulin were also examined.

The experiments were thus conducted similarly to Barres et al. (1992, 1993) with minor modifications as indicated in the Materials and Methods.

Addition of CNTF promoted the survival of 35±2% oligodendrocytes as compared with values of only 15±5% for cells grown in a negative control medium (DMEM in the absence of additives). The extent of survival promoted by CNTF was comparable with that obtained with IGF-1 or insulin (Table 1).

CNTF promotes the generation and maturation of oligodendrocytes in cultures of embryonic optic nerve

To examine whether CNTF was able to promote oligodendro-
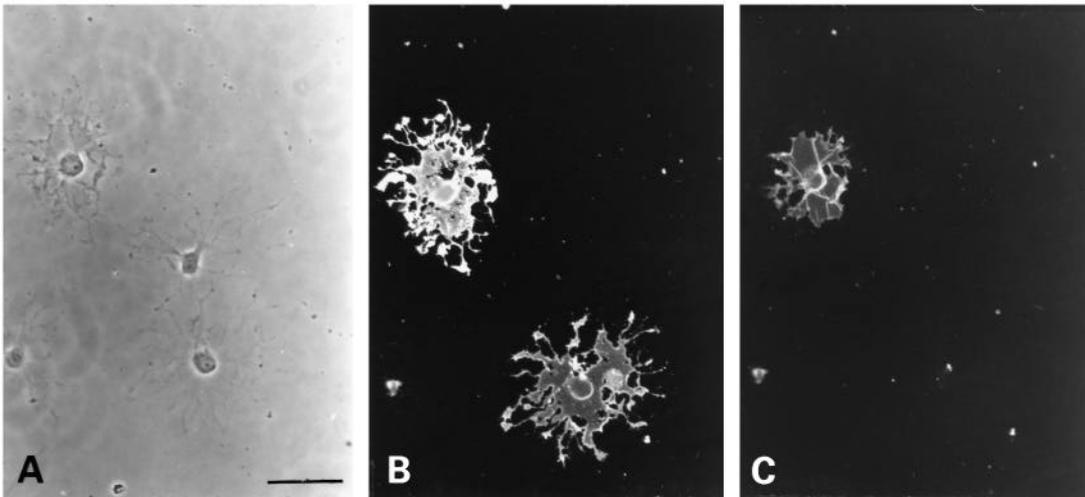
Fig. 2. Identification of newly generated oligodendrocytes by Lucifer Yellow pulse labelling. Purified O-2A progenitor cells were grown at a density of 5000 cells/coverslip in the presence of 10 ng/ml bFGF ± CNTF. CNTF was added at 2 ng/ml, the optimal concentration for generating oligodendrocytes (see Fig. 1). All factors were added daily. 2 days after growth in the various conditions, cells were pulse-labelled with anti-GalC antibodies and Lucifer Yellow (see Results, Materials and Methods). These prelabelled cells were then cultured for an additional 4 days in their original medium, which was daily supplemented with fresh bFGF ± CNTF. After a total of 6 days, cells were stained with anti-GalC and A2B5 antibodies (visualized with rhodamine and coumarin conjugates, respectively). Oligodendrocytes present at the time of initial labelling were Lucifer Yellow$^+$ Rhodamine$^+$, while newly generated oligodendrocytes were Lucifer Yellow$^-$ Rhodamine$^+$. (A) Three cells growing in the presence of bFGF + CNTF. Two of the cells were GalC positive at the end of the experiment (B). Of these two cells, only one was also labelled with Lucifer Yellow (C), indicating that this oligodendrocyte was present in the culture at the time of prelabelling, while the second oligodendrocyte was generated in the 4 days following the prelabelling. The third cell shown in phase contrast could be identified as an O-2A progenitor cell by A2B5 staining with a coumarin-conjugated antibody (coumarin labelling not shown). The scale bar corresponds to 30 μm.

cyte generation and maturation in a situation in which in vitro development has been shown to be similar to that which occurs in vivo, we determined whether application of CNTF to O-2A progenitors growing in heterogeneous cultures of embryonic day 19 rat optic nerve exposed to PDGF promoted differentiation of these cells into oligodendrocytes. Previous studies have demonstrated that the first oligodendrocytes appear in such cultures after an appropriate interval of 2 days (Raff et al., 1988), this being equivalent to the time at which oligodendrocytes can first be isolated from the intact optic nerve (Miller et al., 1985). As the great majority of cells in such cultures are not of the O-2A lineage, this culture system further enabled us to investigate whether any effects of CNTF on O-2A progenitors and oligodendrocytes growing within a complex mixture of cell types resembled effects seen on purified cells. Cells were cultured in the presence of 10 ng/ml PDGF, or PDGF plus CNTF at concentrations of 2 ng/ml or 4 ng/ml. Cultures were immunolabelled after 1, 2 and 4 days of in vitro growth.

