Astrocyte-derived TGF-β2 and NGF Differentially Regulate Neural Recognition Molecule Expression by Cultured Astrocytes

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Abstract. Because of the importance of neural recognition molecules expressed by glial cells to mediate interactions with neurons, growth factors and cytokines known to be functional during morphogenesis and in diseases of the nervous system were studied for their effects on recognition molecule expression by cultured immature and mature astrocytes from several brain regions. In cultures of immature astrocytes, transforming growth factors-β1 (TGF-β1) and -β2 (TGF-β2) and nerve growth factor (NGF) increased expression of the neural adhesion molecule L1, leading to a glia-mediated L1-specific increase in neurite outgrowth of dorsal root ganglion neurons on the astrocyte substrate. L1 expression induced by TGF-β was inhibited by addition of antibodies to NGF, suggesting that TGF-β influences L1 expression by modulating production of NGF by astrocytes. TGF-β1 and -β2 decreased expression of N-CAM by immature astrocytes. Since N-CAM expression was not affected by NGF and antibodies to NGF did not abolish the TGF-β-induced decrease in N-CAM expression, NGF did not appear to be the mediator for regulating expression of N-CAM. Expression of the adhesion molecule on glia (AMOG) was not affected by any factor. NGF and TGF-β2 in latent form, but not TGF-β1 were found in the culture supernatants. Addition of interferon-γ (IFN-γ), interleukin-1β (IL-1β), interleukin-6 (IL-6), platelet-derived growth factor (PDGF), or basic fibroblast growth factor (bFGF) to the cultures did not change recognition molecule expression. Recognition molecule expression by mature astrocytes was not found to be modified by any of the factors tested. In view of the observation that levels of L1 and N-CAM expression correlated with the presence of TGF-β2 and NGF in the culture supernatants of immature astrocytes, an autocrine regulatory mechanism for recognition molecule expression by these cells is suggested to play a crucial role in regulation of neuron–glia interactions.

THE elucidation of the cellular and molecular mechanisms underlying neuronal survival, migration of neuronal cell bodies, and neurite outgrowth is not only pertinent for the understanding of neural development, but also for the understanding of degenerative and regenerative processes in the central and peripheral nervous systems of adult vertebrates. Growth factors and recognition molecules on the cell surface and in the extracellular matrix have both been implicated in morphogenetic processes (for reviews, see Barde, 1990; Schachner, 1990). Neurotrophic factors, on the one hand, influence several cellular properties, among them neuronal survival and neurite outgrowth. On the other hand, neural recognition molecules have been implicated in neuronal migration and neurite outgrowth (for review, see Doherty and Walsh, 1989). Evidence for a direct involvement of recognition molecules in cell survival is missing, although it is likely that they are also implicated in this phenomenon by virtue of their influence on second messenger systems (Schuch et al., 1989) and the cytoskeleton (Pollerberg et al., 1986). Since both growth factors and recognition molecules are believed to mediate important morphogenetic processes, it is plausible to assume that the two types of communication mechanisms between cells are interdependent and influence each other. However, relatively little attention has been given to this interdependence.

First hints linking growth factor activity to recognition molecule expression stem from the observation that the nerve growth factor- (NGF)[1] inducible large external glycoprotein NILE is related, if not identical to the recognition molecule L1 (Block et al., 1985). NGF enhances L1 expression not only in NGF receptor bearing neurons, but also in Schwann cells (Seilheimer and Schachner, 1987). Since L1 mediates neurite outgrowth on other neurites and on Schwann cells, most likely together with N-CAM by an assisted homophilic binding mechanism (Kadmon et al.,

1. *Abbreviations used in this paper*: bFGF, basic fibroblast growth factor; BME, basal modified Eagle's medium; CMF-HBSS, Ca^{2+}- and Mg^{2+}-free HBSS; EGF, epidermal growth factor; GFAP, glial fibrillary acidic protein; IFN-γ, interferon-γ; IL, interleukin; L-TGF-β, latent transforming growth factor-β; NGF, nerve growth factor; TBS, Tris-buffered saline; TGF, transforming growth factor.

1990*a*,*b*), NGF has been proposed as mediator of neurite outgrowth in the lesioned peripheral nervous system of adult mammals (Seilheimer and Schachner, 1988). Since NGF and NGF receptor expression by Schwann cells is upregulated after damage (Heumann et al., 1987*a*,*b*), an NGF-induced increase in L1 expression appears to fit a physiologically meaningful purpose.

Even less information is available on the molecular mechanisms regulating recognition molecule expression in the central nervous system. Although immature astrocytes, for instance, have been shown to promote neurite outgrowth better than mature ones (Fallon, 1985*a*,*b*) and several molecules, among them also L1, have been suggested to mediate neuron–glia interactions by heterophilic binding mechanisms (Bixby et al., 1988; Neugebauer et al., 1988; Grumet and Edelman, 1988; Werz and Schachner, 1988; Drazba and Lemmon, 1990; Smith et al., 1990), knowledge about the regulatory factors underlying recognition molecule expression by either of the interacting partners has remained elusive. We, therefore, decided to investigate the physiological roles of several growth factors in their ability to induce astrocytes to modify recognition molecule expression. Particular attention was given to (*a*) growth factors known to have physiological roles in the central nervous system such as NGF, PDGF, and bFGF and to (*b*) cytokines being produced in diseases of the nervous system and found to affect the function of astrocytes, such as IL-1, IL-6, TGF-β, and IFN-γ (Fontana et al., 1987).

Here we report that TGF-β upregulates L1 and downregulates N-CAM expression by immature, but not mature astrocytes and that this regulation of L1 expression is mediated by NGF.

Materials and Methods

Antibodies

mAbs from rat reacting with the neural recognition molecules L1 and N-CAM and the adhesion molecule on glia (AMOG) from mouse have been described (Rathjen and Schachner, 1984; Goridis et al., 1983; Antonicek et al., 1987). Polyclonal L1 antibodies from rabbits were obtained by immunoaffinity purification using an L1 antigen column (Martini and Schachner, 1986). Polyclonal antibodies from rabbit to mouse liver membranes have been described (Lindner et al., 1983). mAb to NGFβ from mouse and polyclonal antibodies to glial fibrillary acidic protein (GFAP) from mouse were obtained from Boehringer (Mannheim, FRG). Purified anti-TGF-β1 rabbit IgG and control nonimmune rabbit IgG were a gift from Dr. L. Ellingsworth (Collagen Corp., Palo Alto, CA). Purified anti-TGF-β2 IgG from rabbit was a generous gift of the Sandoz Company (Basel, Switzerland). The use of these antibodies has been described (Bodmer et al., 1989). Polyclonal antibodies recognizing TGF-β1 and TGF-β2 (anti TGF-β) were obtained from R & D Systems (Minneapolis, MN). Polyclonal antibodies from goat to vimentin from cultured human foreskin fibroblasts were obtained from Sigma. The 8D9 mAb from mouse against chicken L1 has been described (Lemmon and McLoon, 1986). For neutralization of the activities of TGF-β1 and -β2 and NGF in the astrocyte cultures, TGF-β antibodies were used at 20 μg/ml and NGF antibodies at 2 μg/ml. For all immunocytochemical and immunochemical procedures, antibodies were also used at saturating concentrations.

Goat antibodies to rat and rabbit IgG conjugated with FITC, TRITC, alkaline phosphatase, or horseradish peroxidase were purchased from Jackson Immunoresearch Laboratories (West Grove, PA) and Cappel Laboratories (Malvern, PA).

Factors

Growth factors and lymphokines were used at the following concentrations: nerve growth factor (7s NGF), 100 ng/ml; bFGF, 20 ng/ml; and PDGF, 20 ng/ml. These factors were obtained from Boehringer Mannheim. Recombinant human transforming growth factors β2 and β1 (TGF-β2, TGF-β1), being used at 10 ng/ml, were kindly provided by Dr. Max Schreier (Sandoz Company) and Dr. Michel Palladino (Genentech, San Francisco, CA), respectively. Recombinant human interleukin-1β (IL-1β) and interleukin-6 (IL-6), used at concentrations of 20 U/ml and recombinant human interferon-γ (IFN-γ) used at 20 ng/ml were obtained from Genzyme Corp. (Boston, MA).

