Volume 314, number 3, 471–476 FEBS 11917 December 1992

Uptake and metabolism of fluorescent ceramide analogs by rat oligodendrocytes in culture

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Received 2 September 1992; revised version received 1 November 1992

We studied the metabolism of sphingolipids by oligodendrocytes derived from rat spinal cord by providing lipid vesicles with either *N*-lissamine-rhodaminyl-ceramide (LRh-Cer) or *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-ceramide (NBD-Cer) to the cells cultured in a chemically-defined medium. With both probes the major fluorescent product turned out to be sphingomyelin (SM). Most of LRh-SM was not cell-associated but recovered from the culture medium, probably due to back-exchange to the lipid vesicles. The accumulation of LRh-SM, both in the cells and in the medium, was inhibited in the presence of monensin or brefeldin A, whereas the production of NBD-SM was much less affected by these Golgi perturbing drugs. With LRh-Cer as substrate, LRh-labelled fatty acid (FA), galactosyl- and sulfogalactosyl-ceramides (GalCer and SGalCer) were also formed. NBD-Cer, however, was metabolized to glucosylceramide (GlcCer) and GalCer but not to SGalCer or NBD-FA. These data demonstrate that chemical modifications of ceramide alter its metabolism in oligodendrocytes and that the metabolites of LRh-Cer reflect the glycolipid composition of myelin more closely than those of NBD-Cer.

Ceramide metabolism; Sphingomyelin; Glycolipids of myelin; *N*-lissamine rhodamine; NBD; Rat oligodendrocyte

1. INTRODUCTION

Oligodendrocytes, a type of macroglia, are the cells that synthesize, maintain [1,2] and after trauma regenerate [3] sheaths of myelin membrane around axons in the central nervous system. It has been estimated that during the period of active myelination – in the rat during its third postnatal week – an oligodendrocyte may synthesize and assemble as much as three times its own weight in myelin per day [4].

Compared to plasma membranes of other cell types, mature myelin has a high content of sphingolipids: sphingomyelin (SM, 4 mol% of total lipids), galactosylceramide (GalCer, 16 mol%) and sulfogalactosyl-ceramide (SGalCer, 4 mol%) [4], but only trace amounts of glucosylceramide (GlcCer; [5]). Primary cultures derived from neonatal rat spinal cord offer an interesting model system to investigate the myelin-associated ceramide metabolism and intracellular routing of sphingolipids, because they contain at least 90% oligodendrocytes [6].

Fluorescent ceramide analogs have proven to be powerful tools to follow the synthesis of sphingolipids, subsequent sorting and their transport to the plasma membrane [7–10]. We decided to use ceramide tagged either with *N*-lissamine rhodamine (LRh) or *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) (NBD) as fluorophore. NBD-Cer has been used extensively to characterize sphingolipid trafficking in epithelial cell lines in which SM and GlcCer are the main reaction products [11,12]. The LRh-label has certain experimental advantages over the NBD-probe as discussed previously [13].

To the best of our knowledge this is the first time that fluorescent analogs of ceramide have been applied to study sphingolipid metabolism in oligodendrocytes. Our results with NBD-Cer largely comply with those obtained in other cell systems. LRh-Cer, however, generates a different profile of sphingolipids in oligodendrocytes, resembling more closely that of myelin. In addition, the effects of the Na^+-ionophore, monensin [14], and of brefeldin A, a fungal metabolite disrupting the organization of the Golgi system [15], were evaluated. Collectively, our findings suggest that LRh-Cer

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Abbreviations: BSA, Bovine serum albumin; Cer, Ceramides; FA, Fatty acid; FITC, Fluorescein isothiocyanate; GalCer, Galactosylceramides (galactocerebrosides); GlcCer, Glucosylceramides (glucocerebrosides); HBSS, Hanks balanced salt solution; HPTLC, High-performance thin-layer chromatography; LRh, *N*-lissamine rhodamine; NBD, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-; SGalCer, Sulfogalactosylceramide (sulphatide); SM, Sphingomyelin.

may be the probe of choice to study the topology and routing of sphingolipids in oligodendrocytes.

