A Role for Gangliosides in Astroglial Cell Differentiation In Vitro

Laura Facci,* Stephen D. Skaper,*‡ Marco Favaron,* and Alberta Leon*

*Fidia Research Laboratories, Department of Central Nervous System Research, 35031 Abano Terme, Italy; and
‡Department of Biology, School of Medicine, University of California, San Diego, La Jolla, California 92093

Abstract. Rat cerebral astroglial cells in culture display specific morphological and biochemical behaviors in response to exogenously added gangliosides. To examine a potential function for endogenous gangliosides in the processes of astroglial cell differentiation, we have used the B subunit of cholera toxin as a ganglioside-specific probe. The B subunit, which is multivalent and binds specifically to GM1 ganglioside on the cell surface, induced a classical star-shaped (stellate) morphology in the astroglial cells and inhibited DNA synthesis in a dose-dependent manner. The morphological response was massive and complete within 2 h, with an ED_{50} of 0.8 nM, and appeared to depend on the direct interaction of the B subunit with GM1 on the cell surface. A B subunit–evoked inhibition of DNA synthesis and cell division (ED_{50} = 0.2 nM) was observed when the cells were stimulated with defined mitogens, such as epidermal growth factor and basic fibroblast growth factor. Maximal inhibition approached 80% within 24 h. The effects of the B subunit were unrelated to increases in cAMP. These observations, taken together with previous studies, demonstrate that both endogenously occurring plasma membrane gangliosides and exogenously supplied gangliosides can influence the differentiative state (as judged by morphological and growth behaviors) of astroglial cells in vitro.

GANGLIOSIDES, sialic acid–containing glycosphingolipids (44), are normal membrane constituents, largely localized to the outer leaflet of the plasma membrane and thus presenting their carbohydrate moieties on the surface of the cells (49). They are abundant in neural tissues, and the ganglioside compositions of neuronal and glial cell membranes appear to be different (25). Numerous studies have identified effects of exogenously supplied gangliosides on neuronal cells, both in vitro (11, 26, 27, 38) and in vivo (8, 34, 39, 45). There have been, however, few investigations addressing possible interactions of gangliosides with glial cells.

Rat astroglial cells in vitro have been reported to display two responses to GM1 and other gangliosides. One ganglioside effect is prevention or reversal of the assumption by flat astroglial cells of a stellate (star shaped) morphology under the influence of certain agents (12, 37), which include neuron-released molecules such as neurotransmitters and neuropeptides (29). The other ganglioside effect is that of growth modulation in these cells (23, 36). A possible role for endogenously derived gangliosides in these astroglial cell behaviors was never examined.

Several studies have indicated that the cross-linking of cell surface gangliosides can influence some (41, 42), but not other (9, 10) cellular behaviors. For example, the B subunit of cholera toxin, which is multivalent and binds exclusively to GM1 ganglioside on the cell surface (15), has been reported to induce the proliferation of lymphocytes (42) and growth-arrested, nontransformed 3T3 fibroblasts (41). The B subunit, however, inhibited the growth of rapidly dividing normal 3T3 cells and virally transformed 3T3 cells (41). The molecular basis for these opposing effects of B subunit could conceivably reflect differences in cell metabolic properties, or the amount or distribution of surface GM1 being occupied by B subunit, the latter suggested by Spiegel and co-workers (41). It has been reported that the levels of cell surface gangliosides vary as a function of 3T3 cell growth state (20), and that transformed 3T3 cells have less GM1 than normal 3T3 cells (20).

It is tempting to speculate that the previously observed astroglial cell morphology and growth responses to exogenous gangliosides also depend on the amount or distribution of ganglioside that may be incorporated. At the same time, nothing is known concerning the significance of these findings with respect to the function of endogenous gangliosides in the same astroglial cellular behaviors. Until now, there has been no assessment of the manipulation of endogenous ganglioside in cells displaying well-defined responses to the same exogenous ganglioside molecules. To investigate this question, we have used the B subunit of cholera toxin as a ganglioside-specific probe in order to examine a role for membrane GM1 ganglioside in the regulation of astroglial cell morphology and growth. The results presented here provide the first evidence that manipulation of endogenously oc-

curring plasma membrane gangliosides can influence specific behaviors of astroglial cells in vitro.