In the presence of 2 or 4 ng/ml of CNTF, 18±3% and 24±4%, respectively, of the O-2A lineage cells were oligodendrocytes after 4 days of growth (Fig. 4). In contrast, only 4±2% of the O-2A lineage cells were oligodendrocytes at this time point in cultures exposed to PDGF alone. Thus, CNTF promoted the generation of oligodendrocytes in heterogeneous cultures of embryonic rat optic nerve cells. Differences between cultures receiving PDGF alone versus PDGF + CNTF were not seen, however, after 2 days of in vitro growth.

CNTF application was also associated with the promotion of MBP expression in oligodendrocytes developing in cultures of embryonic rat optic nerve cells. For example, 2±0.3% of the oligodendrocytes expressed MBP in cultures that received 4 ng/ml CNTF for 4 days, while cultures receiving PDGF alone did not contain any MBP$^+$ cells (Fig. 4). Application of CNTF
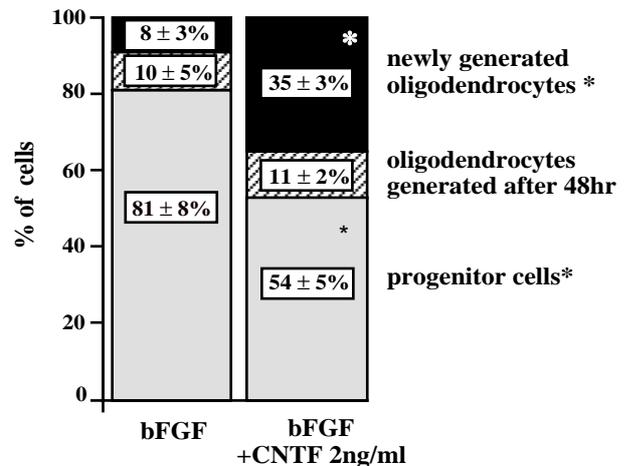
Fig. 3. The increase in the proportion of oligodendrocytes in cultures with CNTF is due to the formation of new oligodendrocytes. Experimental details were as described in the legend of Fig. 2. Fig. 3 depicts the percentage composition of cell populations. The total cell numbers seen after 6 days of growth in the presence of bFGF alone were 522±38 cells and in the presence of bFGF + CNTF the total cell number was 461±14 cells. There were no cell types other than O-2A progenitors and oligodendrocytes present in these cultures. Each value represents the mean ± s.e.m. from two different experiments containing sets of 6 coverslips; * indicates values significantly different from control values.

over 6 days was associated with a more dramatic promotion of MBP expression: cultures receiving PDGF alone still contained no MBP$^+$ cells, while in cultures exposed to 2 or 4 ng/ml of CNTF 11±2% or 17±2% of GalC$^+$ oligodendrocytes were MBP$^+$, respectively (data not shown).

To determine whether the effects of CNTF on promoting

Table 1. CNTF promotes oligodendrocyte survival

| Conditions	Total number of oligodendrocytes (live and dead cells)	% live oligodendrocytes
DMEM	1137±123	15±5
DMEM/IGF [100 ng/ml]	1382±260	43±7*
DMEM/Insulin [1 µg/ml]	1554±74	32±2*
DMEM/CNTF [2 ng/ml]	1755±88	35±2*

Purified O-2A progenitors were grown at a density of 5000 cells/coverslip in the presence of different factors (as indicated), which were added daily. Doses used for CNTF correspond to concentrations which maximally promote oligodendrocyte differentiation (see Fig. 1). After 3 days cells were stained with anti-GalC antibody (to identify oligodendrocytes) and MTT. Live cells were identified as GalC$^+$/MTT$^+$ with intact cell bodies and well-defined processes. Dead cells were identified as GalC$^+$ 'ghosts' with no nuclei and no visible MTT reaction product. The addition of IGF-1, insulin or CNTF was sufficient to support the survival of approximately 40% of the cells ($P<0.001$ as compared with DMEM alone). Oligodendrocytes were the only cell type present in these cultures. Each value represents the mean ± s.e.m. of two different experiments containing sets of 6-8 coverslips, * indicates values that are significantly different from control values.