2. EXPERIMENTAL

2.1. *Animals and chemicals*

Female Wistar rats 16–18 days pregnant were obtained from IFFA Credo (l'Arbresle, France). The chemically-defined culture medium for growth and development of oligodendrocytes has been described [16]. Tissue culture dishes were from Nunc (Roskilde, Denmark). Poly-L-lysine, monensin, brefeldin A and fluorescein isothiocyanate (FITC) dextran were purchased from Sigma (St. Louis, USA) and C_6-NBD-ceramide from Molecular Probes (Eugene, USA). NBD-labelled lipid reference compounds for high-performance thin-layer chromatography (HPTLC) were kindly provided by Dr. G. Van Meer (Medical School, Utrecht University, The Netherlands). C_{12}-LRh-lipids were synthesized as described [17]. HPTLC silicagel-60 plates were from Merck (Darmstadt, Germany).

2.2. *Cell culture*

Oligodendrocyte-enriched cultures derived from spinal cords of 1-week-old rat pups were prepared according to [6]. Glial cells were plated on plastic culture dishes (diameter, 6 cm) in Dulbecco's minimal essential medium (DMEM) with 5% (v/v) newborn-calf serum. After one day the cultures were shifted to chemically-defined medium. After two days in culture, cytosine-1-β-D-arabinoside (10^{-5} M), an inhibitor of mitosis, was added to the cultures to inhibit the proliferation of contaminating astrocytes. Experiments were carried out after four or five days in culture.

2.3. *Preparation of lipid vesicles*

Solutions of egg phosphatidylcholine and fluorescent ceramide (LRh-Cer or NBD-Cer) were mixed in a molar ratio of 85:15, respectively. Organic solvents were evaporated under nitrogen and the lipids were suspended in Hanks Balanced Salt Solution (HBSS), containing 10 mM HEPES and 5 mM EDTA (pH 7.4; 185 μg lipid/ml) by sonication (8 times 99 s, 70 μm amplitude, with equal cooling periods in between; MSE, Soniprep-150, sonifying equipment).

2.4. *Incubation of cells in culture with fluorescent ceramides*

The suspension of lipid vesicles was diluted with chemically-defined medium (devoid of BSA) to the appropriate concentration of labelled ceramide and added to the cells (final volume, 4 ml). Inhibitors: monensin (0.1 μM), brefeldin A (1 μg/ml) or sodium azide/2-deoxy-D-glucose (5 mM/50 mM), were added 30 min before the fluorescent ceramides.

2.5. *Analysis of the fluorescent lipids*

The medium was removed from the culture dish and the cells were washed twice with 0.5 ml HBSS; the wash fluids being added to the medium. Cells were harvested from the culture dish with a rubber policeman and suspended in a small volume (≈ 1 ml HBSS/dish). Lipids were extracted from the cell suspension and from the medium with 2.2 vol. of methanol and 1 vol. chloroform (30 min, 4°C). At this stage, LRh-lipid and NBD-lipid samples were treated differently, as described below.

2.5.1. LRh-lipids

Subsequently, 1 vol. of chloroform and 1 vol. of 0.88% (w/v) KCl were added. The organic phase was dried under nitrogen and the lipids were dissolved in chloroform/methanol, 6:4 (v/v). Aliquots were taken to assay the total cell-associated fluorescence (Fig. 1). HPTLC (Fig. 2) was performed as described [13]. LRh-fluorescent spots were detected under UV-light, scraped off and extracted (twice with 2 ml of chloroform/methanol, 6:4 (v/v)). In case of LRh-SM the silica was extracted thrice with 2 ml of methanol and once with 2 ml chloroform/methanol, 6:4 (v/v). Quantitative extraction was achieved by sonication in a Branson-2200 ultrasonic bath (5 min at room temperature).

2.5.2. NBD-lipids

Control experiments showed that acidification was necessary to extract NBD-fatty acid quantitatively into the lower, organic phase. Therefore, 1 vol. of chloroform and 1 vol. of 0.88% (w/v) KCl/10 mM HCl were added. Aliquots were taken to assay the total cell-associated fluorescence (Fig. 1). HPTLC (Fig. 2) was performed with chloroform/methanol/25% (v/v) NH_4OH/H_2O 70:30:4:1 (by vol.) as the solvent system. NBD-fluorescent spots were detected under UV-light, scraped off and extracted with 2 ml of chloroform/methanol/H_2O (1:2.2:1, by vol.) [18]. In accordance with previous reports (cf. [11]) we also found that the total NBD-fluorescence decreased markedly during the incubation of the cultures with NBD-ceramide (approx. 60% was recovered after 24 h). The levels of the various NBD-metabolites were corrected for this.