Materials and Methods

Materials

Eagle's basal medium (EBM)[1] and penicillin G sodium were purchased from Gibco (Grand Island, NY); fetal calf serum from Seromed (Berlin, Federal Republic of Germany); L-glutamine, poly-L-ornithine HBr (44,000 mol wt), bovine pancreatic insulin, ovalbumin, cholera toxin, and forskolin from Sigma Chemical Co. (St. Louis, MO); bovine plasma fibronectin, bovine pituitary basic fibroblast growth factor (bFGF), and mouse salivary gland epidermal growth factor (EGF) from Bethesda Research Laboratories (Bethesda, MD); B subunit of cholera toxin from List Biological Labs (Campbell, CA); [methyl-^{3}H]TdR (2.0 Ci/mmole) from Amersham International (Buckinghamshire, England); Instagel II from Packard Instrument Co., Inc. (Downers Grove, IL).

Cell Cultures

Primary and secondary cultures of astroglial cells were prepared from cerebra of 1–2-d-old Sprague-Dawley rats as described previously (33). Dissociated cells were cultured in EBM modified to contain 33.3 mM D-glucose, 2 mM L-glutamine, 26.4 mM $NaHCO_3$, 100 U/ml penicillin, and 10% (vol/vol) heat-inactivated FCS. These primary cultures were harvested 10 d later in serum-free modified EBM containing 1% (vol/vol) ovalbumin and diluted to 3.2×10^5 cells/ml in the modified EBM. The secondary cultures to be used experimentally were set up by seeding 50 μl of the final cell suspension into 6-mm plastic culture wells (No. 3070; Falcon Labware, Becton, Dickinson & Co., Oxnard, CA) already containing 50 μl of medium, and which had been precoated with polyornithine and fibronectin (33). Thus, each well started with 16,000 cells, ~12,000 of which attached. All cultures received one medium change at 2 h and another at 24 h. Most cultures were treated and studied beginning 1 d after seeding (D1 cultures) with the new medium containing the agent to be tested. Cultures to be used for morphological evaluation or cell counting were fixed with 2% (vol/vol) glutaraldehyde in EBM, stained with 0.1% toluidine blue, and observed under phase contrast microscopy.

DNA Synthesis

To evaluate DNA synthesis, cultures were pulsed at various times after presentation of B subunit or mitogen with 2 μCi/ml [methyl-^{3}H]TdR (2 Ci/mmole). After a 2-h pulse, cultures were washed twice with 100 μl of ice-cold PBS, 100 μl of cold 5% TCA was added per well, and the cells were kept at 4°C for 20 min. The TCA extract was discarded and each well washed three times with 100 μl of 5% TCA. The cells were dissolved in 100 μl/well of 1 N NaOH, and the NaOH extract transferred to a scintillation vial. Each well was washed with 100 μl of 1 N HCl, and this added to the counting vial. 5 ml of Instagel II were added to each vial, and radioactivity counted in a liquid scintillation spectrometer (Tricarb 460 C; Packard Instrument Co.).

cAMP Measurements

Levels of intracellular cAMP were determined by radioimmunoassay with the use of protocols and kit (No. RPA 509; Amersham International) as described previously for astroglial cells (12). The larger amounts of cellular material needed for cAMP analysis were prepared by seeding 1.2×10^5 primary astroglial cells into 16-mm culture wells (Costar 3524; Data Packaging Corp., Cambridge, MA), coated with polyornithine and fibronectin (12).

Results

B Subunit of Cholera Toxin Induces Morphological Differentiation in Astroglial Cells

Astroglial cells prepared from neonatal rat cerebrum and cultured in serum-free medium maintain a flat, polygonal shape (33). Exposure of these cells to a variety of agents thought to activate cAMP-synthesizing systems (e.g., cAMP analogues, forskolin, cholera toxin, norepinephrine, vasoactive intestinal polypeptide) promotes a rapid (1–2 h) and near complete conversion to a process-bearing (stellate) morphology (12, 29, 30, 37, 48). When the astroglial cells are treated with N^6, $O^{2'}$-dibutyryl cyclic AMP, forskolin, or cholera toxin, the concurrent or delayed administration of GM1 inhibits or reverses the morphological response (37). GM1 does not block the elevation of cAMP that occurs in response to these agents (12).

To further explore this morphological modulation of astroglial cells by gangliosides, the following experiments were carried out. The B subunit of cholera toxin, which is pentavalent and recognizes exclusively ganglioside GM1 on the cell surface (15), was used as a probe to assess a role for endogenous gangliosides in the determination of astroglial cell morphology. When secondary astroglial cells at D1 were treated with 1 μg/ml of B subunit, a massive conversion to the stellate morphology occurred and was complete within 2 h (Fig. 1). The morphology induced by B subunit is indistinguishable from that elicited by cAMP analogues (30, 37) or other cAMP-elevating drugs (12, 29, 48) in these cells. Fig. 2 illustrates the concentration dependence of the B subunit–evoked flat-to-stellate conversion in the astroglial cells. A maximal effect was observed at a B subunit concentration of 250 ng/ml (4.3 nM) or greater, with an ED_{50} of 45 ng/ml (0.8 nM).