Preparation of Immature and Mature Astrocytes from Mouse Brain

Astrocytes were obtained from cerebellum and "rest" brain (brain without cerebellum) of newborn ICR mice and maintained as described (Fischer, 1984). In brief, tissue pieces were incubated in Ca^{2+}- and Mg^{2+}-free HBSS (CMF-HBSS) containing 0.1% trypsin for 15 min at 37°C. Cells were dissociated in 0.05% DNase in CMF-HBSS by pipetting up and down through a fire-polished Pasteur pipette and collected by centrifugation at 420 *g* for 6 min at 4°C. Cells were plated at a density of 2×10^6 cells/25-cm^2 tissue culture flask and maintained in 4 ml defined medium, consisting of basal modified Eagle's medium (BME) with Earle's salts supplemented with 1 mg/ml BSA (Serva Biochemicals, Heidelberg, Germany), 10 μg/ml insulin (Serva Biochemicals), 0.1 mg/ml transferrin (Sigma), 10 nM EGF (epidermal growth factor, Boehringer), 30 nM selenite, and 1% (vol/vol) penicillin/streptomycin (GIBCO/BRL, Gaithersburg, MD).

The culture medium was renewed 24 h after plating or subculture of cells and subsequently every 3–4 d. Subculture of cells was performed as described previously (Fischer et al., 1982; Fischer, 1983). In brief, primary cultures maintained for 4–5 d were incubated with 0.05% trypsin and 0.1 mM EDTA in PBS, pH 7.4 (PBS) for 5–7 min at 37°C. This solution was then exchanged with BME containing Earle's salts and 1 mg/ml soybean trypsin inhibitor (Serva Biochemicals) and the cells were resuspended by gentle pipetting up and down with a fire-polished Pasteur pipette. Cells were then plated at a density of 2×10^6 cells/25 cm^2 in defined culture medium. These cells were designated immature astrocytes according to their marker profile (see below). Immature astrocytes from hippocampus and temporal lobe of newborn mice were cultured exactly as those from cerebellum and "rest" brain.

Mature astrocytes were prepared as described for immature astrocytes in the previous paragraph, except that subcultures were maintained first for 24 h in defined culture medium and then for 48 h in BME containing 10% horse serum and penicillin/streptomycin.

The immature astrocytes were GFAP negative and vimentin positive (Schnitzer et al., 1981; Schnitzer and Schachner, 1981). Maintenance of these immature astrocytes in BME containing Earle's salts and 10% horse serum converted ~95% of all cells into GFAP-positive astrocytes within 2 d of culture (Fischer, 1984), thus underscoring that the population isolated in defined medium is indeed of astrocytic origin. By microscopic analysis, these astrocytes were morphologically quite homogeneous. No macrophage-like cells could be observed. Approximately 1% of all cells were O4 antigen positive and revealed an oligodendrocyte precursor-like morphology (Trotter et al., 1989).

In cultures of mature astrocytes, ~97% of all cells expressed glial fibrillary acidic protein. All cells showed a flat, epithelioid-like morphology. Fibronectin-positive fibroblast-like cells in the mature cultures amounted to <1% of all cells. Macrophage-like cells were not observed.

Cell Lines

The A9 glial cell line was obtained by culturing cells from the cerebellum of 6- to 7-d-old C57Bl/6J mice in defined medium (Fischer et al., 1982). The cell line was maintained for 10 passages before it was taken for the described experiments. The cell line contains immature astrocytes, since cells are vimentin positive but GFAP negative when cultured in defined medium, and switch to GFAP-positive astrocytes after 2 d of maintenance in the presence of 10% horse serum. When cultured in defined medium, the cell line expresses L1.

Mouse neuroblastoma N2A cells were cultured as described (Rathjen and Schachner, 1984).

Immunoprecipitation and Western Blot Analysis

Immunoprecipitation was carried out as described (Faissner et al., 1985). In brief, immature cerebellar astrocytes were maintained for 4–5 d in pri-

mary culture, subcultured and then maintained for 4 d in the presence of 100 ng/ml NGF or 10 ng/ml TGF-β2. Culture supernatants from 7×10^6 astrocytes were collected after these 4 d and cleared at 100,000 *g* for 1 h at 4°C. Cells were then washed twice with CMF-HBSS. Detergent lysates of cells were prepared in 2 ml ice-cold solubilization buffer (20 mM Tris·HCl, 0.15 M NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% Triton X-100, pH 8.0), containing aprotinin and soybean trypsin inhibitor (both at 10 μg/ml). After 10 min on ice, cells were gently scraped off the Petri dish, transferred to test tubes and kept on ice for another 30 min. Detergent lysates were obtained by centrifugation at 100,000 *g* for 1 h at 4°C and taking the supernatant. Immunoprecipitation was carried out with both the detergent lysates and culture supernatants. Supernatants and detergent lysates were mixed with 5 μg of immunoaffinity-purified L1 polyclonal antibody and incubated overnight at 4°C. Preswollen Sepharose–Protein A conjugate (Sigma) prepared as a 10% (vol/vol) suspension in solubilization buffer (120 μl) was then added and incubated for 2 h at room temperature. Sepharose beads were carefully washed by centrifugation through sucrose cushions, resuspended in SDS-sample buffer, boiled for 4 min at 100°C, centrifuged (8,000 *g*, 2 min, 4°C), and supernatants separated by SDS-PAGE (Laemmli, 1970). Proteins were transferred onto nitrocellulose filters. Filters were then blocked for 1 h with 0.3% gelatin in Tris-buffered saline, pH 7.4 (TBS), washed once with TBS and incubated overnight with immunoaffinity-purified polyclonal L1 antibodies (10 μg/ml) at 4°C. After three washes in TBS, the filters were incubated with peroxidase-conjugated goat antibodies against rabbit IgG (at a dilution of 1:1,000) for 2 h at room temperature. After another three washes in TBS, the blots were developed with chloronaphthol solution (3 mg chloronaphthol in 1 ml methanol, 5 ml TBS, and 2 μl H_2O_2).

Enzyme-linked Immunosorbent Assay (ELISA)

Immature astrocytes were subcultured into 96-well flat-bottom microtiter plates (Nunc, Roskilde, Denmark) in defined medium at a density of 2.5×10^4 cells/well. After 24 h, the culture medium was exchanged with defined medium containing the factors. ELISA was carried out 2 or 4 d later. For the ELISA, cells were fixed in 4% formaldehyde in PBS for 20 min at 4°C. After washing in PBS, nonspecific binding sites were blocked in PBS containing 2% BSA for 1 h at room temperature. After another washing step in PBS, first antibodies were added in PBS containing 1% BSA and incubated for 1 h at room temperature. The microtiter plates were then washed and the second, alkaline phosphatase–conjugated antibody, was added in PBS containing 1% BSA for 1 h at room temperature. After three washing steps, the substrate, *p*-nitrophenyl-phosphate (Fluka, Buchs, Switzerland) at 2 mg/ml in 0.1 M glycine buffer, pH 10.4, containing 1 mM $MgCl_2$ and 1 mM $ZnCl_2$, was added, and the absorption at 405 nm was measured in a TiterTek ELISA reader (Flow). All washing steps were carried out with PBS at room temperature.

Expression of recognition molecules on mature astrocytes was carried out after cells had been subcultured, switched to horse serum containing culture medium for 2 d and then maintained for 2 or 4 d in serum containing culture medium in the presence of factors before the ELISA was carried out.

To measure the amount of L1 in the culture supernatant, supernatants were incubated in the 96-well microtiter plates for 1 h at 37°C or overnight at 4°C and the ELISA was carried out on the adsorbed proteins as described. The amount of L1 was determined from a standard curve prepared by addition of a known quantity of L1 to culture supernatants of immature astrocytes from cerebellum maintained in defined medium in the absence of factors.

