Astrocytes in Cell Culture Incorporate GM1 Ganglioside

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ABSTRACT Ganglioside GM1 ^{3}H-labelled at the terminal galactose was added to astrocyte cell cultures. GM1 incorporation was studied in the two typical forms of astrocytes in cell culture of flat and stellate morphology. There was a strong time- and concentration-dependent increase in GM1 incorporation for both cell types of astrocytes. The incorporation of GM1 into the stellate form increased continuously up to 48 h (maximum time studied), while the incorporation into the flat form reached a plateau at the same time. After 2 h of GM1 incubation additional gangliosides appeared; the latter resulted from the metabolism of the GM1 incorporated, indicating that astrocytes in cell culture can biosynthesize more complex gangliosides. To confirm that GM1 was indeed incorporated into astrocytes, two other different approaches were used. Astrocyte cells treated with ^{3}H-GM1 were visualized using autoradiography. The specific marker for GM1, rhodamine-labelled choleratoxin, was used to detect the incorporated GM1 using fluorescence microsocpy. In both cases GM1 treated cells were intensely labelled. These observations indicate that exogenous GM1 ganglioside can also be integrated into the astrocyte membranes as occurs in other types of cells and membranes.

INTRODUCTION

The ability to introduce exogenous gangliosides into cell membranes is one of the more common approaches to studying the biological function of these molecules. Several laboratories have demonstrated that exogenously applied gangliosides may exhibit neuritogenic and/or neuronotrophic properties in nerve cell cultures (Dimpfel et al., 1981; Ferrari et al., 1983; Massarelli et al., 1985; Roisen et al., 1981; Seifert, 1981; Seifert and Förster, 1988; Spoerri and Roisen, 1988; Tsuji et al., 1983). These effects depend on the stable incorporation of ganglioside molecules into the membrane lipid bilayer. This was first shown with tritiated gangliosides as a time- and concentration-dependent process for the neuronal cell line B104 (Morgan and Seifert, 1979).

Further studies both in fibroblasts and in nerve cells (Callies et al., 1977; Facci et al., 1984; Leon et al., 1982; Mancini et al., 1986; Schwarzmann et al., 1983) demonstrated that gangliosides can associate with cell membranes in three different ways: 1) as a loosely attached fraction (water extractable), 2) as a membrane protein associated fraction (trypsin sensitive) and 3) a water- and trypsin-stable fraction embedded in the lipid bilayer of the cell membrane (extractable only by chloroform/methanol treatment).

For glioma cells in culture it has been established that GM1 is functionally inserted into the membrane, since choleratoxin binding to inserted GM1 resulted in an increased level of the intracellular adenosine 3′:5′-cyclic phosphate (cAMP) (Fishman et al., 1980; Skaper et al., 1988; Spiegel, 1988). Exogenous GM1 can also self-associate in the plasma membrane inducing patching and capping (Spiegel et al., 1984). It has been reported that exogenous gangliosides stimulate astroglial cell proliferation (Katoh-Semba et al., 1986) and modulate the morphological changes in these cells produced by cAMP (Facci et al., 1987; Skaper et al., 1986). In all of

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these studies, one might assume that the effects observed were due to a real incorporation of ganglioside into the membrane. However, in a previous study, Asou and Brunngraber (1984a) were unable to demonstrate incorporation of exogenously added GM1 into astrocyte cells by immunocytochemical methods. It seemed unlikely that these cells would be completely different compared with other cell types thus far investigated, since gangliosides are probably inserted into lipid membranes, due to their hydrophobic ceramide part, and have a pronounced effect in astrocytes. Therefore we decided to investigate whether GM1 ganglioside could be incorporated into astrocyte cells in culture, using three different approaches to evaluate this incorporation: biochemical, immunohistochemical, and autoradiography methods.

MATERIALS AND METHODS

Astroglial Cell Cultures

Cultures of rat astroblasts were prepared as described by Kimelberg et al. (1979) by a modification of Seifert and Müller (1984). Cerebral hemispheres of postnatal 2–3 days old rats were dissected, cleaned of their meningeals, and dissociated by trypsinization and by passage through a 1 mm-diameter needle in a small volume of Dulbecco's modified Eagle's medium (DMEM). DMEM supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (FCS) was added to the cell suspension to a final volume of 10 ml/hemisphere. The cells were plated in uncoated petri dishes (diameter, 10 cm, 10 ml/dish) for 7–9 days until the bottoms of the wells were totally covered with astrocytes. After that the cells were trypsinized, replated to a cell number of 0.3×10^7 cells/dish (diameter, 10 cm), and grown again for 2 days in DMEM plus FCS (10 ml/dish).

The medium was then replaced with DMEM (without FCS) for 24 h. At this time point the cell density was about 1×10^7 cells/dish.