Since agents that elicit cAMP elevations in astroglial cells also induce the stellate morphology, a trivial explanation for the effect of the B subunit of cholera toxin is that the preparation used here is contaminated by the A subunit (adenylate cyclase activating). Several lines of evidence, however, show this not to be the case. In the first, the B subunit dose–response obtained with the commercial material was identical to that found using a B subunit preparation (46) kindly provided by Dr. M. Tomasi (Laboratorio di Biologia cellulare e Immunologia, Istituto Superiore di Sanità, Roma). This latter preparation has been biologically characterized as devoid of any adenylate cyclase–activating A subunit (2). Secondly, measurements of intracellular cAMP levels at different times failed to show any increase of cAMP after treatment by B subunit. As Table I illustrates, concentrations of the B subunit up to 20 μg/ml were unable to stimulate cAMP production at any time tested. Cholera toxin at 1 μg/ml, however, caused a 200-fold elevation of cAMP at 1 h in the astroglial cells, tapering off to about an 18-fold stimulation by 16 h. Previous studies have shown this concentration of cholera toxin to elicit a maximal rise of cAMP at about 1 h in astroglial cells (12). Significant, though smaller, increases of cAMP were observed using as little as 5 ng/ml of cholera toxin at 1 h (data not shown). Similar results were obtained in independent experiments using HPLC analysis instead of radio-immunoassay. Thus, the B subunit used in our studies is devoid of any A subunit; the effects of B subunit on astroglial cells are unrelated to increases of cAMP.

To confirm that the observed morphological activity was due to the binding of B subunit to the cells, we measured the ability of cholera toxin to elevate cAMP and its inhibition by the B subunit. The increase in cAMP at 1 h with 5 ng/ml of cholera toxin (59 pmol/10^6 cells vs. 2 pmol/10^6 cells for con-

1. *Abbreviations used in this paper*: bFGF, basic fibroblast growth factor; EBM, Eagle's basal medium; EGF, epidermal growth factor.

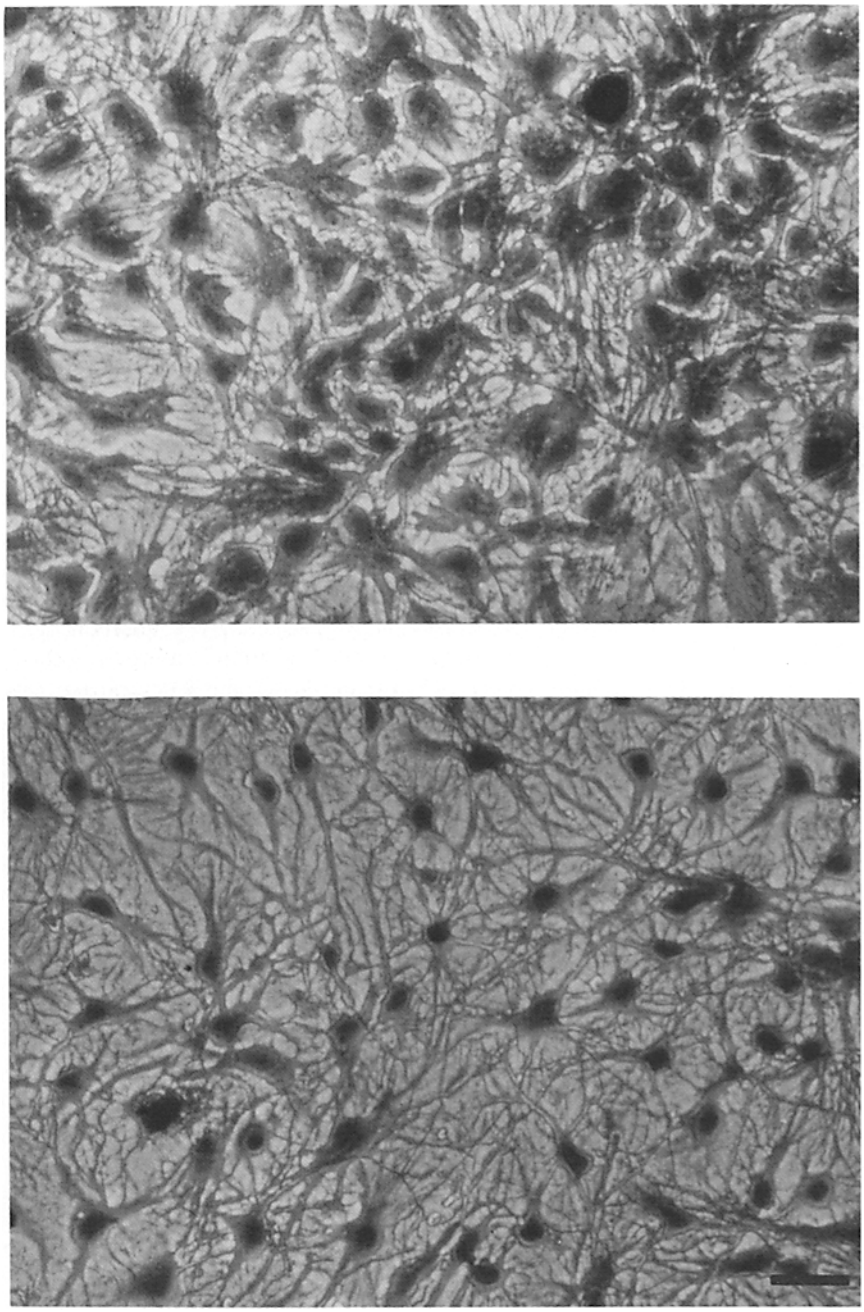
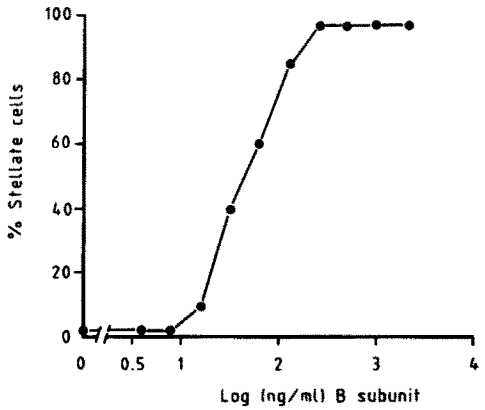