To determine the actual antibody reactivity, background values measured in the absence of primary antibodies were subtracted from the experimental values. All ELISA determinations were carried out in triplicates. For each experimental value, at least three independent experiments were carried out.

Quantification of Neurite Outgrowth

A9 glial cells were cultured on poly-L-lysine-coated glass coverslips (1.6 cm diameter) in defined medium. Purified dorsal root ganglion neurons from 8-d-old chicken embryos (Seilheimer and Schachner, 1988) were then added at a density of 1×10^4 cells/coverslip in defined culture medium (Fischer, 1982). After 4 h antibodies were added in excess over antigen and maintained with the cells in culture for 24 h. Antibodies were IgG fractions of the L1 mAb 324 and 555 (150 μg/ml), polyclonal L1 antibodies that had not been immunoaffinity purified on an L1 antigen column (1 mg/ml), and polyclonal antibodies to mouse liver membranes (1 mg/ml). The mouse liver membrane antibodies react strongly with A9 cells as verified by indirect immunofluorescence. Cultures were then washed and fixed for 20 min at 4°C with 4% formaldehyde in PBS. Neurons were then stained with the 8D9 mouse monoclonal antibody to chicken L1. Peroxidase-coupled goat antibodies to mouse IgG were used to visualize the mAb using chloronaphthol as substrate.

Lengths of neurites were quantitated in the VIDS semi-automatic image analysis system as described (Seilheimer and Schachner, 1988). Only neurites growing over the surface of fully confluent astrocyte monolayers were taken for measurements. Values are derived from two independent experiments carried out in triplicates. For each experiment, 150 neurites were measured.

Indirect Immunofluorescence

Astrocytes cultured on poly-L-lysine-coated glass coverslips were fixed with 4% paraformaldehyde in PBS, washed in PBS, blocked in PBS containing 2% BSA and incubated with the primary antibody for 2 h at room temperature in PBS containing 1% BSA. After washing in PBS, fluorescein- or tetramethylrhodamine-coupled goat antibodies to rabbit, rat, or mouse IgG were used to visualize the primary antibodies.

Northern Blot Analysis

Total RNA was extracted directly from the astrocyte monolayer in 4 M guanidinium thiocyanate followed by phenol/chloroform extraction according to Chomczynski and Sacchi (1987). RNA was quantified by measuring the optical density at 260 nm. Samples containing 2–20 μg of RNA were fractionated on 0.9% agarose/formaldehyde gels, transferred to Hybond-N filters (Amersham International, Amersham, UK) and hybridized with 4×10^6 cpm/ml ^{32}P-labeled in vitro transcripts of L1 cDNA clone K13 (2,447 bp) (Moos et al., 1988). Filters were washed at high stringency (15 mM NaCl, 1.5 mM sodium citrate, 0.1% SDS, pH 7.0, 65°C) and exposed for autoradiography for 27 d using intensifying screens.

Quantification of NGF

For quantitative determination of NGF in the supernatants of cultured astrocytes, an ELISA was performed according to Weskamp and Otten (1987). The detection limit varied in different experiments between 10 and 25 pg NGF/ml culture medium.

Characterization and Quantification of TGF-β

The presence of TGF-β in supernatants of cultured astrocytes was assessed by bioassay using an ovalbumin-specific murine T helper cell line (OVA-7T cells), which is inhibited in its growth by TGF-β1 and -β2 (Wrann et al., 1987; Siepl et al., 1988). Briefly, OVA-7T cells were maintained in the presence of recombinant interleukin-2 (IL-2) in culture medium consisting of Iscove's complete medium (Behring-Hoechst, Marburg, Germany) supplemented with 1 mM L-glutamine and 5×10^{-5} M 2-mercaptoethanol. OVA-7T cells (5×10^3 cells per well), cultured in the 96-well microtiter plates for 72 h, were incubated with IL-2 (25 U/ml) for 72 h and TGF-β1 or TGF-β2 (10^{-8} to 10^{-11} M) or various dilutions of astrocyte-derived culture supernatants and then harvested. 14 h before harvest, 1 μCi (37 kBq) [^{3}H]thymidine was added per well. The concentration of TGF-β1 or -β2 causing 50% inhibition of thymidine incorporation by OVA-7T cells was determined to be ~0.1 ng/ml for both factors. The amount of TGF-β present in the culture supernatants was calculated by using standard curves obtained by blotting the suppression of the IL-2-dependent growth of OVA-7T cells observed with defined concentrations of TGF-β1 and -β2. To determine whether TGF-β was present in the culture supernatants in its latent form, an aliquot from the culture supernatants was acidified with 0.12 M HCl, a procedure known to activate latent TGF-β (L-TGF-β). After 1 h, the solution was neutralized by adding 0.12 M NaOH and 0.025 M Hepes, pH 7.0. This solution was then tested for TGF-β activity in the bioassay. To verify whether TGF-β1 or -β2 are indeed the molecules in the culture supernatants active in the bioassay, antibody neutralization experiments were performed using an IgG fraction of polyclonal antibodies from rabbit that reacted with either TGF-β1 or -β2 (Bodmer et al., 1989).

Results

To evaluate whether astrocytes can be influenced in their expression of recognition molecules by growth factors and lymphokines, the A9 glial cell line was used. These cells were grown in serum-free defined medium as immature