GM1 Treatment of Astrocyte Cells in Culture

A sample of radioactive ganglioside, galactose-6-^{3}H-(N)-GM1 and of unlabelled GM1 were pipetted into a plastic tube and dried in a stream of nitrogen. The residue was resuspended in chloroform:methanol (2:1, by vol), dried again, and dissolved in an appropriate volume of DMEM to obtain the desired concentration carrying 1 µCi/ml of radioactivity. A separate solution was prepared for each of the different ganglioside concentrations employed. Five milliliters of DMEM solution of gangliosides was added to each culture dish, and incubations were carried out at 37°C for different time periods. In the experiments aimed at studying GM1 association at different concentrations (specified under Results), cells were cultured with DMEM containing various concentrations of unlabelled GM1 plus the radioactive GM1.

Autoradiography

For autoradiography experiments the astrocytes were incubated over 24 h in DMEM medium in which GM1 (Fidia, Abano Terme, Italy) was dissolved to reach a concentration of 60 µM. This solution contained 1 µCi/ml of (galactose-6-^{3}H)-(N)-GM1 (7 Ci/mmol, NEN). As the detection of tritiated GM1 implies that the labelled soluble tracer must be completely removed prior to the autoradiography processing and to reduce the background of labelled GM1 binding on the surface of the petri dish, the cells were washed twice with DMEM, trypsinized, and again plated in new dishes for 8 h. After that the cells were rinsed twice with DMEM and twice with phosphate buffer (PBS, 0.1 M; pH 7.4). Cultures were fixed in situ in a 5% solution of glutaraldehyde (in PBS) washed with bidistilled water and air dried. The dry cell layer was coated with L4 film emulsion (Ilford) and exposed for 5 weeks at −80°C. The examination of silver grains was done using a light microscope (interference contrast/bright field).

Histochemical Staining

Choleratoxin (B-subunit, Sigma Chemical Co., St. Louis, MO) was coupled with rhodamine-iso-thiocyanate (RITC) and purified as described by Nairn (1976). Additionally, unspecific binding was removed by incubating the complex-containing fraction with insoluble collagen from bovine for 2 h. For detection of incorporated GM1 with RITC–choleratoxin, the cells were incubated with and without GM1 (60 µM) for 24 h. The cells were washed three times with DMEM, and the moist area was reduced by silicon grease to cover slip size. The remaining cells were incubated with 50 µl of RITC-labelled choleratoxin for 1 h and then washed five times with 50 µl of PBS. After that the cells were covered with mounting medium and fixed in place with a coverslip. Fluorescence microscopy was done using a Zeiss microscope equipped with the filter combination BP 546, FT 580, LP 590.

Immunocytochemistry Method

The procedure was the same as described for the RITC–choleratoxin staining but now the moist area was not only reduced but also divided into two parts (cover slip size). One of the parts was incubated with monoclonal antibody–GM1 (kind gift of Dr. S.P. Mahadik, Columbia University, New York) and the other area with the corresponding myeloma medium as control (50 µl both, 1 h, 37°C). The cells were blocked with rabbit serum (20% in PBS) for 30 min, at room temperature. Then the cells were washed three times with PBS and incubated with the second fluoresceine-isothio-cyanate (FITC)-labelled rabbit-to-mouse antibody for 1 h.

Extraction and Isolation of Ganglioside Fraction

For each ganglioside extraction, astrocytes from two petri dishes (corresponding to 4–5 mg of protein) were used. The cells were rinsed twice with DMEM and three times with PBS, harvested from the dishes, and centrifuged at 14,000 rpm for 15 min using an Eppendorf centrifuge 5415. The pellet was washed with distilled water, centrifuged, and resuspended again with distilled water. An aliquot was taken from this cell suspension for protein estimation (Peterson 1977).

For ganglioside extraction the remaining cells were centrifuged, resuspended with 2 ml of trypsin (2.5 units/ml), and incubated at 37°C for 45 min. The cells were centrifuged again, and gangliosides from the pellet were isolated by a scaled-down method described by Svennerholm and Fredman (1980). All of the ganglioside fractions obtained from the cells were dried and resuspended in chloroform:methanol 2:1 by vol. Aliquots from these fractions were dried, resuspended in water, and their radioactivity was measured in a Packard 2000 CA Liquid Scintillation Analyzer using Aqualuma (J.T. Baker Chemicals, The Netherlands) as liquid scintillation cocktail. Gangliosides were chromatographed using (HPTLC) precoated plates (silica gel 60, Merck, Darmstad, Federal Republic of Germany), and the pattern was evaluated. Chromatograms were developed in chloroform:methanol:0.25% $CaCl_2$ aqueous (55:45:10 by vol) during 1 h, dried and the bands were sprayed with resorcinol reagent (Svennerholm, 1963). Ganglioside patterns were quantified by densitometry using a Shimadzu CS-930 dual wavelength TLC scanner. To determine which bands were radioactive, in some experiments bands from high-performance thin-layer chromatography (HPTLC) were visualized, by iodine vapor, to leave gangliosides intact; the bands were then scraped off from the plates, and the radioactivity was determined in a scintillation counter.