Figure 1. Photomicrographs of secondary rat cerebral astroglial cells treated 2 h with no agent (*top*) or 1 μg/ml B subunit of cholera toxin (*bottom*). Bar, 25 μm.



trol) was inhibited by the addition of B subunit in a dose-dependent manner: 0.5 μg/ml of B subunit reduced the cholera toxin–induced rise to 39 pmol cAMP/10^6 cells, while 1.5 μg/ml B subunit totally inhibited the effect of cholera toxin. Furthermore, presentation of B subunit (20 μg/ml) 20 s be-

Figure 2. Dose responsiveness of the B subunit–induced conversion of astroglial cells to the stellate morphology. DI cultures (16,000 cells seeded/6 mm well) were treated with varying concentrations of the B subunit of cholera toxin for 2 h, fixed, stained, and the percent of stellate cells determined. Values are averaged from three replicate experiments (n = 6).

Table I. Effect of Cholera Toxin and the B Subunit on cAMP Levels in Astroglial Cells

Addition	cAMP		
	1 h	4 h	16 h
	pmol/10⁶ cells	*pmol/10⁶ cells*	*pmol/10⁶ cells*
None	1.9 ± 0.5	1.9 ± 0.6	1.9 ± 0.2
B subunit (20 μg/ml)	1.5 ± 0.1	1.9 ± 0.5	1.8 ± 0.3
Cholera toxin (1 μg/ml)	400 ± 50	142 ± 46	35 ± 4

Astroglial cells were seeded as secondary cultures (1.2×10^5 per well in 24-well plates), and treated at D1 with cholera toxin or B subunit for 1, 4, and 16 h. The cells were then extracted with 5% TCA and cAMP measured. Each value is given as the mean ± SD (n = 6).

fore cholera toxin (0.5 μg/ml) markedly reduced the cAMP elevation of the latter from 110 pmol/10^6 cells to 18 pmol/10^6 cells. These data make it seem likely that the effects of the B subunit are due to its specific binding to the cell surface receptor for cholera toxin, that is, GM1. Analogous results have been obtained in studies demonstrating a proliferative effect of B subunit on lymphocytes (42).

