Volume 281, number 1,2, 39-42 FEBS 09545 April 1991

ADONIS 001457939100253Q

Cellular incorporation and localization of fluorescent derivatives of gangliosides, cerebroside and sphingomyelin

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Received 14 December 1990; revised version received 1 February 1991

Fluorescent dansyl derivatives of 3 natural sphingolipids (gangliosides, cerebroside and sphingomyelin) were shown to be readily taken up by culture cells (HeLa-, MDCK- and primary rat brain cells). A part of the incorporated fluorescent sphingolipids remained associated with the cells after incubation in a culture medium containing serum, showing a cellular integration of these lipids. Microscopical studies indicated a localization of incorporated lipids in distinct subcellular regions; whereas dansyl cerebroside densely stained structures suggestive of the cytoskeleton and the actin filament, dansyl sphingomyelin and dansyl gangliosides were primarily associated with the plasma membrane. The findings are consistent with the current views on the arrangement of sphingolipids in animal cells.

Dansyl sphingolipid; Fluorescent sphingolipid; Cellular incorporation; Tissue culture cell; Plasma membrane; Cytoskeleton

1. INTRODUCTION

Amphipathic lipids in animal cells are generally thought to be organized in a primarily bilayer structure, which contains regions of micellar and hexagonal structures [1]. In addition, lipids are heterogeneously distributed in various subcellular positions. Most of the current knowledge on this topic has been obtained for major membrane constituents like glycerophosphatides and cholesterol. Less studies have been carried out with sphingolipids, especially glycosphingolipids, that are usually minor components of animal cells. However, where they are located in the cell is of great interest because of their implication in many important biological roles such as in cellular recognition, differentiation and receptor interactions [2-7].

When we first prepared dansyl cerebroside, it was shown to be quickly taken up by culture cells [8]. Dansyl sphingomyelin synthesized next was also found to be readily incorporated into culture cells [9]. Both sphingolipids were demonstrated to be involved in specific interactions with some integral membrane proteins by fluorescence energy transfer studies [9]. In the meantime we have prepared dansyl gangliosides that contain the same fluorescent hydrophobic portion as other dansyl sphingolipids. By making use of their visibility in the ultraviolet light, it has been possible to comparatively study the distribution of these sphingolipids after their uptake in three types of animal cells.

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2. MATERIALS AND METHODS

2.1. *Preparation of dansyl sphingolipids*

Dansyl cerebroside and dansyl sphingomyelin were obtained from human brain cerebroside and sphingomyelin as described previously [8-10]. Dansyl gangliosides were prepared essentially as described by Neuhofer et al. through lysogangliosides [11]: from 100 mg of pure brain ganglioside mixture provided by Fidia, Italy, 69 mg of dansyl gangliosides were obtained by selective dansylation and reacetylation of lysogangliosides in a two-phase diethylether-water system.

2.2. *Tissue culture*

Continuous cell lines, HeLa- and MDCK-cells, were maintained in plastic Petri dishes in Dulbecco's medium containing 10% fetal serum. Primary rat brain cells were obtained according to the method of Giller et al. [12]. Briefly, several whole brains of embryonic rats, 20 days old, were pooled and dissociated in Dulbecco's medium by forcing through a nylon needle several times. The dissociated cells were then seeded on polylysin-coated plates and maintained in the same medium at 37°C under an atmosphere of 10% CO_2. The cells were used for experiments after 7 to 10 days, when glial cells and neuronal cells became clearly distinguishable.

2.3. *Incorporation of dansyl sphingolipids*

Dansyl cerebroside and dansyl sphingomyelin were dissolved in 95% ethanol at the concentration of 5 mg per ml. Dansyl gangliosides were also dissolved at this concentration in water. Before treating the cells with these sphingolipids, the plates were washed free of serum and then covered in 1 ml of a culture medium (Dulbecco's medium) without serum. Dansyl sphingolipids could be added in amounts of 10-50 μl without damaging the cells. Thereafter the plates were incubated for 15-60 min. In all cases the cells remained viable after these treatments, as indicated by the ability of these cells to continuously grow and divide.

2.4. *Observation of dansyl sphingolipids incorporated cells*

After incubating the cells with dansyl sphingolipids or after equilibrating the treated cells in a medium with or without serum for the indicated period of time, the cells were washed with phosphate buffered saline and observed with a Zeiss fluorescence microscope. For revealing the actin filaments, cells were kept briefly (10 min) in a serum-free culture medium containing 10 μg/ml of trypsin and in-