RESULTS

Astroglial cells cultured in a serum-containing medium usually assume a flat and epithelial-type morphology (flat astrocytes). Administration of Dibutyryl cAMP (DBcAMP) causes the astroglial cells to convert to a more traditional star-shaped morphology (stellate morphology), as can be seen in Figure 1 (Juurlink and Hertz, 1985; Moonen et al., 1975). As mentioned in the Introduction, three different associations of GM1 can be determined after exogenous administration. All of the

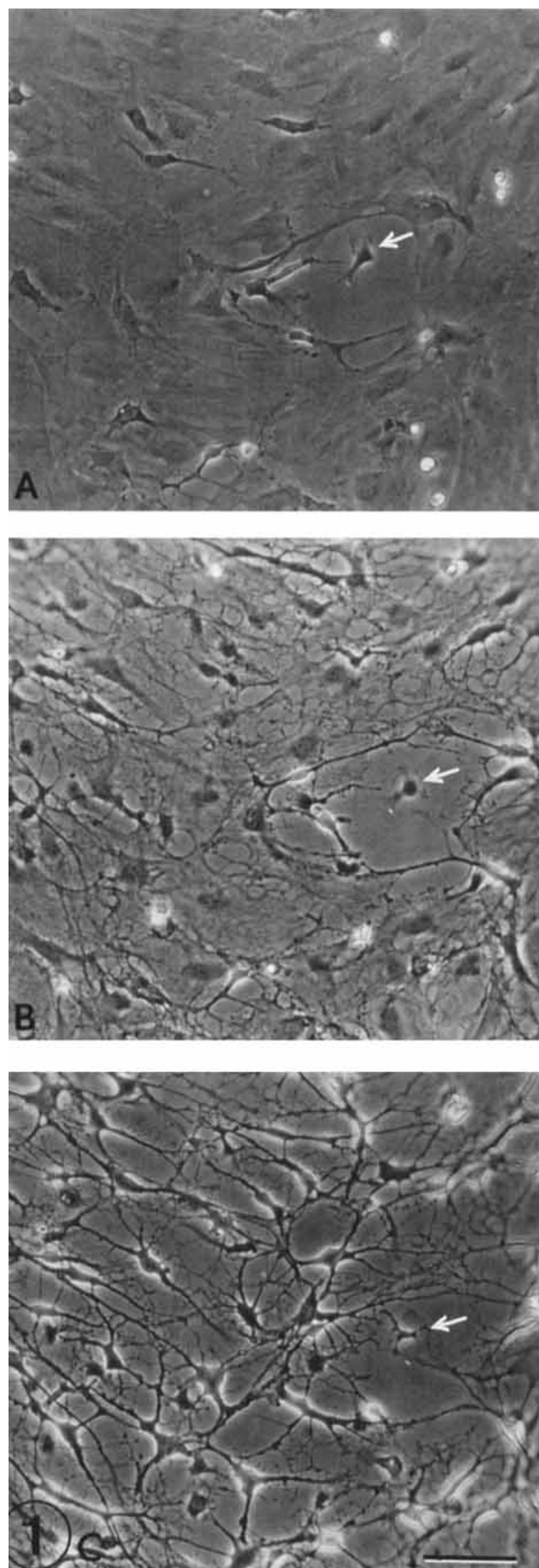

Fig. 1. Morphological changes in astrocytes after treatment with DBcAMP (1 mM concentration). **A:** Astrocytes of 2 day-old rat hemispheres cultured in Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) (10%) for 7–9 days, trypsinized, replated, and grown again for 2 days in the same medium. The medium was then replaced with DMEM in absence of FCS for 24 h. **B:** The same cells (see arrow as a marker) 15 min after addition of DBcAMP. **C:** The cells 150 min after addition of DBcAMP. Bar 100 μm.

experiments described below refer to stably incorporated GM1 (i.e., chloroform:methanol extractable).

As demonstrated in Figure 2 the GM1-incorporation into flat astrocytes was clearly concentration dependent, reaching a plateau at about 60 μM GM1 with 35 nmol GM1/mg of protein incorporated. Based on this result, all further experiments were performed at 60 μM GM1 concentration.

To study the time course of incorporation and to determine if there was a difference between different types of astrocytes, cells were first incubated with or without 1 mM DBcAMP. After 2 h, the DBcAMP-treated cells show stellate morphology. At this time, ^{3}H-GM1 was added, and the cells were incubated for 15 and 30 min, 2, 8, 24, and 48 h. The GM1 incorporation with time was studied for stellate and flat astrocytes. The morphological changes of cells treated with DBcAMP plus GM1 and of cells treated only with GM1 were followed under phase-contrast microscopy over all periods of time.

Figure 3 describes the kinetics of GM1 incorporation (measured as (^{3}H)-GM1) into flat (−DBcAMP) and stellate (+DBcAMP) cells. As shown for the flat astrocytes there was a strong time-dependent increase in GM1 incorporation, from 5 to 35 nmol GM1/mg of protein within the first 8 h, followed by a plateau up to 48 h. In contrast, the incorporation of GM1 into stellate astrocytes (DBcAMP-treated cells) increased continuously up to 48 h, reaching 50 nmol GM1/mg of protein. This difference may simply be due to the morphological change, since the stellate astrocytes exhibit more exposed membrane surface for incorporation of GM1 than do flat astrocytes, although effects of changes in fluidity and other properties of the cell membrane produced by DBcAMP might be also responsible.