MBP expression might reflect a direct action on oligodendrocytes, we again conducted experiments on purified cell populations prepared from optic nerves of 7-day-old rats. MBP expression was examined in cultures of purified O-2A progenitors grown in mitogen-free DMEM-BS ± 2 ng/ml CNTF, the concentration that elicited the maximal generation of oligodendrocytes in cultures of purified O-2A progenitors grown in the presence of bFGF (see Fig. 1). The addition of CNTF to cultures grown in DMEM-BS was associated with an increase in the proportion of oligodendrocytes expressing MBP after 3 days from 19±1% in DMEM-BS alone to 35±5% in DMEM-BS + CNTF (Table 2).

LIF, but not IL-6, promotes oligodendrocyte generation, survival and maturation

Due to the similarity of action that has been observed between CNTF, LIF and IL-6 by other investigators (Taga et al., 1989; Birren and Anderson, 1990; Murakami et al., 1991; Lord et al., 1991; Ip et al., 1992; Barres et al., 1993), we also examined the effects of exposure to LIF and IL-6 on O-2A progenitors.

As with CNTF, LIF was able to induce both increases in the numbers of oligodendrocytes and decreases in the numbers of O-2A progenitors in cultures of purified progenitors growing in the presence of bFGF. Exposure of cells to 4 ng/ml LIF for 6 days had effects similar to those obtained with 2 ng/ml of CNTF (compare Fig. 5 with Fig. 1). No significant effect of LIF was seen at lower doses, nor after 3 days of exposure. Pulse-labelling experiments (as in Figs 2 and 3) confirmed that new oligodendrocytes were being generated in cultures exposed to 4 ng/ml of LIF, with a 4.4-fold increase seen in the numbers of such cells in cultures treated with bFGF + LIF as compared with cultures exposed to bFGF alone (data not shown). No dose of IL-6 examined was associated with an increase in oligodendrocyte number at either 3 days or 6 days, or with generation of increased numbers of oligodendrocytes in pulse-labelled cultures. 