B Subunit of Cholera Toxin Inhibits DNA Synthesis and Growth of Astroglial Cells

Earlier findings from other laboratories have demonstrated that an interaction of the B subunit with endogenous gangliosides results in stimulation or inhibition of proliferation, depending upon the type of cell used and its growth state (41, 42). In view of these growth regulatory effects of B subunit, we examined the effect of B subunit on the proliferation of astroglial cells. Initial experiments with D1 secondary cultures of astroglial cells demonstrated that the B subunit caused a dose-dependent inhibition of DNA synthesis. A concentration of ~60 ng/ml (1 nM) or greater inhibited cell proliferation to a maximum of 85% at 24 h, with an ED_{50} of 12 ng/ml (0.2 nM) (Fig. 3). An identical dose–response curve was obtained using the B subunit of noncommercial origin (data not shown). The B subunit inhibited [^{3}H]TdR incorporation into the cells even when the labeling pulse was increased to 24 h, with B subunit continuously present (50% of control). These effects of B subunit were not associated with loss of cell viability, nor with a reduced transport of [^{3}H]TdR into the TCA-soluble pool of the cells (data not shown).

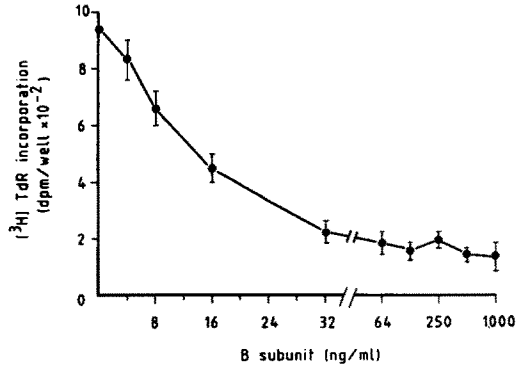


Figure 3. Concentration dependence of B subunit inhibition of DNA synthesis in astroglial cells. Cultures of secondary astroglial cells at D1 (16,000 cells seeded/6 mm well) were exposed to various concentrations of the B subunit of cholera toxin for 24 h, and assayed for [^{3}H]TdR incorporation into DNA during the last 2 h. Each value is the mean ± SD (n = 6, two experiments).

The inhibitory effect of the B subunit was also observed when astroglial cells were stimulated with either EGF or bFGF, at high or low cell densities. Table II illustrates the results. The B subunit (1 μg/ml) reduced DNA synthesis at 24 h by 80–85% without mitogen addition, irrespective of initial cell seeding density. In the presence of 10 ng/ml EGF (and 5 μg/ml insulin), proliferation was stimulated ~4.5-fold at 24 h. Polypeptide growth factors active on astroglial cells generally require insulin (6, 28, 31, 36). The growth-stimulating effect of EGF was essentially abolished in the concurrent presence of 1 μg/ml B subunit, although not to the level seen in control cultures with the B subunit. Similar results were obtained with bFGF; however, this latter mitogen was less effective than EGF. Insulin alone is reported to have no effect on DNA synthesis in cultured astroglial cells (6, 31, 36).

The antiproliferative effect of the B subunit was confirmed by direct cell counting and autoradiography. The increase in cell numbers elicited by EGF or bFGF at 48 h was reduced in the presence of B subunit by 85 and 45%, respectively, of the mitogen-stimulated values relative to control (Table III). In addition, autoradiographic analysis revealed fewer [^{3}H]TdR-labeled nuclei after a 24-h exposure to B subunit. As Table IV illustrates, the percent of labeled nuclei in cultures without mitogen decreased from 10 to 6% with B subunit, while the value of 28% in EGF-treated cultures was reduced to 10%. These results substantiate the data on direct cell number measurements in Table III. The inhibitory action of the B subunit on astroglial cells stimulated to grow with EGF or bFGF most likely is not a consequence of an interference by B subunit of mitogen binding to the cells. When cells were first exposed to the mitogen for 24 h and then to B subunit, the same inhibition was observed. For example, 48-h control cultures incorporated [^{3}H]TdR at 554 dpm/h, with EGF (plus insulin) yielding 2,445 dpm/h. Addition of 1 μg/ml B subunit after 24 h of EGF/insulin treatment, in the continued presence of the mitogen, decreased [^{3}H]TdR labeling to 642 dpm/h 24 h later. Similar results were obtained with bFGF. Thus, B subunit is still effective when added after a prior mitogen activation.