The typical ganglioside pattern of astrocytes in culture is shown in lane 7 (C) of the Figure 4. As can be seen, the characteristic GM3 content and the lack of appreciable amounts of polysialogangliosides were found both in flat and stellate astrocytes. No differences were observed in the ganglioside pattern of these two different forms.

To confirm that the measured radioactivity of incorporated ^{3}H-GM1 corresponds to ganglioside GM1, the extracted ganglioside fractions were analyzed on HPTLC plates.

Figure 4 shows that a significant GM1 incorporation can be detected as early as 15 min; the increase in the incorporation with time was observed for both cell types. At later time points (8, 24, and 48 h) additional bands, corresponding with GM2 and GD1a, appeared; these bands resulted from the metabolism of the incorporated GM1. Since the terminal galactose residue was tritium-labelled in ^{3}H-GM1, only this and GD1a can be radioactive; thus the GM2 produced by splitting off the galactose terminal was not radioactive. Almost all the radioactivity was found in the GM1 band, GD1a only contained a very small percentage with respect to GM1 (2–3%).

Since GM3 seems to stay constant at all times in both types of cells, we have used this as internal standard. The densitometric areas for GM1 and GM3 were mea-

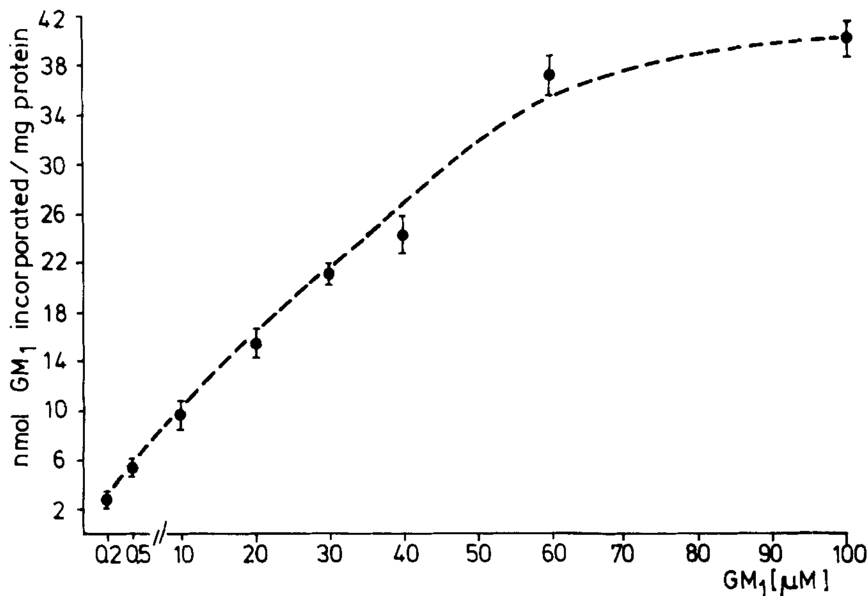

Fig. 2. Concentration dependence of GM1 incorporation (nmol/mg protein) into flat astrocytes in Dulbecco's modified Eagle's medium (DMEM) without serum after 24 h incubation at given GM1 concentration (μM). Cells were treated, and gangliosides were extracted, as described in Materials and Methods. Points are the average of four different experiments done by duplicates. The data represent the trypsin-stable pool of GM1, which has been incorporated into the membrane.

sured for all the time points, and the ratio GM1/GM3 was calculated. Figure 5 shows essentially the same difference in the kinetics of the incorporated GM1 for the two types of cells, as found when the incorporated GM1 was related to protein (Fig. 3).

These results indicate that GM1 ganglioside was incorporated in a time- and concentration-dependent manner in both types of astrocytes. To confirm these results by using different approaches, we studied GM1 incorporation into flat astrocytes, using a monoclonal

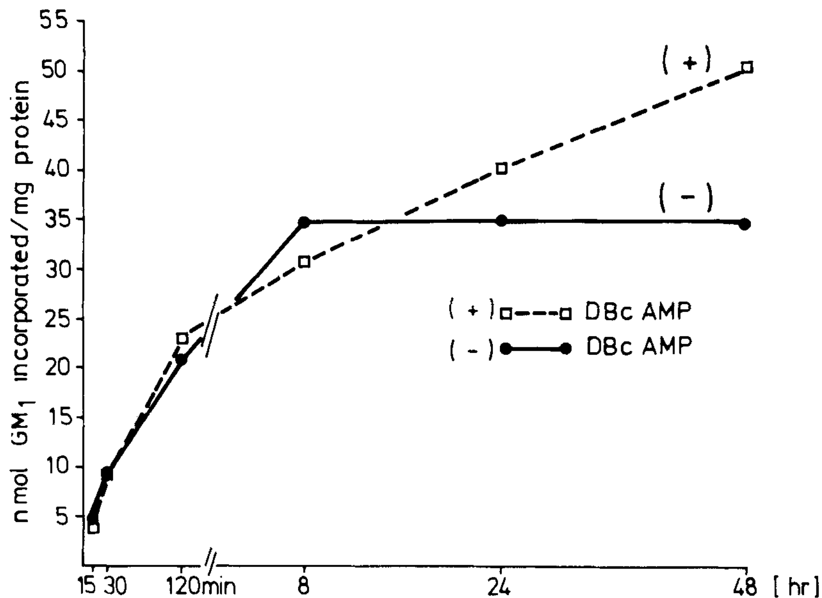

Fig. 3. Time dependence of GM1 (trypsin-stable) incorporation (nmol/mg protein) at 60 μM GM1 concentration in Dulbecco's modified Eagle's medium (DMEM) into astrocytes: (−) in absence of DBcAMP, flat astrocytes; (+) in presence of DBcAMP, stellate astrocytes.