6 days of application of lower doses of IL-6 was associated, however, with a reduction in numbers of O-2A progenitors (Fig. 5B). Neither LIF nor IL-6 induced expression of GFAP.
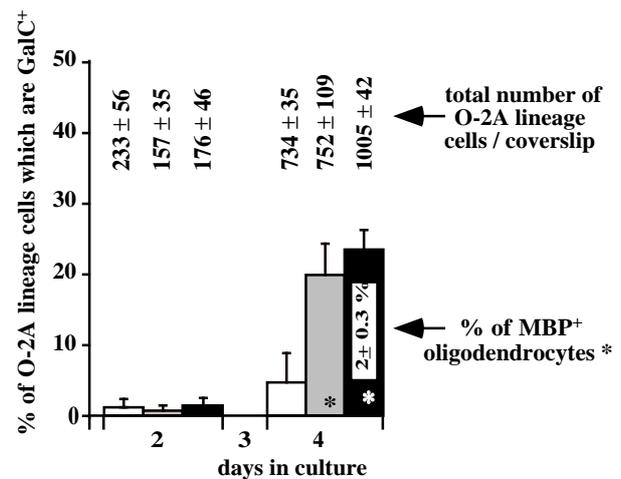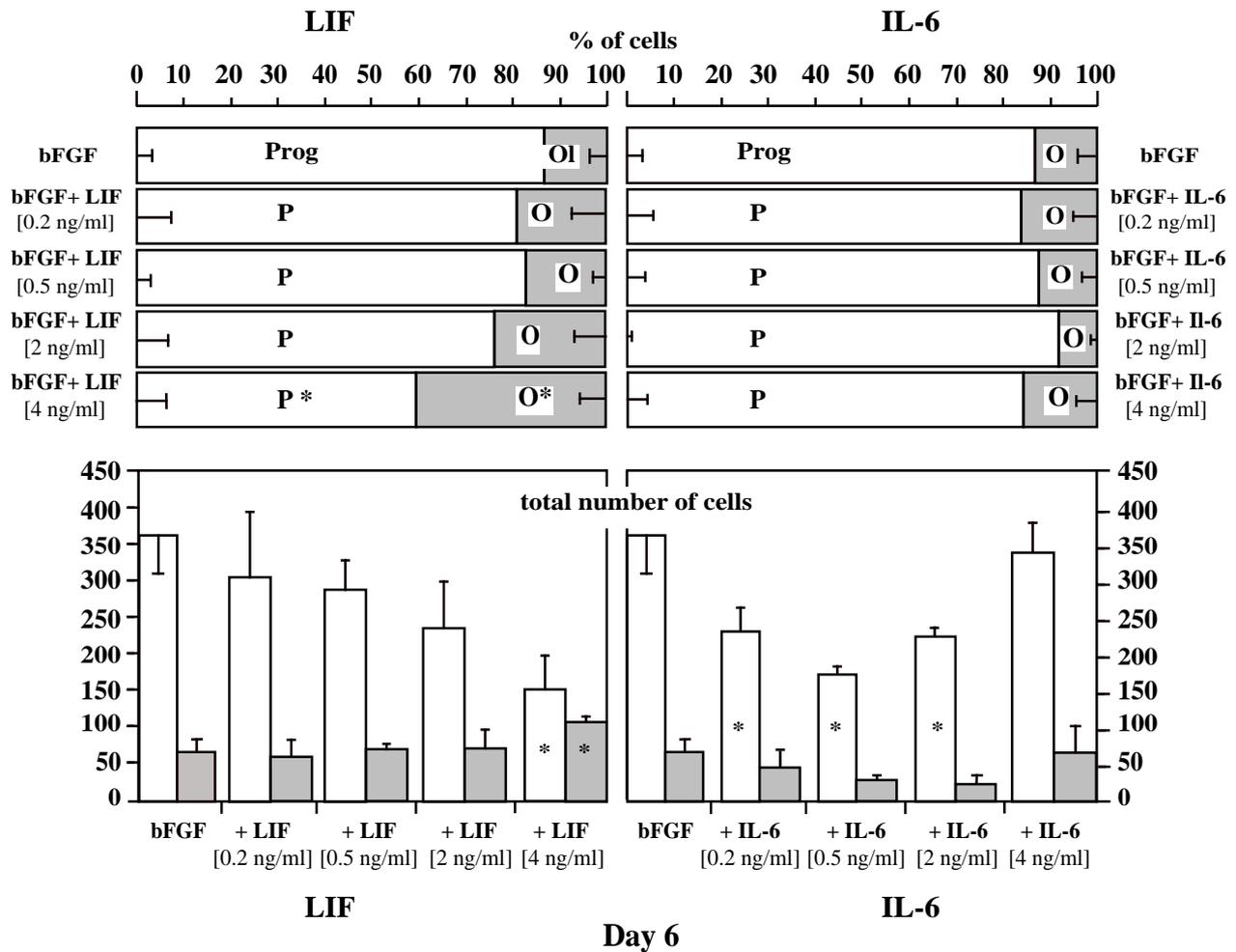

Fig. 4. CNTF promotes the generation of oligodendrocytes in cultures derived from optic nerves of embryonic rats. CNTF was added daily to cultures of optic nerves prepared from E19 rats and plated at a density of 8000 cells/well. Cells were grown in the presence of 10 ng/ml PDGF ± CNTF (2 ng/ml or 4 ng/ml: open bars, PDGF alone; shaded bars, + 2 ng/ml CNTF; solid bars, +4 ng/ml). After 2 and 4 days of in vitro growth the cells were immunolabelled to identify O-2A progenitors, MBP$^-$ oligodendrocytes and MBP$^+$ oligodendrocytes. As shown, the application of CNTF for several days enhanced the generation of oligodendrocytes ($P<0.001$), and the expression of MBP in the population of oligodendrocytes ($P<0.001$), but did not alter the timing of the first appearance of oligodendrocytes in the cultures. Cultures grown in PDGF (open bars) alone contained less oligodendrocytes and no MBP$^+$ oligodendrocytes at day 4. The remaining O-2A lineage cells were A2B5$^+$ progenitor cells. The total number of O-2A lineage cells is indicated as mean ± s.e.m. above the bars. The value of the bars represents the mean ± s.e.m. from 2 different experiments with sets of 4 coverslips; * indicates values significantly different from control values.