B Subunit-evoked Inhibition of DNA Synthesis Is a Time-dependent Process

The inhibition of astroglial cell proliferation elicited by the B subunit of cholera toxin was also time dependent. A maximal inhibition of 90% in control cells was seen at 24 h, whereas the B subunit inhibited DNA synthesis in EGF-stimulated cells by 75% at 24 h (Fig. 4). In the latter case, B subunit was maximally effective at 15 h (80% inhibition) although not significantly different from 20 and 24 h. Between 24 and 48 h, the inhibitory effect of the B subunit began to decrease. DNA synthesis was inhibited by 45–50% at 48 h, and by only 25% at 72 h in untreated cells (no inhibition with EGF at this time). Representation of B subunit at 72 h failed to initiate any additional inhibition alone or with mitogen, although the cells remained responsive to a further stimulation by EGF or bFGF, again suggesting that the B subunit does not exert its inhibitory effects on cell prolifera-

Table II. Inhibition of DNA Synthesis in Astroglial Cells by the B Subunit of Cholera Toxin

Additions	[^{3}H]TdR incorporated			
	16,000 cells		4,000 cells	
	− B Subunit	+ B subunit	− B subunit	+ B subunit
	dpm/h/well	*dpm/h/well*	*dpm/h/well*	*dpm/h/well*
None	1,050 ± 140	171 ± 27	406 ± 18	77 ± 10
EGF	5,077 ± 423	1,305 ± 220	1,670 ± 248	422 ± 68
bFGF	3,448 ± 435	1,557 ± 325	1,187 ± 185	441 ± 46

Astroglial cells were seeded as secondary cultures in 96-well tissue culture plates in 0.1 ml EBM at 4 × 10^3 or 16 × 10^3 cells per well. After 1 d, 0.1 ml of EBM was added containing 10 ng/ml EGF or bFGF (and 5 μg/ml insulin), and 1 μg/ml of B subunit where indicated. Incorporation of [^{3}H]TdR was determined during the last 2 h of the 24-h incubation. Values are the mean ± SD (n = 8, two experiments).

tion via an interference with growth factor binding to the cells.

Discussion

The results presented here demonstrate that the B subunit of cholera toxin can induce a pronounced morphological change and inhibit DNA synthesis in rat cerebral astroglial cells in vitro. This latter response to the B subunit was also seen when astroglial cells not rapidly dividing were stimulated with defined mitogens, such as EGF or bFGF. The only identified receptor for the B subunit is the ganglioside GM1 (7, 15, 42). Studies from other laboratories have shown that the B subunit is able to stimulate lymphocytes by directly binding to GM1 on the cell surface (42). The molecules of B subunit bind only to the oligosaccharide chains of GM1 exposed on the cell surface (15, 17, 22). In this study, the observed effects of B subunit also appear to be due to the binding of B subunit to the cells, as the ability of cholera toxin to stimulate cAMP levels was completely inhibited by the addition of B subunit. The B subunit, however, was equally effective in promoting the morphological conversion of the astroglial cells.

Astroglial cells cultured in the absence of serum display a flat, polygonal morphology (33). Administration of cAMP analogues or cholera toxin (among other agents) causes the cells to convert to a stellate (star shaped) morphology (12, 29, 30, 37, 48), suggesting a possible mediation through intracellular cAMP (12, 48). Treatment of these cells with the B subunit of cholera toxin elicited a morphological change that was indistinguishable from that observed with activation of cAMP-synthesizing systems (12, 37). The B subunit used here is devoid of any cAMP-elevating A subunit, in accord with other studies (41, 42). These results raise the possibility that the effects of cholera toxin on astroglial cell morphology may be due, at least in part, to the binding of its B subunit

Table III. The B Subunit of Cholera Toxin Reduces Mitogen-induced Cell Number Increases

Treatment (48 h)	Cells/mm^2
Control	446 ± 63
EGF	905 ± 100
EGF + B subunit	604 ± 90*
bFGF	862 ± 101
bFGF + B subunit	603 ± 98*

Secondary astroglial cells at D1 (16,000 cells seeded/6 mm well) were treated with the indicated agents for 48 h: 1 μg/ml B subunit: 10 ng/ml EGF or bFGF (plus 5 μg/ml insulin). Cells were fixed, stained, and counted under phase microscopy. Values are the mean ± SD (n = 8, two experiments).
* $P < 0.05$ compared with mitogen without B subunit (Student's t test).

Table IV. Autoradiographic Analysis of the Antiproliferative Effect of the B Subunit of Cholera Toxin

Treatment	Percent labeled nuclei
None	10 ± 2
B subunit	6 ± 1
EGF	28 ± 4
EGF + B subunit	10 ± 3

Secondary astroglial cell cultures at D1 (1.2 × 10^5 cells seeded/16 mm well) were treated for 24 h with 1 μg/ml B subunit, 10 ng/ml EGF (plus 5 μg/ml insulin), or both. Cultures were pulsed with 2 μCi/ml [^{3}H]TdR for the entire 24-h period, and then processed for autoradiography as described elsewhere (1). Each value is taken from analysis of three cultures (300–400 cells scored in each condition), and is the mean ± SD.