2.6. *Fluorimetry*

LRh-lipids (in chloroform/methanol, 6:4 (v/v)) were excited at 560 nm and their fluorescence was measured at 575 nm. NBD-lipids were assayed in the same solvent system (460–520 nm) or in chloroform/methanol/H_2O 1:2.2:1 (by vol.) at a wavelength pair of 465–530 nm. The concentration of LRh-ceramide in chloroform/methanol (6:4, v/v) was determined spectrophotometrically at 566 nm (molar extinction coefficient, $95 \cdot 10^3 \cdot l \cdot mol^{-1} \cdot cm^{-1}$ [17]). The fluorimetry was carried out with a Perkin Elmer Luminescence Spectrometer LS-50.

3. RESULTS AND DISCUSSION

The aim of this study was to investigate the metabolism and intracellular routing of sphingolipids in cultured oligodendrocytes using fluorescent ceramide analogs. We compared LRh and NBD as fluorescent labels. LRh-sulphatide was used recently in metabolic studies with fibroblasts [19] and with oligodendrocytes [13], while NBD has been used extensively to study the metabolism, transport and sorting of (glyco)sphingolipids in a variety of cell types [18,20–22] . Specifically, NBD-Cer has been shown to label the Golgi compartment of the cell [23]. Monensin [14] and brefeldin A [15] have been used as tools to obtain insight into intracellular traffic events of lipids.

Long-term incubations of oligodendrocyte-enriched cultures [6] were performed with vesicles containing phosphatidylcholine and either LRh-Cer or NBD-Cer. The fluorescent metabolites present, both in the cells and in the medium, were analysed. Fig. 1A shows for both probes that an initially linear relationship between added and cell-associated fluorescence is followed by saturation kinetics at higher concentrations. Relatively more LRh-Cer than NBD-Cer associates with or is taken up by the cells: after 24 h the level of cell-associated fluorescence reached with LRh-Cer was about twice that with NBD-Cer as substrate (Fig. 1B). The mechanism of incorporation was further investigated by incubating the cells under energy-depleted (5 mM azide/50 mM deoxyglucose [24]) or low-temperature (4°C; 60–90 min) conditions that block endocytosis. The internalization of the fluid-phase marker FITC-dextran [25] in oligodendrocytes in culture was blocked effectively under these conditions . On the other hand, in the presence of either NBD-Cer or LRh-Cer, cell-associated fluorescence did not decrease nor did the intracellular