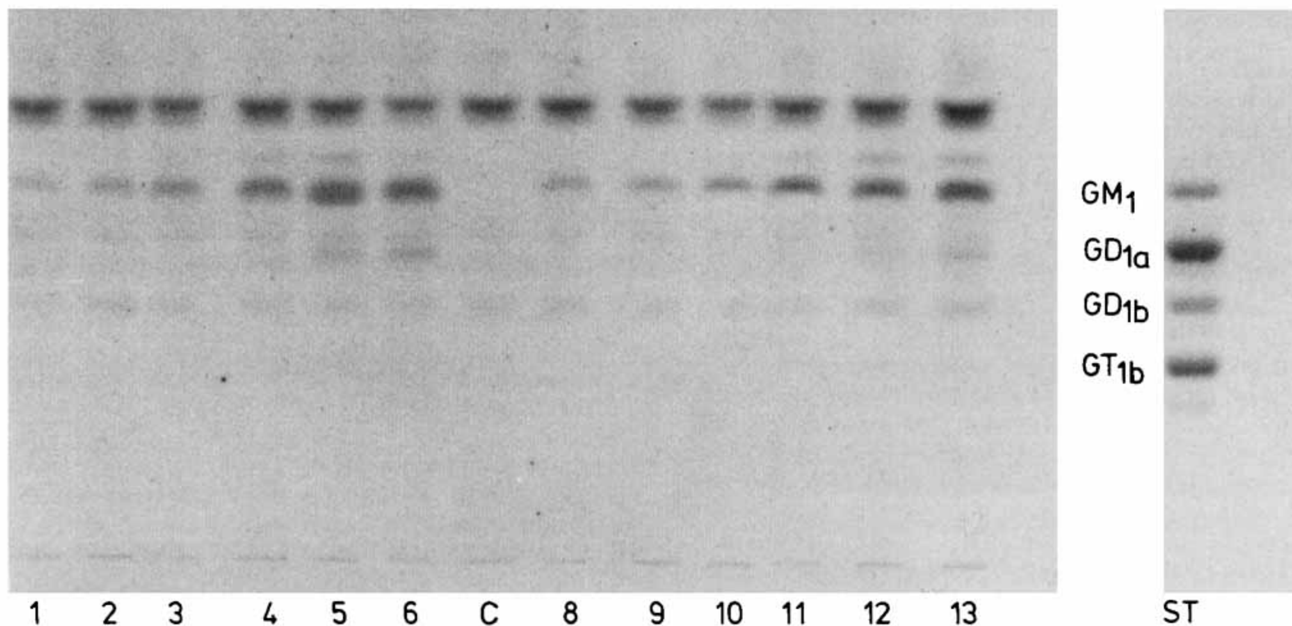

Fig. 4. High-performance thin-layer chromatography (HPTLC) of total gangliosides (trypsin-stable) from astrocytes treated with and without DBcAMP in the presence of GM1. Lanes 1–6: Cells treated with DBcAMP (stellate morphology) 15 and 30 min and 2, 8, 24, and 48 h after GM1 incubation. Lane 7 (C): astrocytes cultured without any treatment as a control. Lanes 8–13: cells without DBcAMP (flat morphology) 15 and 30 min and 2, 8, 24, and 48 h after GM1 incubation. ST: ganglioside mix, as a standard. The band of the cells that travels like GD1b did not stain with resorcinol reagent and therefore is not considered to be a ganglioside. Note that after 8 h (Lanes 4 and 11) GM2 and GD1a can be visualized.