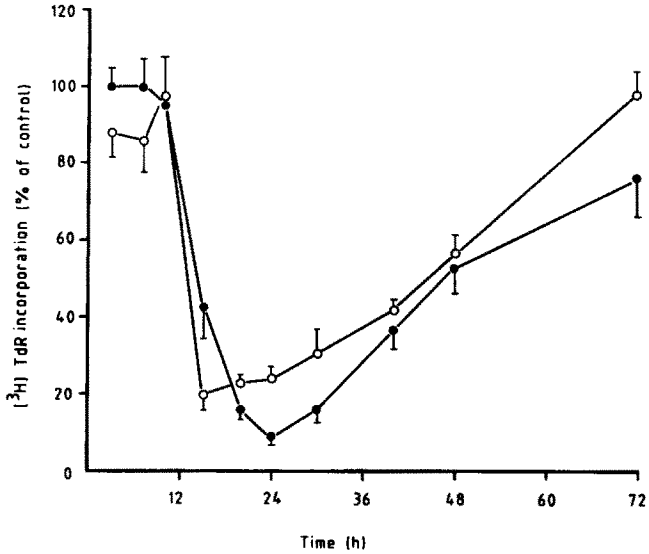


Figure 4. Time dependence of inhibition of DNA synthesis in astroglial cells induced by the B subunit of cholera toxin. Cultures of the secondary astroglial cells (16,000 cells seeded/6 mm well) at D1 were treated without and with 1 μg/ml of B subunit, in medium only (●) or with the addition of EGF (10 ng/ml) and insulin (5 μg/ml) (○) for various time periods. The cells were pulsed with [^{3}H]TdR in the last 2 h of each period. Results are expressed as the percent inhibition in comparison to control cultures (no B subunit) for each time (mean ± SD, n = 6).

to cell surface GM1 and not only to its ability to activate adenylate cyclase. Since the maintenance of the stellate morphology in astroglial cells is critically dependent on a stable microtubular network (5), the action of B subunit implies that cross-linking of surface GM1 molecules is translated to events at the level of the cytoskeleton.

In this study, we also found that the B subunit can inhibit DNA synthesis in cultured astroglial cells that are not rapidly dividing. Maximal inhibition of DNA synthesis was observed 24 h after B subunit introduction, reaching ~20% of control levels. Furthermore, the stimulation of DNA synthesis and cell number increases induced by EGF or bFGF was inhibited to an equal extent, and was found whether the mitogen was presented concurrently or 24 h before B subunit. The B subunit-evoked inhibition of DNA synthesis at 24 h was reflected in a decreased percentage of labeled nuclei at 24 h, and a smaller increase in actual cell numbers in the presence of mitogen at 48 h. These anti-proliferative effects of the B subunit on astroglial cells, as with the morphological effects, are unrelated to increases of cAMP.

In several other studies, the B subunit of cholera toxin has been reported to stimulate DNA synthesis in resting lymphocytes (42) and quiescent 3T3 cells (41), but to inhibit the growth of rapidly dividing normal 3T3 cells and transformed 3T3 cells (41). From these observations, the above authors concluded that the ability of the B subunit to stimulate the division of resting cells is a general phenomenon, with endogenous gangliosides playing a role in the regulation of both positive and negative signals for cell growth. In contrast the present studies demonstrate that, at least for astroglial cells and independent of growth state, B subunit inhibits DNA synthesis. While most experiments were performed by seeding the astroglial cells directly in a serum-free medium, other experiments were done in which cells were seeded at low density in 24-well culture plates in serum-containing medium to allow continued growth, and B subunit subsequently added in the absence of serum after different times. In this latter case inhibition was also observed, irrespective of whether the cells were still actively dividing or had ceased to show further increases in number (Skaper, S. D., L. Facci, and M. Favaron, unpublished observations). The suggestion that the opposing responses of 3T3 fibroblasts to B subunit may be related to the amount or distribution of surface GM1 being occupied (41) is based upon the observations that transformed 3T3 cells have less GM1 than normal 3T3 cells (16, 20), and that the levels of cell surface gangliosides increase as normal 3T3 cells reach confluency (20). The failure of astroglial cells to display similar opposing growth responses to the B subunit may result for a variety of reasons. For example, astroglial cells may possess differences in their membrane ganglioside composition in comparison with normal 3T3 cells. Treatment of the former cells with neuraminidase to convert polysialogangliosides to GM1 produces a severalfold increase in membrane GM1 content, as measured by binding of a B subunit-horseradish peroxidase complex using ELISA (Favaron, M., S. D. Skaper, and L. Facci, unpublished observations). The inhibitory response of DNA synthesis to B subunit, however, remains after neuroaminidase treatment. Differences between astroglia and 3T3 cells may also result from variations in the metabolic state of the two cell types when used or different mechanisms whereby B subunit acts.