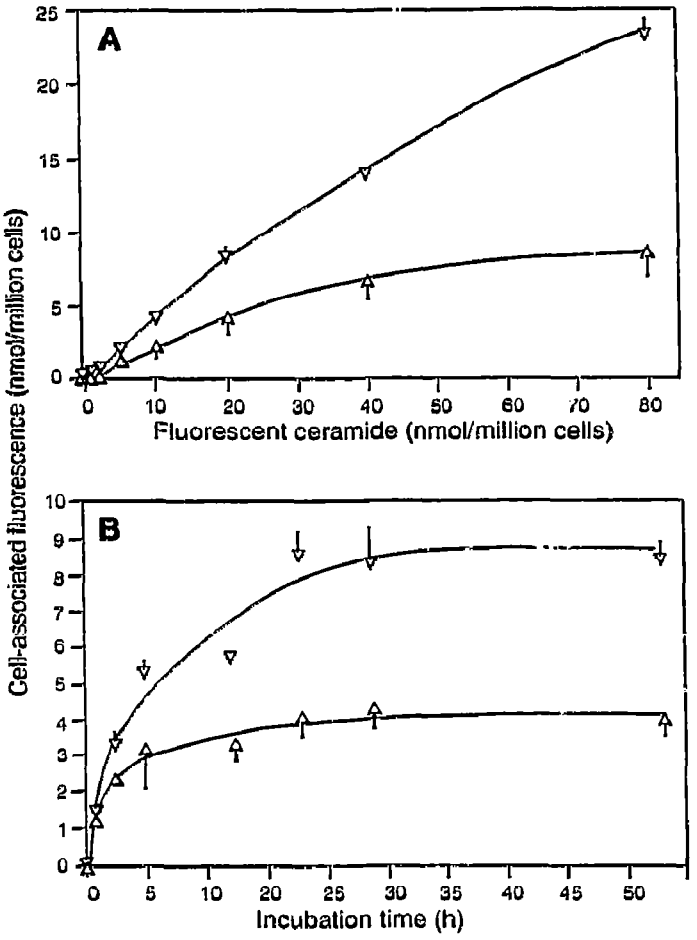

Fig. 1. Association of fluorescent analogs of ceramide with oligodendrocytes in primary culture. (A) Cells were incubated (24 h, 37°C) with varying amounts of lipid vesicles containing 15 mol% LRh- (▽) or NBD- (△) labelled ceramide. (B) Cells were incubated (various times, 37°C) with a constant amount of lipid vesicles (equivalent to 20 nmol of LRh- (▽) or NBD- (△) ceramide per million cells). This experiment was repeated three times with similar results.

labeling pattern change as inspected by confocal scanning laser microscopy using a described procedure [13]. These findings suggest that both LRh- and NBD-Cer (as was described in another cell type for the latter [23]), do not enter the cell via endocytosis, which is an energy- and temperature-dependent process, but more likely associate with the plasma membrane and diffuse from there into the cell.

Fig. 3 shows the accumulation of SM which appeared to be the major metabolite formed (cf. Fig. 2): 63% (with LRh-Cer) and 87% (with NBD-Cer) of the total metabolites after 24 h of incubation. The rate of SM accumulation was much higher with NBD-Cer than with LRh-Cer as substrate (approx. 40× at 5 h and 15× at 24 h). This difference may reflect the affinity of SM synthase for the two fluorescent ceramide analogs and/or their intracellular distribution. The latter option is supported by the observation that NBD-Cer labels preferentially the Golgi compartment [23], the main site of SM synthesis [26–29]. We observed a more diffuse distribution of LRh-Cer throughout the cell and concentration of the probe along cytoskeletal elements (cf. [13,21]).

Most of the fluorescent SM was recovered from the culture medium. This might be caused by back-exchange of SM from the plasma membrane to lipid vesicles present in the medium (see [30]). Therefore, we investigated whether this process was also operative under our experimental conditions (Table 1). Cultures were incubated for 24 h with LRh-Cer-containing vesicles (condition A), during which period LRh-SM distributed almost equally between cells and medium (44 and 56%). After further incubation of the cells in fresh medium without LRh-containing vesicles (condition B), only a small part (12%) was recovered from the medium. On the other hand, when fresh medium plus natural-ceramide containing vesicles was added (condition C), much more (35%) SM was found in the medium. These results suggest that the ceramide-containing lipid vesicles supplied to the medium can pull fluorescent SM out of the cells.

A high rate of galactolipid synthesis (GalCer and SGalCer) is a characteristic feature of oligodendrocytes [1], whereas virtually no GlcCer is found in myelin membranes [5]. In our experiments, glycolipids (GalCer, SGalCer and GlcCer) represented only a modest fraction of the total fluorescent metabolites of LRh- and NBD-Cer (Fig. 4). The overall rate of glycolipid production was 50% more with NBD-Cer than with LRh-Cer and – as was observed with SM (Fig. 3) – glycolipids were also found in the culture medium. Surprisingly, with NBD-Cer as substrate, both NBD-GlcCer and NBD-GalCer were formed, whereas NBD-SGalCer was not produced. In contrast, LRh-Cer was not converted into LRh-GlcCer, but rather into LRh-GalCer and LRh-SGalCer.