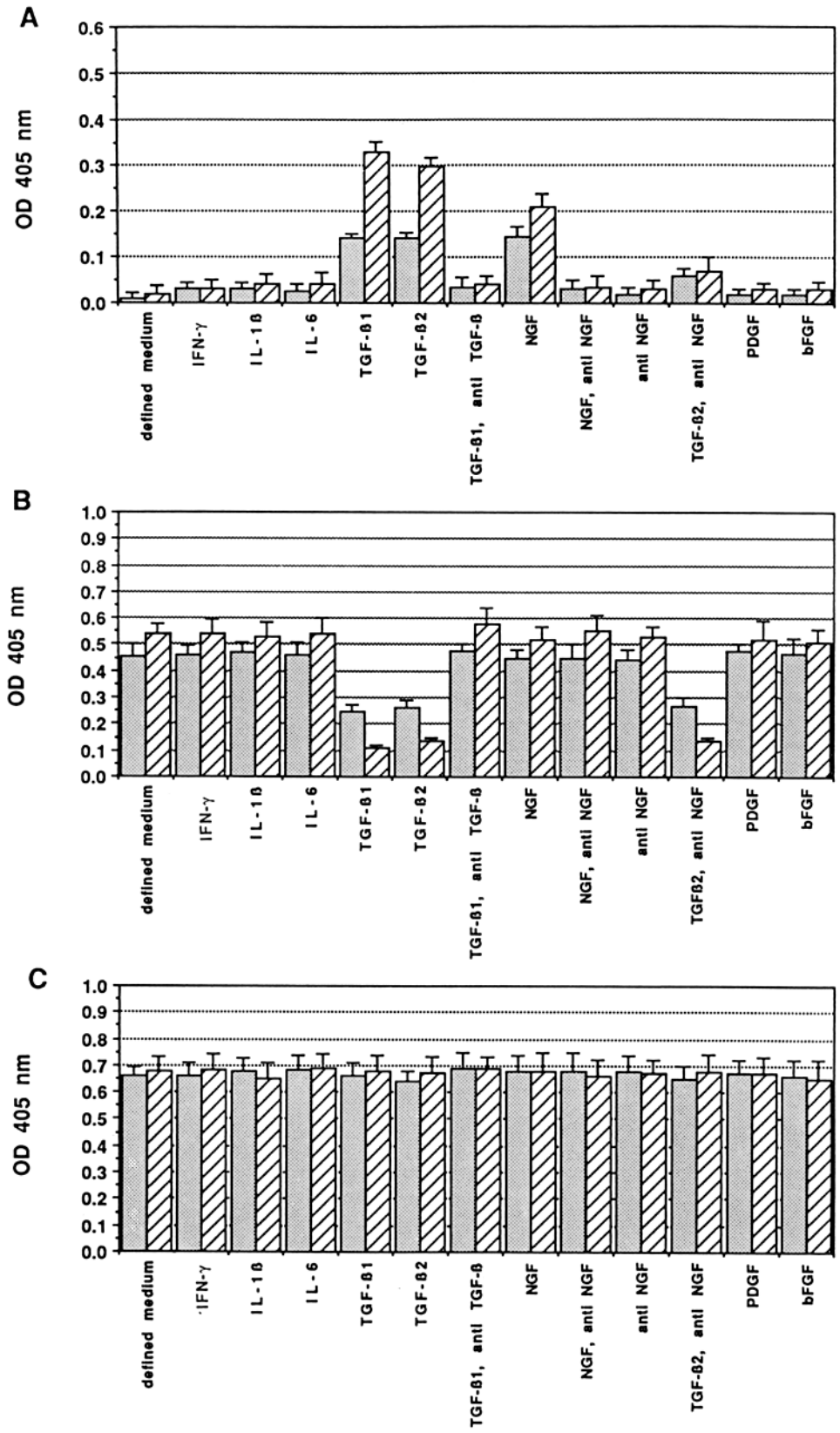

Figure 1. Effect of various growth factors, cytokines and antibodies on the expression of the recognition molecules L1 (*A*), N-CAM (*B*), and AMOG (*C*) by immature astrocytes from cerebellum of newborn mice as measured by ELISA. Cells were exposed to the additives for 2 (▨) or 4 (▨) d. Antibodies to TGF-β (anti-TGF-β) neutralize the bioactivity of both TGF-β1 and -β2. The values given represent the optical density at 405 nm ± SD.

GFAP-negative and vimentin-positive glial cells that could be converted to mature, GFAP-positive astrocytes by maintenance in serum-containing medium. The effect of growth factors and cytokines on the expression of recognition molecules at the cell surface was quantified by ELISA. We chose growth factors and cytokines which have been found in the central nervous system during development and/or after brain damage in adulthood. Interferon-γ (IFN-γ), interleu-

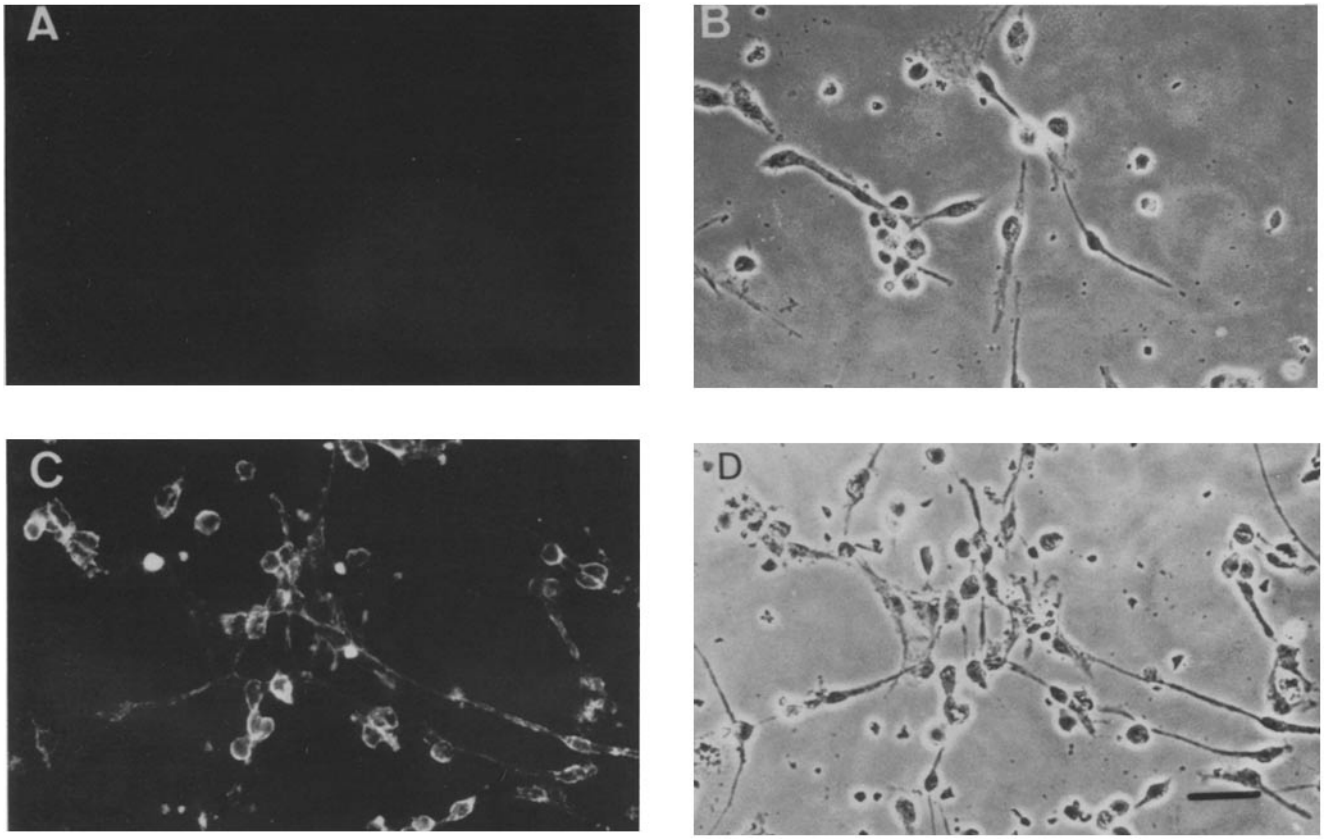

Figure 2. Immunocytological localization of L1 on the cell surface of cultured immature cerebellar astrocytes from newborn mice in the absence (*A*) and presence (*C*) of NGF for 4 d. *B* and *D* are the corresponding phase contrast micrographs to fluorescence images *A* and *B*, respectively. Bar, 10 μm.

kin-1β (IL-1β) and interleukin-6 (IL-6), transforming growth factors-β1 (TGF-β1) and -β2 (TGF-β2), nerve growth factor (NGF), PDGF, and bFGF were tested. Since the initial observations with this cell line indicated that immature, but not mature A9 cells responded to TGF-β1 and -β2 by upregulation of the neural adhesion molecule L1, downregulation of N-CAM and indifference in the expression of the adhesion molecule on glia (AMOG), we decided to investigate the influence of these factors in primary cultures of astrocytes.

No influence of the growth factors or cytokines on recognition molecule expression by primary cultures of mature astrocytes from different brain regions could be detected under the culture conditions used in this study. In the following, only the effects on immature astrocytes will therefore be described.

Expression of Recognition Molecules by Immature Cerebellar Astrocytes

In primary cultures of immature cerebellar astrocytes in defined medium, no L1 was detectable (Fig. 1 *A*). TGF-β1 and -β2 increased the expression of L1 after 2 and 4 d of maintenance in the presence of the factors (Fig. 1 *A*). The increase was larger after 4 ($\sim$10-fold) than after 2 d ($\sim$4-fold) (Fig. 1 *A*). The effects of TGF-β1 and -β2 could be abolished by an antibody to TGF-β that neutralizes the bioactivity of both TGF-β1 and -β2 (see Fig. 1 *A* for TGF-β1). NGF also increased expression of L1 after 2 (approximately fourfold) and 4 (approximately sevenfold) d (Figs. 1 *A* and 2). This effect was neutralized by antibodies to NGF (Fig. 1 *A*). Addition of antibodies to NGF to the culture for 2 or 4 d in the absence of added NGF did not change the expression of L1 over control levels. Cell numbers were not changed in the cultures under the influence of TGF-β1, TGF-β2, or NGF. To investigate whether TGF-β1 and -β2 are able to influence L1 expression by modulating NGF synthesis by these cells, TGF-β2 was added to the cultures in the presence of antibodies to NGF. No significant increase in L1 expression was observed under these circumstances. Maintenance of cells in the presence of IFN-γ, IL-1β, and IL-6, PDGF or bFGF also did not lead to detectable levels of L1 expression.