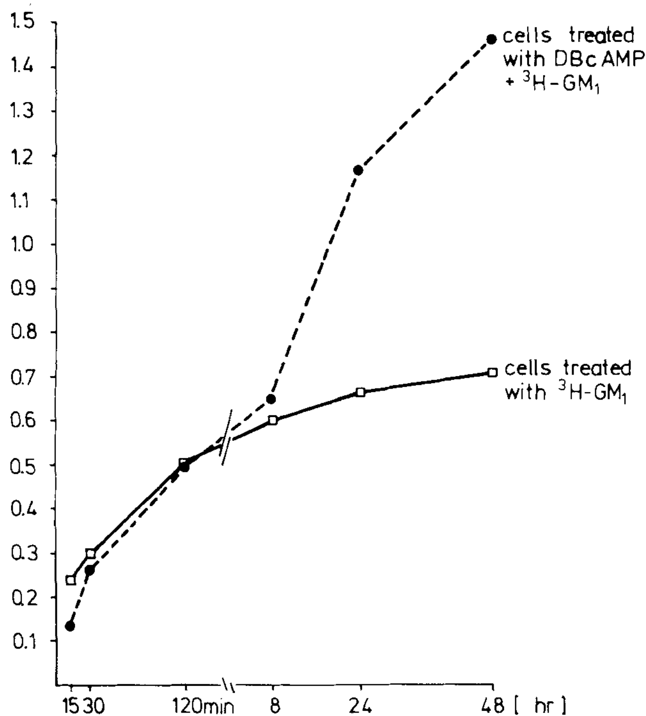

Fig. 5. Kinetics of GM1 incorporation (trypsin-stable) into flat astrocytes (□——□, without DBcAMP) and into stellate astrocytes (●– – –●, with 1 mM DBcAMP) in Dulbecco's modified Eagle's medium (DMEM) at 60 μM GM1 concentration. The ratio of the densitometric area of GM1 and GM3 was done. The data are the average of four independent determinations. SEM was less than 10%.

antiserum against GM1. Immunocytochemistry did not give positive results due to a lack of specificity, (i.e., non-specific staining of cells, whether or not they were treated with GM1). Since this monoclonal antiserum does not cross-react with other gangliosides, it may have cross-reacted with other epitopes in glycoproteins.

Therefore we decided to use the specific binding of choleratoxin (B-subunit) to GM1. Rhodamine-labelled choleratoxin B-subunit (RITC-ChTx) was added to astrocyte cell cultures (flat form). Figure 6 shows that in cultures treated with GM1 nearly all of the astrocytes were intensely stained. Astroglial cells cultured without GM1 treatment revealed the absence of RITC-ChTx binding, in contrast with the intense binding always observed in treated cells. The surface of the untreated astrocyte cells showed no binding of conjugate, even at the highest concentration tested.

The detection of trypsin-stable incorporated GM1 was not possible by staining with RITC-ChTx due to technical reasons: trypsinization and replating in serum-free medium causes only poor attachment of cells (as can be seen for autoradiography, Fig. 7C,D). These cells were loosely attached and therefore were lost during the incubation with RITC-ChTx and the following washing steps.

Finally, we used autoradiography for direct visualization of the incorporated ^{3}H-GM1. As Figure 7 illustrates, after incubation of astrocytes with ^{3}H-GM1, the cells were clearly labelled; grains can be seen all over the astrocytes surface. To reduce the background and at the same time to prove stable ganglioside incorporation, the astrocytes were trypsinized and replated. Figure 7C,D shows that the cells still contained ^{3}H-GM1 and were distinctly labelled, while the background was reduced.

DISCUSSION

The data presented in this paper clearly demonstrate that, as known from other cell systems (see references in the Introduction), living astrocytes in culture are able to incorporate GM1 ganglioside in a time- and concentration-dependent manner. Furthermore, both flat astrocytes and stellate astrocytes (DBcAMP treated) incorporate exogenously added GM1 ganglioside.

There was a greater incorporation in cells that were treated with DBcAMP, compared with untreated cells. Because the former cells undergo profound morphological changes to the stellate cell type, this might be accompanied by an expansion of cell membrane and therefore lead to greater exposure of surface. This then might lead to an increased incorporation of GM1, as observed in these experiments.

In a previous paper, Asou and Brunngraber (1984a) reported that astrocytes derived from newborn rat brain did not incorporate ganglioside GM1. The procedures, to identify GM1 incorporation, used biotinylated avidin peroxidase and anti-serum against GM1. Neither technique detected GM1 on the surface of the astrocytes. Since we have opposite results, a more likely explanation for their failure to visualize incorporated GM1 was probably due to technical considerations.

It has been reported that the stellation response to DBcAMP was blocked by the concurrent presence of GM1 and DBcAMP (Skaper et al., 1986). We did not observe this effect. The difference might be explained by the different coating of the culture dishes used in those experiments or by other variables of cell culture conditions.

The ganglioside pattern of the astrocyte cells in culture observed was in accordance with those obtained by other authors (Asou and Brunngraber, 1983, 1984b; Dreyfus et al., 1980; Robert et al., 1975). It is interesting that the ganglioside pattern differs totally from the pattern reported for astrocytes in vivo (Hamberger and Svennerholm, 1971; Ledeen, 1983). Ganglioside compo-

Fig. 6. Detection of GM1 in astrocytes membranes with rhodamine-isothio-cyanate (RITC)-labelled choleratoxin (B-subunit). **A:** Astrocytes incubated in Dulbecco's modified Eagle's medium (DMEM) (24 h) without GM1, phase contrast. **B:** The same cells in fluorescence. **C:** Astrocytes incubated in DMEM plus GM1 (60 μM), phase contrast. **D:** The same cells in fluorescence. Bar 50 μm.