The effects of the vesicular membrane flow inhibitors monensin [14] and brefeldin A [15] on the production of SM were evaluated. Review of the literature [14,15] indicates that these drugs can elicit multiple cellular events and that their overall effect depends on the cell type studied. Oligodendrocytes did accumulate less SM in the presence of monensin or brefeldin A (Fig. 3). This effect was much more pronounced with LRh-Cer than with NBD-Cer as substrate. With the latter analog a clear inhibition by monensin was observed only after a lag-time of about 5 h whereas brefeldin A did not affect the cell-associated SM. Parenthetically, we may note that treatment with monensin or brefeldin A did not affect the total cell-associated fluorescence nor the viability of the cells in culture. With regard to the formation of glycosphingolipids, we found that brefeldin A induced an increase of NBD-GlcCer from 1.1 nmol/million cells to 3.3 nmol/million cells, whereas monensin induced an increase to 1.6 nmol/million cells. The parallel, monensin-induced decrease of NBD-SM (Fig. 3)

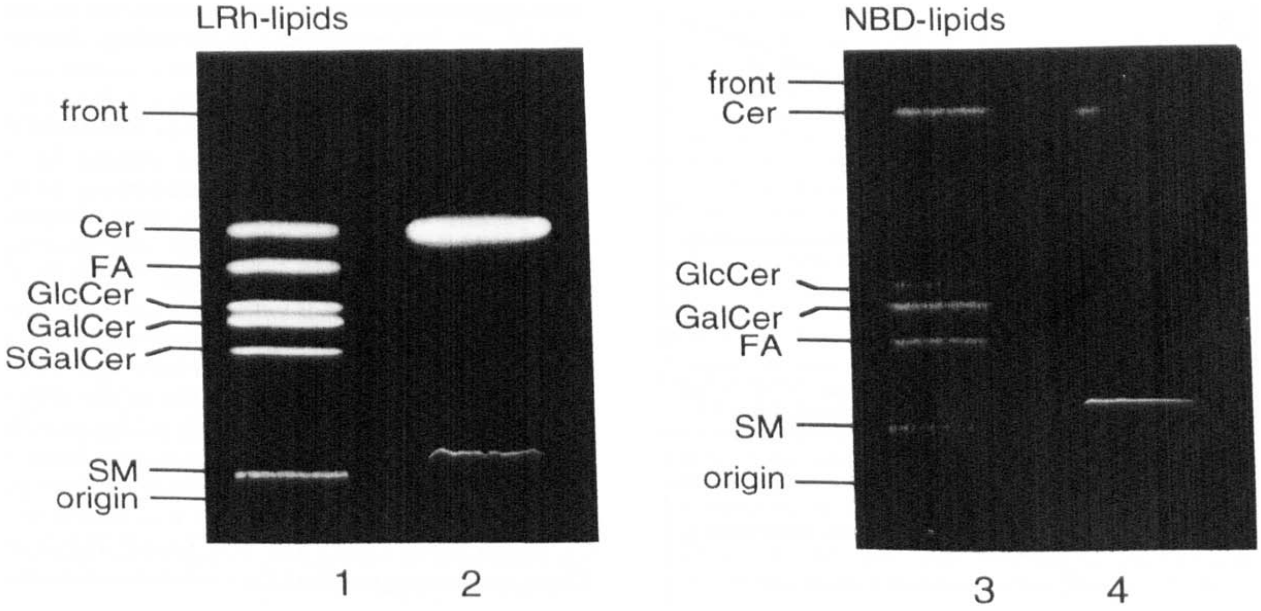

Fig. 2. HPTLC chromatogram of LRh- and NBD-labelled metabolites. Oligodendrocytes were incubated with LRh- or NBD-Cer containing vesicles for 18 h at 37°C. Fluorescent cell-associated lipids were extracted and chromatographed on HPTLC-plates with chloroform/ethyl acetate/*n*-propanol/methanol/0.25% (w/v) KCl, 25:25:25:16:9 (by vol.) for LRh-lipids and chloroform/methanol/25% (v/v) NH_4OH /H_2O, 70:30:4:1 (by vol.) for NBD-lipids, as solvent system. The chromatogram was photographed under UV light. Lane 1: LRh-lipid standards; lane 2: cell-associated LRh-lipids; lane 3: NBD-lipid standards; lane 4: cell-associated NBD-lipids. The respective Rf values (average of six HPTLC chromatograms) are: LRh-Cer 0.74, LRh-FA 0.65, LRh-GlcCer 0.46, LRh-GalCer 0.44, LRh-SGalCer 0.32 and LRh-SM 0.10 for LRh-lipids, and NBD-Cer 0.91, NBD-GlcCer 0.48, NBD-GalCer 0.43, NBD-FA 0.34 and NBD-SM 0.13 for NBD-lipids. It is noteworthy that in the presence of cellular lipids, both NBD-SM and LRh-SM show a higher relative mobility compared to their respective standards. After extraction of the cellular fluorescent-SM from the silica and subjecting it again to HPTLC, the Rf values of the fluorescent-SM equalled that of their standards. The identity of NBD-glycosphingolipids was checked on sodiumborate (2.5%, w/v)-impregnated HPTLC-plates (Rf of NBD-GalCer 0.11 and NBD-GlcCer 0.37).