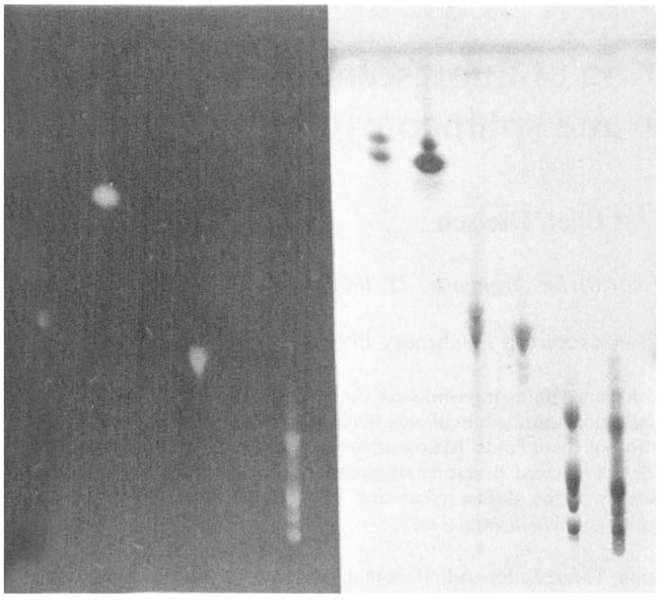

Fig. 1. Thin layer chromatogramme of dansyl sphingolipids and their parent compounds. Developing solvents: chloroform/methanol/0.25% KCl solution, 65:35:8. Left: fluorescent lipids visualized on thin layer plate under UV light. Right: the same plate stained by spraying with 5% sulfuric acid and heating at 150°C. (1) Dansyl cerebroside; (2) dansyl sphingomyelin; (3) dansyl gangliosides; (4) cerebroside (upper and lower spots represent components with fatty acids differing in chain length; (5) dansyl cerebroside; (6) sphingomyelin (upper and lower spots represent components with different chain length); (7) dansyl sphingomyelin; (8) gangliosides; (9) dansyl gangliosides.

cubated at 37°C until the cells rounded up partially without being detached from the plate.

2.5. *Thin layer chromatography of dansyl sphingolipids*

Dansyl sphingolipids were chromatographed on silica gel plates using the solvent system chloroform/methanol/0.25% KCl solution (65:35:8) and viewed under ultraviolet light or stained by spraying the plates with a 5% solution of sulfuric acid and heating at 150°C as described previously [13].

3. RESULTS AND DISCUSSION

Fig. 1 shows a comparison between the starting sphingolipids and their fluorescent derivates. This figure also indicates that all ganglioside species in the starting material were dansylated. On thin layer chromatographically, dansyl sphingolipids obtained behaved similarly to their parent compounds, showing that polarity was not influenced much by dansyl derivatization. Three types of culture cells (HeLa-, MDCK- and primary rat brain cells) were seen to become very rapidly labelled when incubated in a medium containing these lipids. With all cells used, the ease of uptake was increasing in the order of dansyl gangliosides, dansyl cerebroside and dansyl sphingomyelin (shown for MDCK-cells in Table I). Different proportions of incorporated fluorescence remained associated with the cells when incubated in a medium containing 10% serum: with dansyl cerebroside, less fluorescence remained associated with the cells than with dansyl sphingomyelin and dansyl gangliosides. Table I shows these results for MDCK-cells. With HeLa- and primary brain cells, the results were very similar.

Because of the structural similarity, dansyl sphingolipids may be expected to behave similarly to their natural counterparts when taken up in cells. Microscopical examination showed that dansyl sphingolipids were differently incorporated into distinct subcellular regions of the cells. Typically, dansyl cerebroside labelled the intracellular part of cells (Fig. 2 A,B). Fluorescence pattern of these cells at a high magnification was suggestive of the cytoskeleton structure (Fig. 2A) described by Osborn and Weber [14] and by Franke et al. [15]. After treatment of these cells with trypsin, they exhibited long fibers reminiscent of the ac-

Table I

Cell-associated fluorescence* after incubation of dansyl sphingolipids treated MDCK-cells in a medium without or with 10% serum.

	Dansyl-gangliosides	Dansyl-cerebroside	Dansyl-sphingomyelin
After labelling[1]	15	46	86
in medium without serum[2]	16	48	86
in medium with serum (once)[3]	7	12	22
in medium with serum (twice)[3]	6	6	16
in medium with serum (thrice)[3]	6	2	15

*Relative intensity of fluorescence (with 145 as maximum). [1]To label cells, 20 μl of an ethanolic (or aqueous) solution of fluorescent lipids (containing 100 μg of these lipids) was added to the culture medium (devoid of serum) that covered the cells. The plates were then incubated for 1 h at 37°C before washing. [2]Labelled cells were further incubated without sphingolipids in Dulbecco's medium without serum for 30 min at 37°C. [3]Labelled cells were further incubated without sphingolipids in Dulbecco's medium with 10% serum, once, twice and thrice successively, each time for 30 min at 37°C.