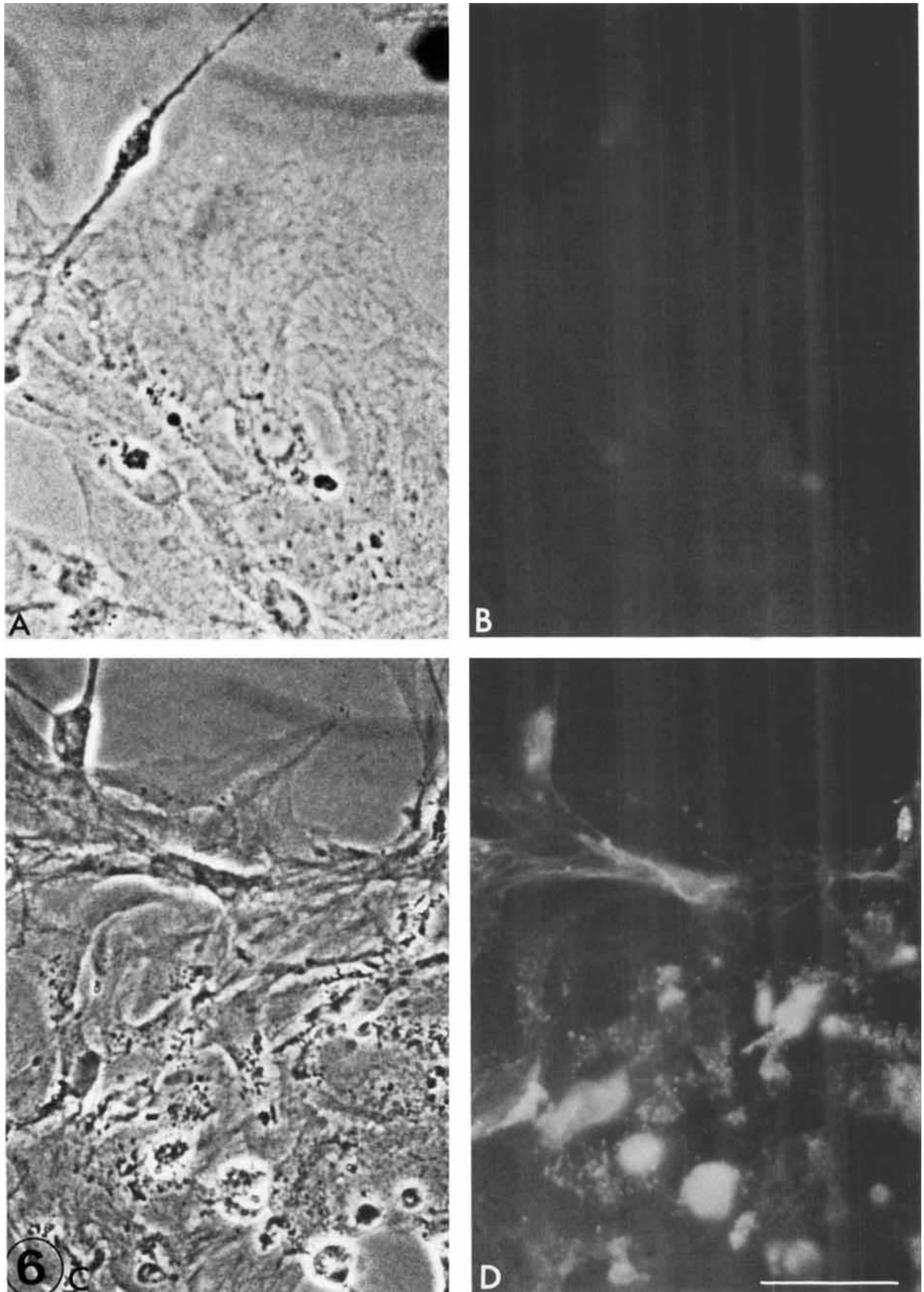

Fig. 6.