In contrast to L1, expression of N-CAM was readily detectable on immature cerebellar astrocytes (Fig. 1 *B*). TGF-β1 and -β2 reduced the expression of N-CAM by $\sim$50% after 2 d of maintenance in vitro and by $\sim$80% after 4 d over control levels. NGF, antibodies to NGF, IFN-γ, IL-1β, IL-6, PDGF, or bFGF did not change the basal level of N-CAM expression. When TGF-β2 was added to the cultures in the presence of antibodies to NGF, the reduction of N-CAM levels was the same as in the absence of antibodies to NGF, suggesting that N-CAM expression, unlike L1 expression, was not influenced by TGF-β2 via modulating expression of NGF.

The levels of expression of AMOG by immature cerebellar astrocytes were not changed under any of the conditions tested (Fig. 1 *C*).

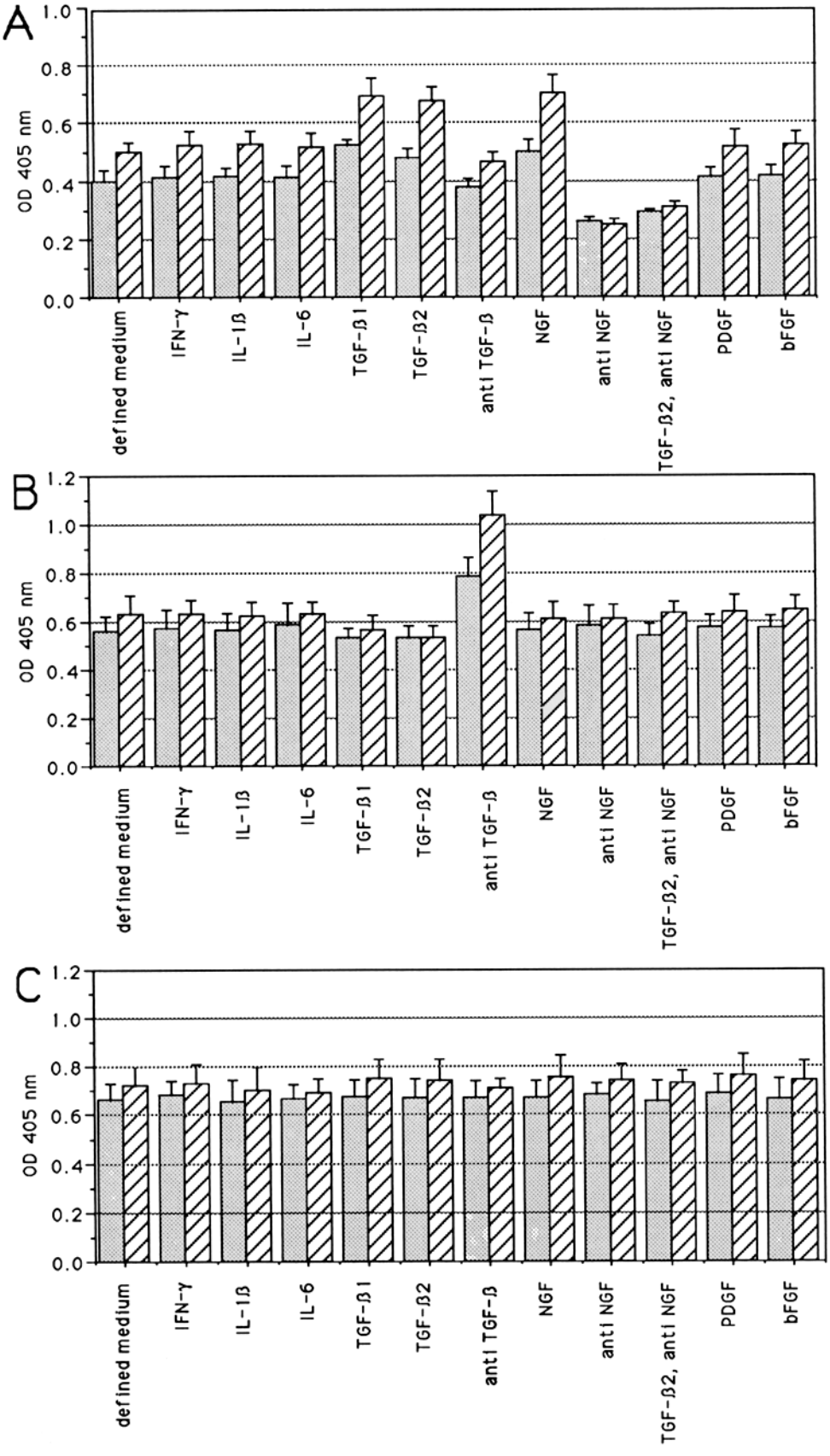

Figure 3. Effect of various growth factors, cytokines and antibodies on the expression of the recognition molecules L1 (*A*), N-CAM (*B*), and AMOG (*C*) by immature astrocytes from "rest" brain (total brain minus cerebellum) from newborn mice as measured by ELISA. Cells were exposed to the additives for 2 (▨) or 4 (▨) d. Antibodies to TGF-β (anti TGF-β) neutralize the bioactivity of both TGF-β1 and -β2. The values given represent the optical density at 405 nm ± SD.