and increase of NBD-GlcCer may indicate a shift from sphingophospho- towards sphingoglycolipid synthesis. A similar effect of monensin was reported in a radioactive tracer study of ceramide in human fibroblasts [31]. Contrarily to this, however, we found that the synthesis of glycolipids from LRh-Cer was not altered by either monensin or by brefeldin A. So, although these drug-induced effects cannot be fully explained, they do support the notion – suggested previously for other cell types (see [26,32–34] for a more extensive discussion) – that SM synthase does not or not completely co-localize with the enzymes catalysing the synthesis of GlcCer, GalCer and SGalCer.

With LRh-Cer as substrate LRh-FA was found to be a cell-associated reaction product (Fig. 4). LRh-FA accumulated also in the culture medium but its quantification after HPTLC was not feasible because LRh-Cer was present in a very large excess in the medium (cf. Fig. 2). In contrast, with NBD-Cer as substrate, NBD-FA was detected neither in the cells nor in the medium. Only in the presence of monensin, NBD-FA accumulated in the medium (about 1 nmol/million cells in 24 h) and lower amounts were recovered after treatment with brefeldin A.

In conclusion, these results demonstrate that the uptake and further metabolism of the two fluorescent ceramide analogs (C_{12}-LRh and C_6-NBD) by rat spinal cord oligodendrocytes in culture differ not only quantitatively, but also qualitatively: (i) less NBD-Cer than

Table I

Back-exchange of LRh-SM from the cells to the lipid vesicles in the medium

Incubation conditions	Accumulation of LRh-sphingomyelin			
	Cell-associated		Medium-associated	
	%	(pmol/million cells)	%	(pmol/million cells)
(A) 24 h in medium + LRh-Cer-vesicles	44 ± 5	(87 ± 4)	56 ± 5	(111 ± 5)
(B) as (A) and 24 h in fresh medium	88 ± 2	(90 ± 2)	12 ± 2	(13 ± 0.2)
(C) as (A) and 24 h in fresh medium + Cer-vesicles	65 ± 6	(64 ± 4)	35 ± 6	(35 ± 2)

Oligodendrocyte-enriched cultures were incubated (24 h, 37°C) with LRh-Cer-containing lipid vesicles (condition (A)). Subsequently, the cultures were washed twice with HBSS and incubated (24 h, 37°C) in fresh medium without (B) or in the presence of normal ceramide-containing vesicles (C). Values are means ± S.D. (n = 3).