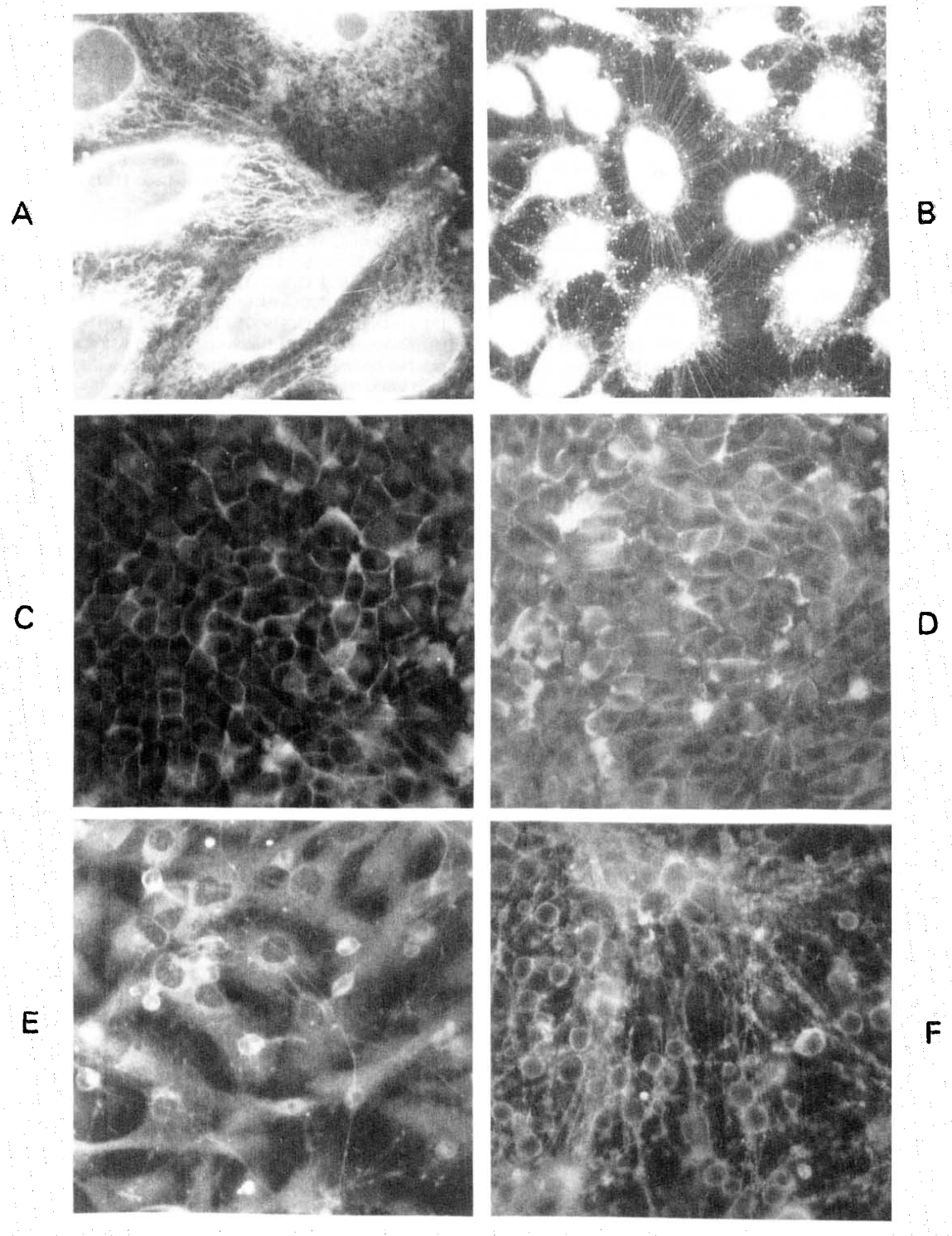

Fig. 2. Labelling of HeLa-, MDCK- and primary rat brain cells with dansyl cerebroside, dansyl sphingomyelin and dansyl gangliosides. A. HeLa-cells labelled with dansyl cerebroside, × 2400. B. HeLa-cells labelled with dansyl cerebroside after mild treatment of cells with trypsin, × 2400. C. MDCK-cells labelled with dansyl sphingomyelin, × 300. D. MDCK-cells labelled with dansyl ganglioside, × 300. E. Rat brain cells labelled with dansyl cerebroside, × 300. F. Rat brain cells labelled with dansyl gangliosides, × 300.

tin filament (Fig. 2B) described by Pollack et al. [16]. These results are consistent with the findings of Sakakibara et al., who showed that cytoskeleton structures of culture cells were specifically labelled by fluorescein-tagged anti-cerebroside antibodies [17,18]. In contrast to dansyl cerebroside, dansyl sphingomyelin

and dansyl gangliosides labelled the boundary of these cells indicative of the plasma membrane (Fig. 2 C,D). This agrees with the general assumption, that sphingomyelin and gangliosides are components of the plasma membrane. The results were comparable with rat brain cells: the glial cells were stained internally by dansyl cerebroside (Fig. 2E), whereas the plasma membrane and the neuronal processes were labelled by dansyl gangliosides and dansyl sphingomyelin (Fig. 2F).

In this study, 3 naturally occurring sphingolipids were derivatized into dansylated compounds with the aim to comparatively study their intracellular distribution after incorporation into culture cells. It is of particular interest, that these lipids were differently incorporated into subcellular regions coinciding with the locations of natural sphingolipids according to current views. Further studies are underway to explore other uses of these dansyl sphingolipids, such as for fluorescence energy transfer measurements in subcellular structures [9]. Studies of glycolipid metabolism like the one carried out by Tettamanti [19] may also be complemented by the use of these highly detectable fluorescent derivatives.

Acknowledgements: We are thankfull to DFG Hu 317/1-2 and Fonds der Chemischen Industrie for financial support.

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