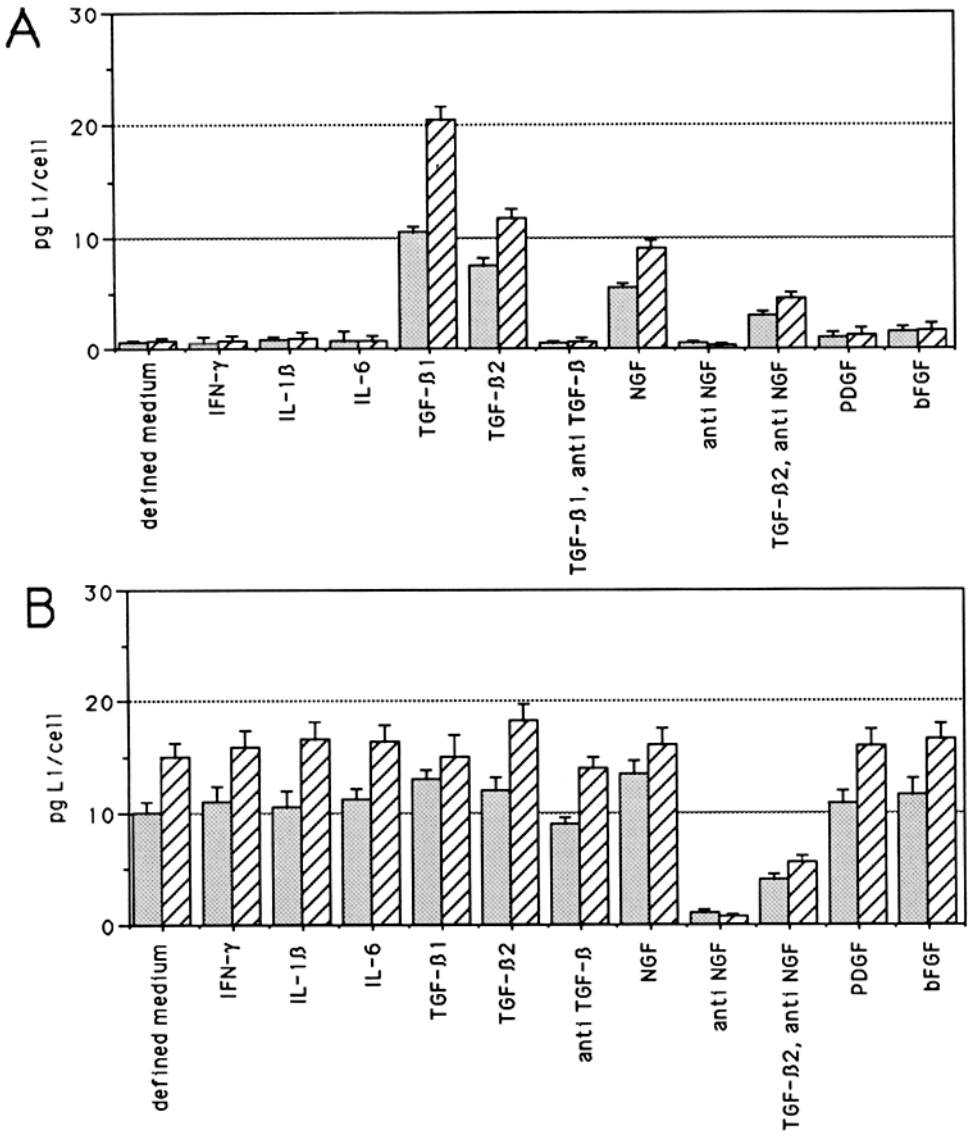

Figure 4. Effect of various growth factors, cytokines, and antibodies on the presence of L1 in the culture supernatants of immature astrocytes from cerebellum (*A*) and "rest" brain (total brain minus cerebellum) (*B*) from newborn mice as measured by ELISA. Culture supernatants were collected after 2 (▨) or 4 (▨) d of culture in the presence of the additives and L1 was quantified by ELISA using a standard curve. Antibodies to TGF-β (anti TGF-β) neutralize the bioactivity of both TGF-β1 and -β2. The values given represent the amount of L1 (picograms/cell) ± SD.

Expression of Recognition Molecules by Immature "Rest" Brain Astrocytes

Similar to immature cerebellar astrocytes, immature astrocytes from "rest" brain (total brain minus cerebellum) were influenced in their expression of recognition molecules by TGF-β1 and -β2 and NGF (Fig. 3 *A*). However, "rest" brain astrocytes, in contrast to cerebellar astrocytes, expressed already detectable levels of L1 in the absence of factors (Fig. 3 *A*). The relative increase in L1 expression over control levels was thus less than for cerebellar astrocytes. After 2 d of maintenance in TGF-β1 or -β2, cells hardly showed an increment in L1 expression. After 4 d, an increment by a factor of ~1.5 was seen. Similar relative increases were observed for NGF. A significant inhibition in L1 expression was, however, observed after culture in the presence of antibodies to NGF. Cell numbers were not changed in the cultures under the influence of TGF-β1 or -β2, NGF or antibodies to NGF. As for cerebellar astrocytes, the TGF-β2-induced increase in L1 expression could be reduced by addition of antibodies to NGF to the levels seen in the presence of antibodies to NGF alone. Surprisingly, the basal expression of L1 was hardly inhibited by antibodies to TGF-β1 and -β2, possibly suggesting that other than TGF-β-mediated mechanisms predominantly regulate L1 expression. IFN-γ, IL-1β, IL-6, PGDF, and bFGF did not change the basal level of L1 expression in these cultures.

The expression of N-CAM by the immature "rest" brain astrocytes was not modulated by addition of TGF-β1 and -β2 nor by any of the other tested factors (Fig. 3 *B*). Addition of antibodies to TGF-β1 and -β2 increased the expression of N-CAM, indicating that these astrocytes produce endogenous TGF-β, which, when being removed from the culture by the antibodies, alleviates the suppression of N-CAM expression.

The expression of AMOG by immature "rest" brain astrocytes was not changed under any of the conditions tested (Fig. 3 *C*).

Similar results were obtained with astrocytes from hippocampus and temporal lobe of newborn mice (not shown).

Expression of L1 in Supernatants of Immature Cerebellar and "Rest" Brain Astrocytes

The relative levels of L1 expression in the culture supernatants of immature cerebellar astrocytes correlated with the

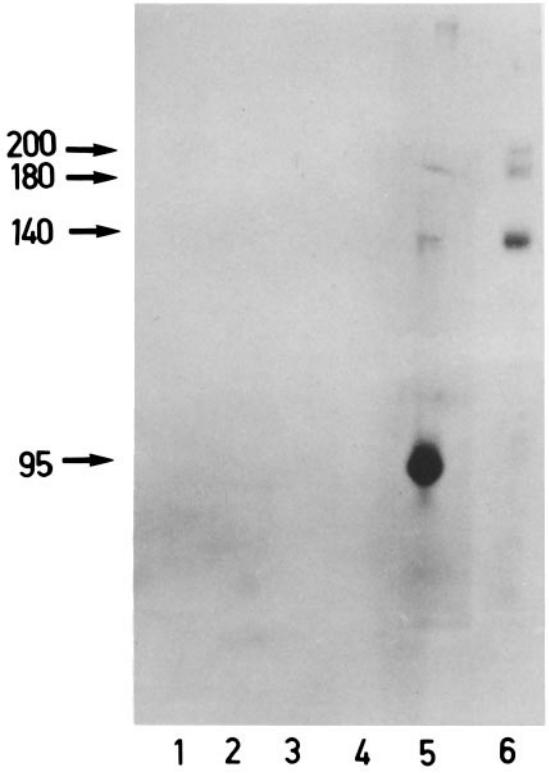

Figure 5. Western blot analysis of culture supernatants (lanes *1*, *3*, and *5*) and detergent lysates (lanes *2*, *4*, and *6*) of immature astrocytes from cerebellum of newborn mice cultured in defined medium (lanes *3* and *4*), after exposure to TGF-β2 for 4 d (lanes *1*, *2*, *5*, and *6*) using polyclonal L1 antibodies (lanes *3–6*) or no primary antibody (lanes *1* and *2*). Supernatants and detergent lysates were first immunoprecipitated using polyclonal L1 antibody and the immunoprecipitates were then used for Western blot analysis again using polyclonal L1 antibody. Molecular weight markers are indicated in kilodaltons at the left margin.

relative levels of L1 expression on cultured cells (Fig. 4 *A*). TGF-β1 and -β2 and NGF, but not IFN-γ, IL-1β, IL-6, PDGF, and bFGF increased the expression of L1. An ~30-fold increase in L1 expression was seen after 4 d of maintenance in TGF-β1 and an ~15-fold increase was seen after 2 d in TGF-β2 or NGF. The effects of TGF-β were specifically inhibitable by antibodies to TGF-β. Furthermore, antibodies to NGF abolished most of the TGF-β2-induced increase in L1 expression.

In cultures of immature "rest" brain astrocytes, no significant increase in L1 levels could be observed in the supernatants after addition of TGF-β1 and -β2 or NGF (Fig. 4 *B*). Antibodies to NGF completely abolished expression of L1, whereas antibodies to TGF-β1 and -β2 did not significantly reduce the expression of L1. When TGF-β2 and antibodies to NGF were added to the cultures simultaneously, a significant inhibition (approximately twofold) of L1 expression could be observed. These observations support the notion that L1 expression by these cells is dependent on NGF and that culture-endogenous levels of NGF are sufficient to elicit elevated levels of L1 expression in the culture supernatant.

Molecular Forms of L1 in Culture Supernatants and Detergent Lysates of Immature Cerebellar Astrocytes

The molecular forms of L1 in culture supernatants and detergent lysates of immature cerebellar astrocytes were determined by immunoprecipitation with polyclonal L1 antibodies and subsequent Western blot analysis with polyclonal L1 antibodies (Fig. 5). As expected from the ELISA, no L1 could be detected in the supernatants and detergent lysates of cells maintained in the absence of TGF-β2. In detergent lysates of cells treated with TGF-β2, a major component at 140 kD could be seen, while the 180- and 200-kD components were less prominent. In the supernatants of the TGF-β2-treated cells, a prominent 95-kD component, probably resulting from proteolytic degradation, and the less prominent bands at 140 and 180 kD were seen. Also, some high molecular L1-immunoreactive material was detectable in the supernatants, probably representing molecular aggregates that were not dissociated by SDS-PAGE. It is interesting that in the immunoprecipitation step the supernatants, but not detergent lysates yielded bands that were not L1 immunoreactive by subsequent Western blot analysis.