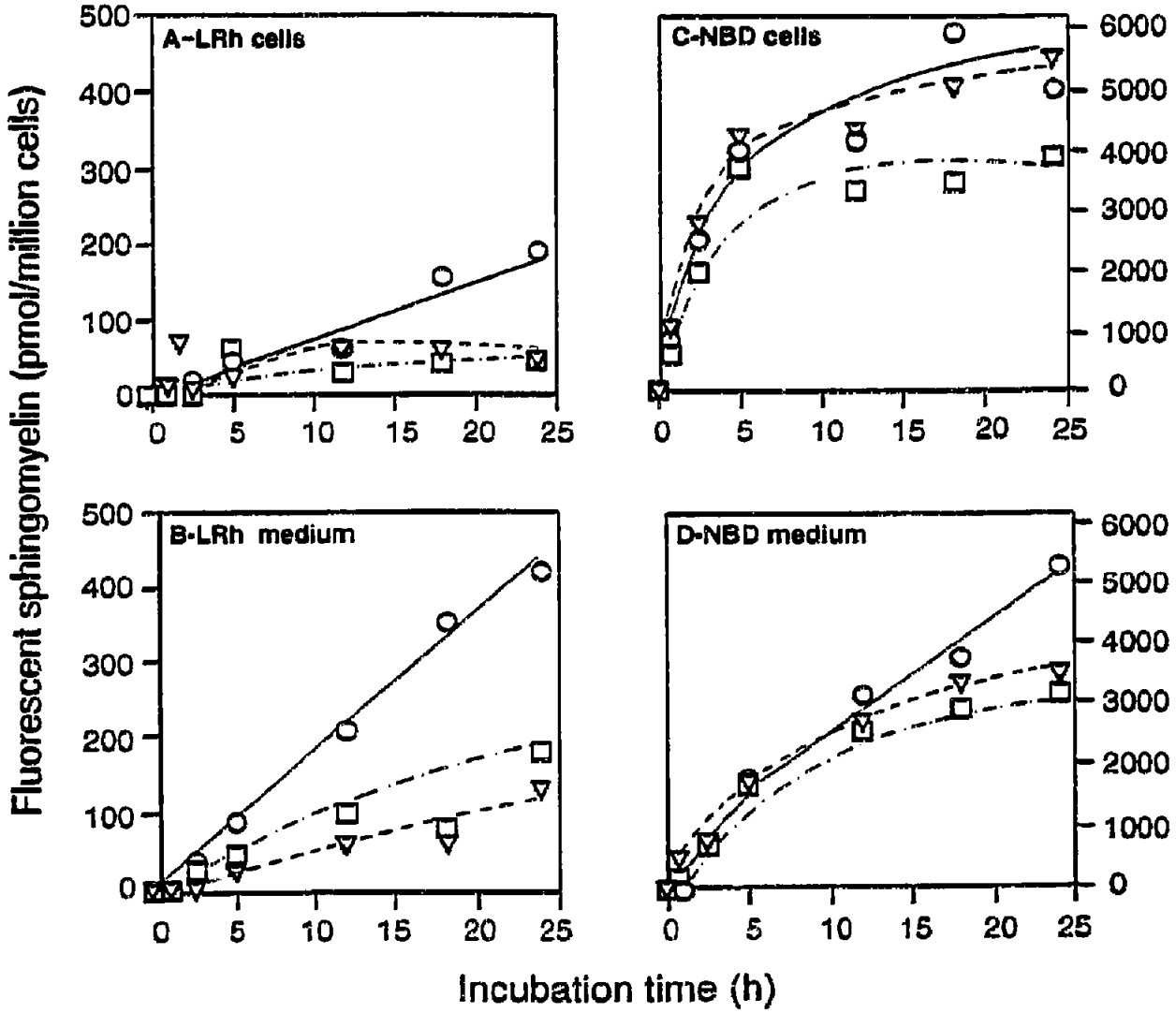

Fig. 3. The production of labelled SM with LRh- or NBD-analogs of ceramide as substrate. Cultures were incubated with lipid vesicles (see legend to Fig. 1B) in the absence (○—○) or presence of inhibitors: monensin (□-·-□) or brefeldin A (▽---▽). The NBD- and LRh-lipids present in the cells and in the medium were extracted and the amount of fluorescent SM was estimated. One typical experiment out of three is shown.

LRh-Cer is taken up (Fig. 1) but much more NBD-Cer is metabolized, especially into NBD-SM (Fig. 3); (ii) LRh-Cer is converted into galactolipids (GalCer, SGal-Cer) and not into glucolipid (GlcCer), whereas with NBD-Cer as substrate, NBD-GlcCer and NBD-GalCer but no NBD-SGalCer are formed (Fig. 4); (iii) accumulation of fluorescent FA is observed with LRh-Cer, but not with NBD-Cer; and (iv) monensin and brefeldin A appear to affect differentially the conversion of the two substrates into SM (Fig. 3) and into other metabolites. Hence, we postulate that the physico-chemical properties of the probes (polarity and molecular mass) are responsible for the observed differences, although we cannot exclude the possibility that the length of the acyl-chain linking the fluorophore to the sphingosine-base (C_6 in case of NBD an C_{12} for LRh-) influences the metabolic properties of the ceramide analogs to some extent.