Table 2. CNTF promotes oligodendrocyte maturation

	DMEM-BS	DMEM-BS CNTF [2 ng/ml]
Total number of oligodendrocytes	1069±174	995±141
Total number of MBP$^+$ oligodendrocytes	204±36	345±49*
% MBP$^+$ oligodendrocytes	19±1	35±5*

O-2A progenitor cells purified from optic nerves of P7 rats were grown at a density of 3000 cells/coverslip in the conditions indicated and factors were added daily. Cultures were immunolabelled after 3 days to examine the expression of MBP, a marker of oligodendrocyte maturation. There was a 2-fold increase in the proportion and number of MBP$^+$ oligodendrocytes in cultures grown in the presence of CNTF ($P<0.001$) as compared with those grown in DMEM-BS alone. All MBP$^+$ cells were also GalC$^+$ and the remainder of the cells were GalC$^+$/MBP$^-$. These were the only cell types present in the cultures. Each value represents the mean ± s.e.m. from two different experiments containing sets of 5-6 coverslips; * indicates values that are significantly different from control values.

Application of LIF, but not of similar concentrations of IL-6, to cultures of O-2A progenitors growing in chemically defined medium (and thus differentiating into oligodendrocytes) promoted both oligodendrocyte survival (as in Table 1) and expression of MBP (as in Table 2). LIF (4 ng/ml) was as

Fig. 5. LIF, but not IL-6, can promote the generation of oligodendrocytes in cultures of purified O-2A progenitors. Experiments were conducted as in Fig. 1. The only cultures in which there was a significant increase in the proportion of O-2A progenitors differentiating into oligodendrocytes were cultures receiving 4 ng/ml of LIF for 6 days, although reductions in numbers of O-2A progenitors were seen in cultures treated with 0.2, 0.5 and 2 ng/ml of IL-6 or 4 ng/ml of LIF; * indicates values significantly different from control values.

effective as 2 ng/ml of CNTF at promoting oligodendrocyte survival, with 40±2% of oligodendrocytes surviving at the time point examined. IL-6 application (10 ng/ml) was associated with survival of 23±7% of oligodendrocytes at this time point, a value not significantly different from the value of 15±5% obtained with DMEM alone.

Application of 4 ng/ml of LIF for 3 days to cultures of cells differentiating into oligodendrocytes in DMEM-BS (as in Table 2) promoted MBP expression in 39±4% of oligodendrocytes, a value comparable to that obtained by application of 2 ng/ml of CNTF. In contrast, only 25±4% of oligodendrocytes in cultures exposed to IL-6 expressed MBP, a value not significantly different from the 19±1 % of MBP+ oligodendrocytes seen in cultures grown in DMEM-BS alone.

As in the above experiments, application of LIF to cultures of embryonic optic nerve cells growing in the presence of PDGF enhanced oligodendrocyte generation and maturation, while application of IL-6 to these cultures was without effect (as shown in Fig. 6).

CNTF and LIF can interact with extracellular matrix produced by endothelial cells to generate type-2 astrocytes

As the results that we obtained by studying purified O-2A lineage cells are in contrast with previous studies suggesting that CNTF promotes differentiation of O-2A progenitors into type-2 astrocytes in heterogeneous cultures of optic nerve cells (Hughes et al., 1988; Lillien et al., 1990; Lillien and Raff, 1990), we next examined the response of purified O-2A progenitors to growth in the presence of CNTF, LIF or IL-6 and extracellular matrix produced by endothelial cells (EnMx). This combination of factors induces O-2A progenitors to undergo complete differentiation along the type-2 astrocyte pathway (Lillien et al., 1990). Purified O-2A progenitors were grown on coverslips coated with EnMx (see Materials and Methods) and cultured in the presence of CNTF, LIF or IL-6.