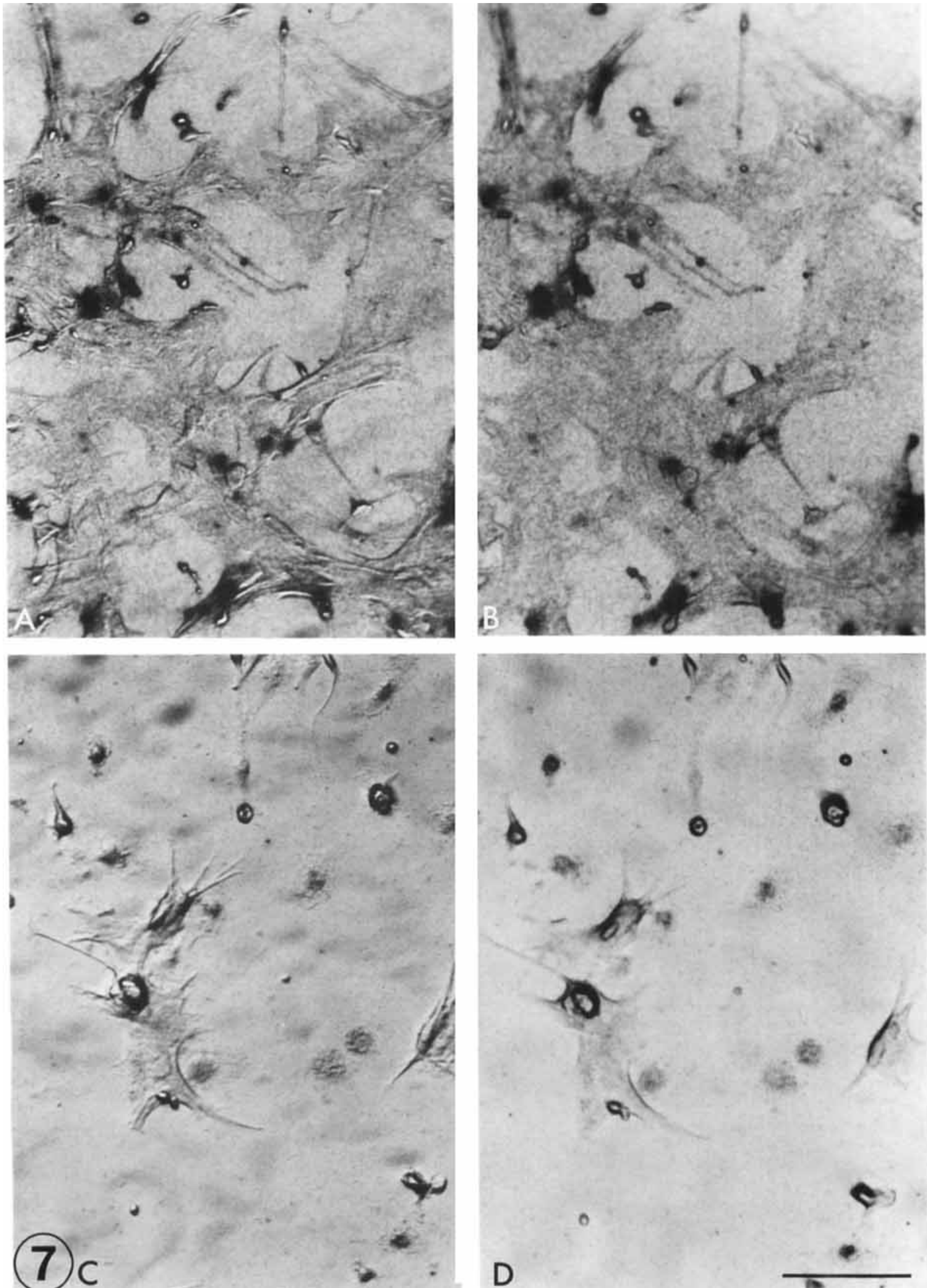

Fig. 7.

sition of astrocytes is a controversial problem. In view of the simple ganglioside pattern of astrocytes in culture, it seems that the complex pattern obtained with glial fractions might be the result of contamination by materials rich in polysialogangliosides (for example nerve endings) or that the astrocytes in culture lose the capacity to make other gangliosides.

Holmgren et al. (1973), using choleratoxin binding to the plasma membrane of astroglial cells in vivo, showed that these cells had very weak binding compared with that of the neuronal cell membrane, suggesting that only the plasma membrane of neuronal cells has a high ganglioside concentration and a complex pattern. Landa and Moscona (1985) found that Müller-cell-derived gliocytes express the same composition as retinal neurons, but they become altered after some time in culture. These results suggest that in vivo resting glial cells might exhibit a different ganglioside pattern compared with that of proliferating glia in cell culture.

The findings described here have raised interesting questions about the astrocyte cell system. The fact that GM1 was incorporated and that after time GD1a appeared as a metabolic product demonstrates that these cells can biosynthetize gangliosides with more carbohydrates; it also indicates that the enzymatic machinery to make more complex polysialo-gangliosides seems to be present in the cells. The normal astrocyte cell cultures, however, revealed that the only gangliosides expressed in their membranes are GM3 and in a minor proportion GD3. In this cell culture system, astrocytes constitute more than 95% of the cell population (unpublished observation). It would be of interest to find out if the capacity to make other gangliosides is a phenomenon that depends on the exogenous environment or trophic factors secreted by other cells.

Alternatively, it could be a process that depends on the culture medium or on conditions that inhibit the synthesis of other gangliosides. This possibility is also suggested by a recent report from Kim et al. (1986) demonstrating that GM1 ganglioside was found in most of the glial fibrillary acidic protein (GFAP)-positive astrocytes in a culture where neuronal cells and fibroblast-like astrocytes were present. The fact that GM1 can be stably incorporated into astrocytes in cell culture provides a good model system that can be used to study the various processes of cell–cell recognition and cell–cell interaction where membrane components can play a role. Certainly, this is an interesting system for studying regulation of ganglioside biosynthesis.

ACKNOWLEDGMENTS

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Fig. 7. Autoradiography of astrocytes after 24 h incubation with GM1 (60 μM in Dulbecco's modified Eagle's medium [DMEM]) plus ^{3}H-GM1 (1 μCi/ml). A: Cells visualized using interference contrast. B: Cells in bright field. C: Cells after trypsinization and replating, interference contrast. D: Astrocytes after trypsinization in bright field. All the cells were fixed in 5% glutaraldehyde, and the dishes were air dried. The film emulsion was L4 (Ilford), and time exposition was 5 weeks at −80°C. Bar 100 μm.

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