Northern Blot Analysis of the mRNA Species Expressed by Immature Cerebellar Astrocytes

Whereas immature cerebellar astrocytes did not express L1-specific mRNA (Fig. 6), treatment with NGF for 4 d revealed a 6-kb message by Northern blot analysis which is equivalent in size to that seen in brain of 1-d-old mice or neuroblastoma N2A cells (Fig. 6 and Tacke et al., 1987). When the filters were rehybridized with an actin probe, a single band at 2 kb was visible with equal intensity in all lanes (not shown). The band hybridizing at 28S in total RNA from

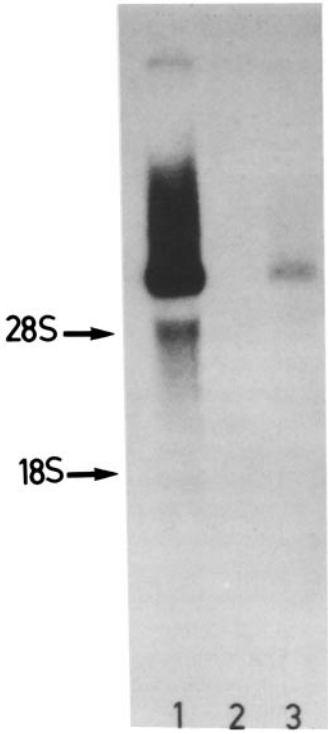

Figure 6. Northern blot analysis of RNA from N2A neuroblastoma cells (lane *1*) and immature cerebellar astrocytes in the absence (lane *2*) or presence of NGF for 4 d (lane *3*). Equal amounts of total RNA (20 μg per lane) were fractionated on agarose/formaldehyde gels, transferred to Hybond-N filters and hybridized with ^{32}P-labeled in vitro transcripts of the L1 cDNA clone K13. Positions of 28S and 18S ribosomal RNA are indicated at the left margin.

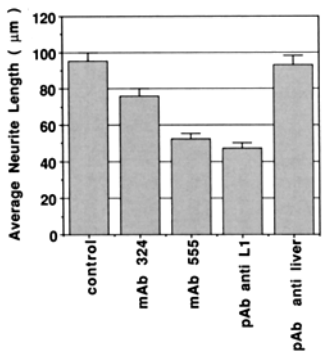

Figure 7. Effects of polyclonal and monoclonal antibodies against mouse L1 on neurite outgrowth from dorsal root ganglion neurons of 8-d-old chicken embryo on monolayers of L1-positive immature mouse A9 glial cells after 24 h. The histogram illustrates the average lengths of neurites (mean ± SEM) of neurons grown in the absence (*control*) and presence of L1 mAbs 324 (*mAb 324*) and 555 (*mAb 555*), polyclonal L1 antibodies (*pAb anti L1*) and polyclonal antibodies to mouse liver membranes (*pAb anti liver*).

N2A cells probably represents nonspecific hybridization to ribosomal RNA.

Determination of Neurite Outgrowth-promoting Properties of L1 Expressed by Immature Cerebellar Astrocytes

To determine whether the L1 molecule expressed by immature astrocytes is indeed functional for neurite outgrowth, the effects of mono- and polyclonal L1 antibodies were investigated in a neurite outgrowth test (Fig. 7). Since L1 has been shown to act by a homophilic or assisted homophilic binding mechanism (Kadmon et al., 1990*a*,*b*), it was necessary to prove that antibody-mediated modifications of neurite outgrowth were due to inhibition of the astrocyte-derived L1. To this aim, neurons were taken from chicken and cultured on L1-positive mouse A9 astrocytes in the absence and presence of antibodies specific for mouse L1. Two mAbs reacting with distinct epitopes on mouse L1 and polyclonal antibodies specific for mouse L1 inhibited neurite outgrowth on immature A9 glial cells, while polyclonal antibodies to mouse liver membranes, which strongly reacted with the A9 glial cells, did not interfere with neurite outgrowth.

Determination of NGF and TGF-β1 and -β2 in Supernatants of Cultured Astrocytes

NGF was neither detectable by ELISA in supernatants of 2-d-old cultures of immature astrocytes obtained from cerebellum or "rest" brain, nor in 2- or 14-d-old cultures of mature "rest" brain astrocytes (not shown). However, after 4 d in culture, high levels of NGF were detectable in the supernatants of immature "rest" brain astrocytes (33×10^{-5} pg NGF/cell; 132 pg NGF/ml; or 1×10^{-12} M NGF) (Table I). The culture medium of immature cerebellar astrocytes showed only approximately one-fifth of this level of expression (6.4×10^{-5} pg NGF/cell; 26 pg NGF/ml; or 2×10^{-13} M NGF) (Table I). Thus, the amount of L1 expressed by immature astrocytes correlated with the levels of NGF synthesized by these cells. Likewise, antibodies to NGF were most effective in reducing basal L1 expression in those cultures which produced high levels of NGF (see Fig. 4 *B*).

Expression of TGF-β was tested in the culture supernatants using a sensitive bioassay (Table I). It was found that immature astrocytes from both cerebellum and "rest" brain secreted TGF-β2, but not TGF-β1 in a latent form requiring acidification to gain bioactivity. Highest levels were detected in supernatants of immature astrocytes from "rest" brain (1.6×10^3 pg TGF-β2/ml; or 6.4×10^{-11} M TGF-β2), which also showed highest levels of NGF production.

Discussion

Results obtained in the present study indicate that astrocyte-derived TGF-β2 and NGF differentially regulate the expression of some neural recognition molecules by cultured immature astrocytes from different brain regions. The specificity of the effects of TGF-β and NGF was probed for in several ways. First, of the growth factors tested, namely interferon-γ (IFN-γ), interleukin-1β (IL-1β), interleukin-6 (IL-6), transforming growth factors β1 (TGF-β1) and -β2 (TGF-β2), NGF, PDGF, or bFGF, only TGF-β1 and -β2 and NGF showed a significant increase in the expression of L1 by immature astrocytes in culture. Second, changes in L1 expression were seen by several methods, i.e., ELISA, indirect immunofluorescence, immunochemistry, and Northern blot analysis. Third, neither TGF-β1 or -β2 nor NGF altered the cell number or cell morphology of cultured astrocytes over the time periods studied and care was taken to make comparisons under identical conditions. Fourth, the expression of another recognition molecule, N-CAM was downregulated in the presence of TGF-β1 and -β2 and not affected by NGF. Furthermore, levels in expression of the adhesion molecule on glia were not modified by any of the factors tested in this study. Fifth, only immature, but not mature astrocytes responded to any of the factors by altered levels of recognition molecule expression under the conditions of this study. Sixth, antibodies to TGF-β and NGF abolished some of the effects seen with these factors.

The TGF-β-mediated increase in L1 expression by immature cerebellar astrocytes appears to be due to the direct or indirect regulation of NGF expression by TGF-β1 and -β2. This could be shown by adding TGF-β1 or -β2 to the cultures in the presence of antibodies to NGF leading to a complete abolishment of the TGF-β-induced increase in L1 expression. This was not only observed for L1 surface-bound on astrocytes, but also for the soluble form of L1 detectable in the culture supernatant. A similar regulatory mechanism is likely to be operant in cultures of immature astrocytes from "rest" brain (total brain minus cerebellum), where basal levels of L1 expression appear to be increased due to the synthesis of TGF-

Table I. Determination of the Concentrations of L1, NGF, and TGF-β in Supernatants Taken from Cultures of Immature Astrocytes from Cerebellum and "Rest" Brain

Antigen or growth factor	Immature astrocytes	
	From cerebellum	From "rest" brain
L1	0.5	15
NGF	6.4×10^{-5}	33×10^{-5}
L-TGF-β1	$<1.6 \times 10^{-8}$	$<1.6 \times 10^{-8}$
L-TGF-β2	6.2×10^{-4}	2.7×10^{-3}
TGF-β1/TGF-β2	$<1.6 \times 10^{-8}$	$<1.6 \times 10^{-8}$

Concentrations (picograms/cell) of L1, NGF, latent TGF-β (L-TGF-β) and bioactive, free TGF-β were determined in the culture supernatants after 4 d of culture. The ratio of volume of culture medium to cell number was the same for all supernatant samples taken. Values are from two independent experiments carried out in triplicate for L1 and NGF, and from a representative experiment for the TGF-β determinations.