When cells were grown on EnMx in DMEM-BS in the presence or absence of CNTF or LIF, no cells with the antigenic phenotype (i.e., GFAP+A2B5+) of type-2 astrocytes
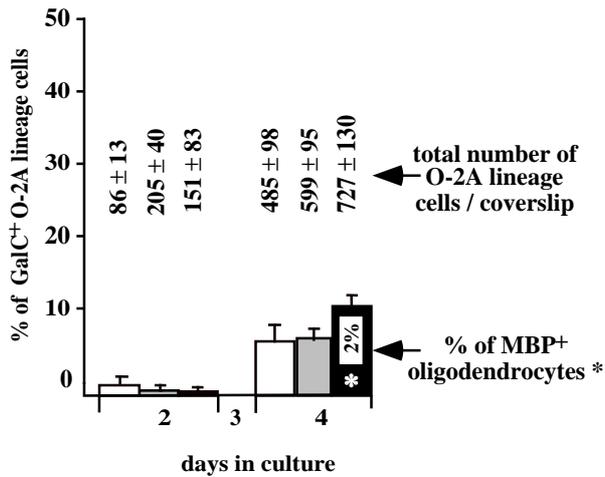
Fig. 6. LIF can enhance the generation and maturation of oligodendrocytes in cultures of embryonic rat optic nerve. Experiments were conducted as in Fig. 4, but LIF (4 ng/ml) or IL-6 (10 ng/ml) was applied to the cultures instead of CNTF. Addition of LIF for 4 days was associated with a significant increase ($P<0.001$) in oligodendrocyte number and proportion of oligodendrocytes expressing MBP; * indicates values significantly different from control values.

could be detected after 2 days (data not shown). After one more day in culture, $26\pm6\%$ of the cells exposed to EnMx in DMEM-BS alone were type-2 astrocytes and the remaining cells were O-2A progenitor cells (Fig. 7). This value was enhanced by addition of 0.5 ng/ml and 4 ng/ml CNTF to $60\pm9\%$ and $75\pm2\%$ type-2 astrocytes, respectively. Addition of LIF at a concentration of 0.5 ng/ml or 4ng/ml led to the generation of $66\pm7\%$ and $73\pm2\%$ type-2 astrocytes, respectively. In all cases the remainder of cells were O-2A progenitor cells; no oligodendrocytes were seen in these cultures. IL-6 had no effect on the generation of type-2 astrocytes. At no time point examined in these experiments did we see induction of GFAP expression in the absence of EnMx, nor did we see expression of GFAP in cultures exposed to factors for only 1 day (data not shown).

DISCUSSION

We have found that CNTF and LIF are pleiotropic modulators of development in the O-2A lineage. Both molecules enhanced the generation of oligodendrocytes in cultures of dividing O-2A progenitors. CNTF and LIF also promoted oligodendrocyte maturation, as determined by expression of MBP, and could promote oligodendrocyte survival to an extent comparable with IGF-1 or insulin. In addition, LIF and CNTF both promoted the differentiation of O-2A progenitors into type-2 astrocytes but only when applied in the presence of extracellular matrix (EnMx) derived from cultures of endothelial cells.

CNTF and LIF promoted the generation and differentiation of oligodendrocytes in several different experimental conditions. These proteins enhanced the generation of oligodendrocytes in cultures of purified O-2A progenitor cells growing in bFGF (this being the simplest system for which it is possible to ask about direct effects on progenitors; Mayer et al., 1993), thus indicating a direct action of CNTF and LIF on O-2A pro-
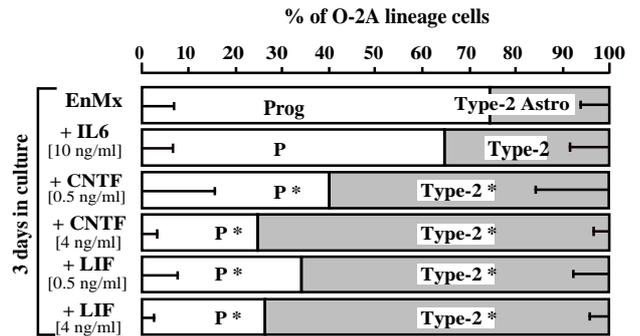
Fig. 7. CNTF and LIF enhance the generation of type-2 astrocytes in the presence of extracellular matrix produced by endothelial cells (EnMx). Purified O-2A progenitors were plated at a density of 3000 cells/coverslip and grown for 3 days on coverslips coated with EnMx in the presence of CNTF (0.5 and 4 ng/ml), LIF (0.5 and 4 ng/ml) or IL-6 (10 ng/ml); factors were added daily. Open bars represent the proportions of A2B5^{+} progenitor cells, while shaded bars show the proportions of A2B5[+]/GFAP[+] type-2 astrocytes. These two cell types were the only cells present in the cultures. In the presence of EnMx on its own, ~25% of all cells express the antigenic phenotype of type-2 astrocytes after 3 days. Additional application of CNTF or LIF leads to an increase in the proportion of type-2 astrocytes up to $78\pm2\%$. Il-6 had no significant effect on type-2 astrocyte development compared to EnMx alone. The total number of cells were in the range of 400-450 cells/coverslip. The values are the mean ± s.e.m. from 2 separate isolations containing sets of 4-6 coverslips; * indicates values significantly different from control values.