The mechanism(s) by which the B subunit is able to regulate the morphology and growth of astroglial cells is unknown. Interaction of membrane proteins with specific ligands can alter biochemical events in some cells. For example, B lymphocytes are activated by antibodies that cross-link membrane immunoglobulins. This cross-linking leads to enhanced inositol phospholipid metabolism and rapid increases in diacylglycerol (3), elevation of intracellular free calcium concentration, and phosphorylation of proteins associated with the plasma membrane and cytoskeleton (21). The growth-stimulatory action of the B subunit for resting 3T3 cells is reported to be associated with a rise of intracellular calcium that depends upon external calcium and increased membrane potential, but no enhancement of phosphoinositide turnover (40). It is also possible that gangliosides may modulate protein phosphorylation (4, 18, 24, 47), which in turn can serve as a signal to modulate both the cytoskeleton and cell growth. These parameters remain to be examined in the present system. Since, however, the only known action of the B subunit is to bind to GM1 ganglioside on the cell surface, the expected cross-linking and subsequent aggregation of GM1 by the multivalent B subunit appears to be relevant to elicit these cellular behaviors.

It is tempting to speculate that the same series of events are involved in the B subunit-induced change in astroglial cell morphology and inhibition of proliferation. Preliminary observations suggest that both the morphological and biochemical responses to the B subunit are linked to cytoskeletal stability at the level of the microtubules (Skaper, S. D., M. Favaron, and L. Facci, unpublished observations). An ability of endogenous gangliosides to modulate cytoskeletal organization would need to be reconciled with the fact that these lipid components do not span the cell membrane. One explanation for a cytoskeletal connection of gangliosides might be found in the interaction of gangliosides with one or more integral membrane proteins, which in turn interact with cytoskeletal proteins directly, i.e., a bridging membrane protein that binds in a *cis* fashion to GM1 at the outer cell surface and in a *trans* fashion to a cytoskeletal-organizing protein at the inner plasma membrane. Recent observations concerning cooperativity of GM1-dependent with protein-dependent adhesion and neuritogenesis of neuroblastoma cells lend support to this idea (32). In addition, there are several reports of possible association of gangliosides with transmembrane proteins and cytoskeletal elements, based on their resistance to mild detergent extraction (19, 35, 43) and on capping in lymphocytes (22).

The possible relationship between the opposing effects of B subunit versus exogenous GM1 raises an important issue, as it may be relevant to the reported therapeutic effects of exogenously administered GM1 in lesion models of the nervous system. For example, one could speculate that, in vivo, exogenously added GM1 competes with the endogenous, membrane-bound GM1 for some unidentified endogenous GM1-binding substance (an analogue of B subunit). Morphometric analysis has shown that the stellation transformation induced in cultured astroglial cells by treatment with cAMP analogues resembles the reactive astroglial cells which characteristically form in the vicinity of stab wounds in the brain (13, 14). The mechanism underlying this reactive gliosis is still not known. Some balance between cell surface ganglioside levels and these responses may exist. By binding cell

surface GM1 with a specific ligand one may tip the balance in one direction, and by adding exogenous gangliosides one may tip the balance in the opposite direction. The actual situation may not be so straightforward, however, as the added ganglioside could also undergo membrane incorporation, perhaps working against the effects of free ganglioside. This astroglial culture system may still provide a useful model for investigating a role for gangliosides in gliosis; such studies are presently in progress.

Taken together, these and past (12, 23, 36, 37) observations demonstrate that both endogenously occurring plasma membrane gangliosides and exogenously supplied gangliosides can play a role in the morphological and biochemical differentiation of astroglial cells in vitro. Thus, these astroglial cell cultures may provide a model system in which one can study a role for gangliosides as membrane transducers of signals for cytoskeletal-linked events. The differences in membrane concentration and distribution of gangliosides may influence membrane fluidity, with such changes in mobility being linked to cytoskeletal proteins either physically or chemically.

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