β2 and, probably as a consequence, also of NGF by these cells, as shown by antibody inhibition experiments and by direct quantitation of the levels of endogenous factors in the culture supernatants.

The TGF-β-induced decrease of N-CAM expression by immature astrocytes does not, in contrast to L1, depend on NGF as mediator, since neither NGF nor antibodies to NGF altered the TGF-β-induced decrease in N-CAM expression. These results are noteworthy in the context of previous observations on the expression of L1 and N-CAM by cultured Schwann cells (Seilheimer and Schachner, 1987). In these cultures, NGF induced an increase in L1, but not in N-CAM expression. Furthermore, basal levels of L1 expression by Schwann cells could be reduced by addition of antibodies to NGF to the cultures. Thus, Schwann cells and immature astrocytes show a similar susceptibility in their regulation of L1, but not N-CAM expression, by a neurotrophic factor that affects neurite outgrowth. The influence of NGF on immature astrocytes is the first known physiologically relevant effect of NGF on astrocytes and the first more prominent response of any nonneuronal cell to NGF. It is not known which type of NGF receptor mediates this effect. We have observed immunoreactivity of polyclonal antibodies against REX (Weskamp and Reichardt, 1991), the recombinant, baculovirus expressed extracellular domain of the rat NGF receptor (Radeke et al., 1987), on immature cerebellar astrocytes and A9 glial cells (Saad, B., G. Weskamp, F. Reichardt, and M. Schachner, unpublished observations). It remains to be seen whether the subclass of high-affinity NGF receptors of the trk gene family (Klein et al., 1991; Hempstead et al., 1991) is also expressed by astrocytes.

The molecular forms of L1 expressed by immature astrocytes as a consequence of the action of NGF are similar to those expressed by neurons. Cell-bound L1 occurred in the predominant forms of 200, 180, and 140 kD, while the forms detectable in the supernatants were the proteolytic degradation products described previously (Sadoul et al., 1988). A hitherto undetected form with an apparent molecular weight of 95 kD, probably also a proteolytic degradation product, was always seen in the supernatants. Addition of protease inhibitors to the cultures did not prevent the occurrence of this fragment (unpublished observations). Furthermore, the NGF-induced mRNA of immature cerebellar astrocytes hybridizing to the L1 riboprobe displayed a size of 6 kb as seen in early postnatal brain and cultured N2A neuroblastoma cells (Tacke et al., 1987).

Finally, the NGF-induced molecular components of L1 on astrocytes are indeed functional, since antibodies reacting specifically only with the astrocyte-derived and not with the neuron-bound L1, were able to reduce neurite outgrowth. These observations underscore the functional significance of the previously unknown expression of L1 by immature astrocytes. Again, a striking similarity between the mechanisms used by Schwann cells and immature astrocytes is apparent. The suggestion that neurite outgrowth on mature, GFAP-positive astrocytes in culture is mediated by a heterophilic mechanism (Bartsch et al., 1989; Smith et al., 1990; Drazba and Lemmon, 1990) points to the possibility that neuron-derived L1 may use different molecular mechanisms, i.e., homophilic and heterophilic ones, for glia-mediated neurite outgrowth. It is presently not known at which developmental stage glial cells express L1 in vivo (Persohn and Schachner, 1987; Bartsch et al., 1990). However, whenever astrocytes have been found to be associated with L1 immunoreactivity by electron microscopy in the developing intact tissue, it was always located at contact sites with neurons, thus making the decision impossible whether the neuronal or glial cell surface contributes to this immunoreactivity.

Knowledge of the site of TGF-β synthesis in the brain is of value for the understanding of the cellular mechanisms underlying TGF-β-dependent influences. In immunohistological studies, TGF-β immunoreactivity was closely associated with tissue derived from mesenchyme, including the meninges, but not in the brain tissue proper of embryonic or adult mice (Heine et al., 1987). By in situ hybridization, TGF-β3 transcript-containing cells have been described also in the meninges, choroid plexus and the olfactory bulb, while cells containing TGF-β1 and -β2 mRNA were not detectable in other parts of the brain (Wilcox and Derynck, 1988; Pelton et al., 1990). In vitro, however, immature astrocytes are capable of synthesizing TGF-β and it will be important to determine whether they can also do this in vivo. Interestingly, cultured astrocytes secrete TGF-β2, but not TGF-β1. Most of the cultured cell lines so far investigated have been observed to express TGF-β1, but not TGF-β2 (Derynck et al., 1988). In fact, cultured human glioblastoma cells have been described to secrete only TGF-β2, but not TGF-β1 (Bodmer et al., 1989) and TGF-β2 was first purified and its cDNA sequenced from a glioblastoma cell line (Wrann et al., 1987; de Martin et al., 1987). However, unlike glioblastoma cells, astrocytes secrete TGF-β2 in a latent form which requires acidification in order to become biologically active. This feature has been described for all cell types that synthesize TGF-β (for review, see Lyons and Moses, 1990). In vivo, latent TGF-β may be activated by proteases or by a deglycosylation step enabling the release of the biologically active 25-kD TGF-β homodimer being noncovalently associated with the amino-terminal glycopeptide portion of the TGF-β precursor molecule (Lyons and Moses, 1990). Interestingly, supernatants of lymphocytes activated in vitro contain only TGF-β1 in its latent form (Lucas et al., 1990) and antibodies to the bioactive 25-kD TGF-β1 homodimer augment the proliferation of lymphocytes. A comparable situation is provided in the present study: in the absence of detectable bioactive TGF-β in the culture supernatant, antibodies to TGF-β modified the expression of N-CAM by immature "rest" brain astrocytes. Thus, there is evidence for a form of TGF-β which is activated from latent TGF-β in cultured cells by yet undefined mechanisms.

Besides production of TGF-β, cultured astrocytes have been found to secrete also PDGF, IL-1 and IL-6 (Richardson et al., 1988; Frei et al., 1988; Malipiero et al., 1990), which were, however, not found to affect the expression of the recognition molecules investigated in the present study. Recently, IL-1 and IL-6 have been shown to enhance secretion of NGF by Schwann cells and astrocytes, respectively (Lindholm et al., 1987; Frei et al., 1989). The observation that these cytokines do not affect expression of recognition molecules by both mature and immature cultured astrocytes in the present study remains presently unexplained. It is possible, however, that the induction of NGF by IL-1 and IL-6 is quantitatively less pronounced than that by TGF-β. Indeed, TGF-β was found to be a much more potent inducer of NGF production than IL-1 and IL-6 (unpublished observations). Nevertheless, the inability of antibodies to TGF-β to significantly influence L1 expression by immature "rest" brain astrocytes

may be due to the action of endogenous IL-1 or IL-6. Finally, astrocytes in culture may be influenced in their expression of recognition molecules, but the effects could be too small to be detected by the methods used.

The cellular and molecular mechanisms regulating the expression of TGF-β and NGF by immature astrocytes are presently unknown. Interestingly, as for Schwann cells, NGF appears to act on astrocytes via an autocrine mechanism via NGF receptors, both of which are increased in expression after damage and removal of neuronal contact in the peripheral nervous system (Heumann et al., 1987*a*,*b*). The influence of TGF-β on neural recognition molecule expression adds to the list of functions of this cytokine, which affects growth and differentiation of various cell types. TGF-β has a profound effect on the production and deposition of extracellular matrix constituents and their cellular receptors as well as on proteases and their inhibitors (for review, see Roberts et al., 1990). TGF-β has recently been shown to increase expression of N-CAM by cultured fibroblasts (Roubin et al., 1990). Thus, TGF-β not only contributes to cell-to-extracellular matrix, but also cell-to-cell interactions by modulating the expression of recognition molecules. This property may be of interest in both development and repair processes of the nervous system (Eccleston et al., 1989; Nichols et al., 1991). It remains to be seen by which cellular and molecular mechanisms TGF-β and NGF exert their effects on the expression of L1 and N-CAM in normal development and under pathological conditions in vivo.

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