genitors. We also observed an enhanced generation and maturation of oligodendrocytes developing in cultures of embryonic rat optic nerve cells grown in the presence of PDGF, a culture system that allows reconstitution of the normal timing of oligodendrocyte generation in vitro (Raff et al., 1988). The effects of CNTF and LIF were not simply a secondary consequence of an inhibition of O-2A progenitor division, as the total number of O-2A lineage cells present in embryonic optic nerve cultures was not decreased by the addition of these factors (Figs 4, 6). Moreover, survival of oligodendrocytes and expression of MBP was enhanced in cultures growing in chemically defined medium (DMEM-BS), in which no division occurs in this lineage (Raff et al., 1983b; Noble and Murray, 1984); these observations also indicate that the effects of CNTF and LIF are unlikely to be secondary to a simple inhibition of cell division and provide further evidence that these factors act directly on O-2A lineage cells. Although CNTF and LIF specifically may have inhibited division of purified O-2A progenitors growing in the presence of bFGF (Figs 1, 5), even in these cultures, any such effects appeared to be distinct from the promotion of differentiation. For example, only cultures exposed to 2 ng/ml or 4 ng/ml of CNTF for 6 days contained an increased number of oligodendrocytes, even though cultures treated with CNTF doses from 0.2 to 4 ng/ml contained fewer progenitor cells than control cultures exposed to bFGF alone. In addition, IL-6 application was associated with a reduction in progenitor number in cultures of purified cells exposed to bFGF, but was not associated with increases in the number of oligodendrocytes.

Our observations that CNTF promotes oligodendrocyte gen-

eration and maturation contrast strikingly with previous studies suggesting that CNTF promotes differentiation of O-2A progenitors into type-2 astrocytes (Hughes et al., 1988; Lillien et al., 1990; Lillien and Raff, 1990). In our experiments, it was only when O-2A progenitor cells were grown in the presence of EnMx that the type-2 astrocyte pathway was promoted by CNTF or LIF. The ability of CNTF and LIF to enhance differentiation of O-2A progenitors along either of these alternative pathways suggests that these proteins are able to enhance the process of differentiation per se, while the actual path of differentiation promoted is dependent upon the presence of other factors. Although previous studies have reported that CNTF, LIF and IL-6 induce a transient GFAP expression in O-2A progenitor cells (Hughes et al., 1988; Lillien et al., 1990; Barres et al., 1993), we saw no induction of GFAP expression by these proteins in any of our experiments, even when earlier time points were examined (unpublished observations) and even though the cells that we studied were fully competent to differentiate into type-2 astrocytes. Although we do not know why cells in our experiments did not exhibit GFAP expression in response to CNTF, LIF or IL-6, there are a number of differences in experimental details between our studies and those described in previous reports. For example, Lillien et al. (1990) examined GFAP expression in heterogeneous cultures prepared from 1-day-old rats, while we studied purified progenitors, thus allowing for the possible contribution of inductive signals from other cell types present in the cultures. Although Barres et al. (1993) found a transient induction of GFAP expression in a small proportion (<5%) of purified O-2A progenitors after 1 day, these cells were exposed to fetal calf serum during the immunopanning procedure. In contrast, our cells are never exposed to fetal calf serum, which can itself induce GFAP expression (Raff et al., 1983a,b).