LRh-Cer rather than NBD-Cer is our probe of choice to study metabolism and intracellular routing of sphingolipids in oligodendrocytes, because: (i) With LRh-Cer the fluorophore is quantitatively recovered, even after 24–48 h incubations, whereas up to 40% of NBD-fluorescence is lost under such conditions; (ii) the composition of myelin lipids is reflected more closely by the products of LRh-Cer metabolism than is the case with NBD-Cer.

Acknowledgements: This investigation was supported by 'Vrienden MS Research' and the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for Scientific Research (NWO). The authors would like to thank A.

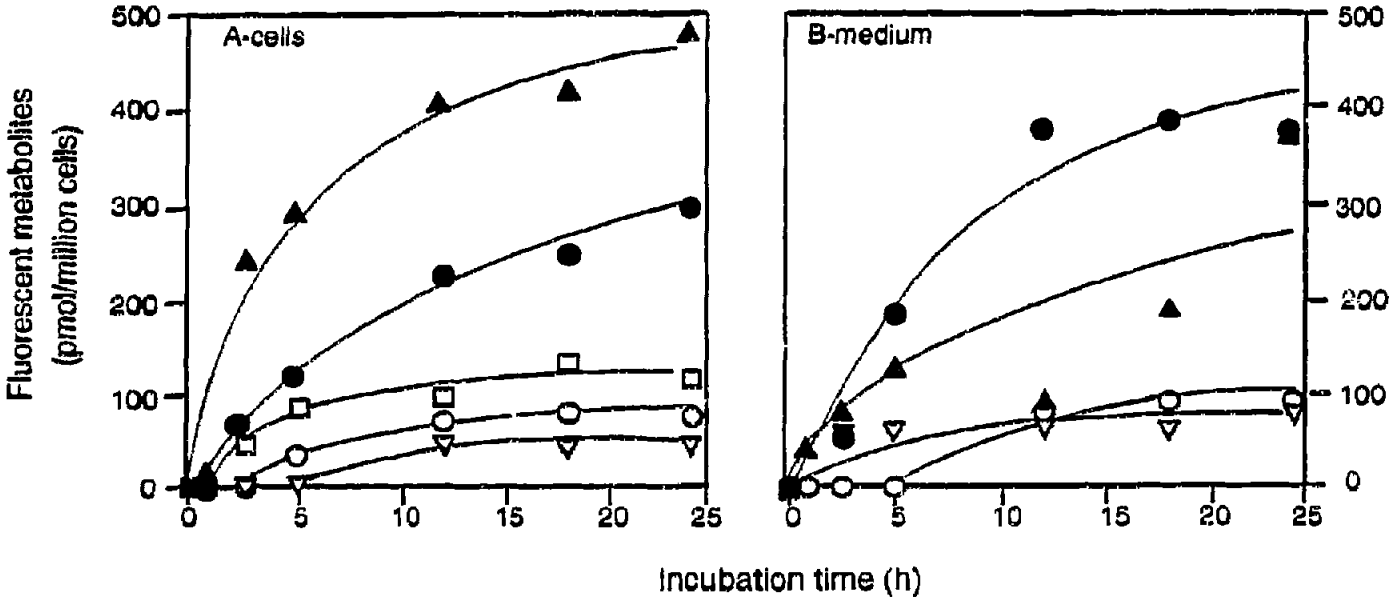

Fig. 4. The accumulation of fluorescent glycosphingolipids and fatty acid. Glial cell cultures were incubated with lipid vesicles (see legend to Fig. 1B) containing LRh-Cer (open symbols) or NBD-Cer (closed symbols). The corresponding fluorescent lipid metabolites in the cells (panel A) and the medium (panel B) were analysed: NBD-GlcCer (▲); NBD-GalCer (●); LRh-GalCer (○); LRh-SGalCer (▽) and LRh-FA (□). The levels of NBD-SGalCer, NBD-FA and LRh-GlcCer were below the level of detection (≤10 pmol/million cells). Data of a representative experiment are shown.

van der Molen for his diligent assistance in the analytical work and W. Hage (Hubrecht Laboratory of Developmental Biology, Royal Netherlands Academy of Arts and Sciences, Utrecht) for his expert technical assistance with confocal scanning laser microscopy.

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