Although some of the results that we obtained are in agreement with recent independent reports from Louis et al. (1993) and Barres et al. (1993), our findings both extend and differ from these other studies. For example, the results of Louis et al. (1993) showed a promotion of oligodendrocyte survival and enhancement of MBP expression by application of CNTF to heterogeneous CNS cultures and to cultures of the GC4 O-2A progenitor cell line. The failure to see increases in numbers of MBP+ cells in these studies suggested, however, that the effect of CNTF may have been to enhance MBP expression in cells that had already achieved this degree of maturation. In contrast, we observed that CNTF and LIF both caused actual increases in the numbers of MBP expressing cells. Furthermore, Louis et al. (1963) did not examine the effects of LIF or IL-6, nor did they identify the promotion of oligodendrocyte generation and maturation seen in our own studies. Of particular interest in the studies of Louis et al. (1993), however, was the finding that CNTF can protect oligodendrocytes against killing by tumor necrosis factor; in agreement with our other results, we have found that LIF is also effective in this regard while IL-6 has no protective activity (M. M. and M. N., unpublished observations). In contrast to the lack of effect of IL-6 in our studies, Barres et al. (1993) have reported that IL-6, LIF and CNTF all promote survival of oligodendrocytes. The doses of IL-6 needed effectively to promote survival in the studies of Barres et al. (1993) were, however, an order of magnitude greater than those needed for LIF or CNTF.

Our observations that the effects caused by CNTF and LIF on O-2A progenitor cells and oligodendrocytes were similar are at least in part consistent with a variety of recent studies demonstrating similar effects of CNTF, LIF and IL-6 (Arakawa et al., 1990; Birren and Anderson, 1990; Taga et al., 1989; Lord et al., 1991; Murakami et al., 1991; Murphy et al., 1991; Oppenheim et al., 1991; Ip et al., 1992). Our studies suggest, however, that these molecules may be subtly different from each other in their effects. For example, IL-6 exposure was associated with reductions in progenitor numbers in cultures of purified cells grown in the presence of bFGF, but did not promote generation of either oligodendrocytes or type-2 astrocytes. In addition, concentrations of CNTF that had no apparent effect on oligodendrocyte generation were associated with reductions in progenitor numbers in similar cultures, while LIF was only associated with a reduction in progenitor numbers in cultures that also showed increased generation of oligodendrocytes. While we do not yet know whether these results mean that varying effects are produced by different concentrations of ligand, as mediated perhaps through receptors with different binding affinities, we would note that we have also found that the effects of bFGF on DNA synthesis and differentiation in cutures of purified O-2A progenitors vary with different bFGF concentrations (Mayer et al., 1993).

Several effects of purified astrocytes on the division and differentiation of O-2A progenitors in vitro have been reported (Noble and Murray, 1984; Raff et al., 1988; Lillien et al., 1988, Aloisi et al., 1988, Dutly and Schwab, 1991; Mayer et al., 1993); two of these effects may involve CNTF. First, Lillien and colleagues reported that a CNTF-like protein secreted by purified cortical (type-1) astrocytes promoted differentiation of O-2A progenitors into type-2 astrocytes when applied together with extracellular matrix derived from endothelial cells (Lillien et al., 1988, 1990). Second, several studies indicate that medium conditioned by purified cortical (type-1) astrocytes (ACM) promotes the differentiation of purified O-2A progenitors into oligodendrocytes in the presence of bFGF (Aloisi et al., 1988; Dutly and Schwab, 1991, Mayer et al., 1993), an effect similar to the one that we have now observed for CNTF and LIF. As cortical astrocytes appear to produce CNTF (Stöckli et al., 1991), it may be that this protein is at least partially responsible for such effects. If CNTF is responsible for the activity of cortical astrocytes in our assays, or contributes to the modulation of differentiation during optic nerve development, then it must be that this apparently cytosolic molecule (Lin et al., 1989; Stöckli et al., 1989) is either released from degenerating cells or is released from healthy cells by a nonconventional release mechanism (see, e.g., Kostura et al., 1989; Rubartelli et al., 1990; Belin et al., 1989).

Although we do not know whether CNTF and/or LIF modulate development of the O-2A lineage in vivo, the in vitro experiments discussed above suggest several influences of possible importance during development or injury. The most likely role that we would suggest for CNTF or LIF at this time is as a promoter of the generation, survival and differentiation of oligodendrocytes during development. The peak period for the generation of oligodendrocytes and for the initiation of myelination in the optic nerve begins 1 week after birth (Skoff et al., 1976 a,b), at about the same time that CNTF mRNA first appears in this tissue (Stöckli et al., 1991). LIF mRNA is also detectable in the visual cortex and superior colliculus of the

postnatal rat CNS (Yamamori, 1991). Thus, there are at least some grounds for believing that these molecules are produced in vivo during the period when myelination is proceeding most rapidly.

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