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Association of Gangliosides to Fibroblasts in Culture: A Study Performed with G_{M1} [^{14}C]-labelled at the Sialic Acid Acetyl Group

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The preparation of a G_{M1}-ganglioside (GM1) [^{14}C]-labelled in the sialic acid residue is reported. This can be obtained by re-*N*-acetylation in the presence of [1-^{14}C]-acetic anhydride, of a GM1 derivative de-*N*-acetylated specifically on the sialic acid residue by alkaline hydrolysis of GM1 with tetramethylammonium hydroxide. The radiolabelled GM1 is utilized to investigate the binding properties and the mode of interaction of GM1 with cultured fibroblasts. Three different forms of association (one "serum-removable", one "trypsin-removable" and one "trypsin-stable") have been recognized to occur in a way that depended on cell culture conditions (presence or absence of fetal calf serum), ganglioside concentration (from 5×10^{-9} M to 10^{-4} M) and incubation time (up to 24 h). Some metabolic modifications of GM1 during the period of high cell viability were also investigated.

Gangliosides are assumed to play a role in receptor phenomena and biotransduction of membrane mediated information [1]. An approach recently introduced to study the functional implications of gangliosides consists of the addition of exogenous ganglioside to various *in vivo* and *in vitro* systems followed by determination of the biological effects exerted [2, 3]. These effects are assumed to derive from insertion of ganglioside into the cell plasma membrane with resulting modifications of the membrane properties and behaviour [4, 5]. However, experiments both *in vivo* [6] and *in vitro* [7] showed

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Abbreviations: GM1, G_{M1}-ganglioside, II^{3}NeuAc-GgOse$_4$Cer; FCS, fetal calf serum; EMEM, Eagle's Minimum Essential Medium with Earle's salts; PBS, Dulbecco phosphate buffered saline without calcium and magnesium.

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that exogenously administered gangliosides penetrate into cells. Therefore it cannot be excluded that some of the above effects are due to interactions of penetrated gangliosides, or of their metabolic products, with intracellular structures and processes.

In order to investigate the molecular aspects of these phenomena, gangliosides are required that carry high specific radioactivity. It is also important that these compounds be isotopically labelled in order to behave identically to the natural gangliosides. Methods have been developed for tritium labelling of the gangliosides most usually examined (GM1 and G_{M2}-, G_{D1a}- and Fucosyl-G_{M1}-gangliosides) at the C-3 of the long chain base [8, 9], or at the C-6 of the terminal galactose or *N*-acetylgalactosamine of GM1, G_{M2}- and G_{D1}-ganglioside) [9-12]. However, the availability of gangliosides isotopically [^{14}C]-labelled and highly radioactive would be extremely convenient. Particularly, studies that implicate a metabolic processing of exogenous gangliosides would be greatly facilitated by the use of gangliosides carrying the [^{14}C]-label in the sialic acid moiety. In fact this form of labelled ganglioside (for instance GM1) is obviously superior to that radioactively-labelled in the terminal galactose when studying the initial steps of the degradative process. In addition, in contrast to gangliosides labelled in the ceramide portion, it is possible to follow the catabolism of gangliosides relatively easily, from the passage of ganglioside to neutral glycosphingolipid. Furthermore, the use of gangliosides labelled in the sialic acid residue, in combination with the same ganglioside labelled in the ceramide moiety, should enable differentiation of the metabolites produced exclusively by the catabolic process from those metabolites which arise from subsequent anabolic re-utilization of the degradation products (lactosylceramide, glucosylceramide, ceramide, long chain base).

Higashi and Basu [13] recently reported a method for the [^{14}C]-labelling of glycosphingolipids in the acetyl group of hexosamines, which, when applied to gangliosides, introduced the radioactivity in the acetyl group of both sialic acid and *N*-acetylhexosamine. We now describe the preparation of ganglioside GM1 [^{14}C]-labelled with a high specific radioactivity in the sialic acid acetyl group. This compound was used to compare the binding of GM1 to human fibroblasts, grown in monolayer culture, in the presence or absence of fetal calf serum. With these studies, the experimental conditions for investigating the relationships between binding and metabolic events encountered by exogenous gangliosides in fibroblasts were assessed.

Materials

Tetramethylammonium hydroxide pentahydrate was purchased from Aldrich (Brussels, Belgium); [1-^{14}C]-acetic anhydride (111 mCi/mmol), from Amersham International (UK); *N*-acetylneuraminic acid (NeuAc), bovine pancreas trypsin (type III:2) and bovine serum albumin from Sigma Chemical Co. (St. Louis, MO, USA); 0.05% trypsin-0.02% EDTA in special salt solution, Eagle's Minimal Essential Medium with Earle's salts (EMEM), Hank's solution, Dulbecco phosphate buffered saline with no calcium and magnesium (PBS), from Flow Laboratories (Irvine, UK); fetal calf serum (FCS) was from NABI (Miami, FL., USA).

Methods

Preparation of Gangliosides

Gangliosides G_{M1}, G_{M2}, G_{M3} (abbreviation according to Svennerholm [14]) were prepared and structurally characterized as previously described [15]. Their purity was greater than 99%. The lipid moiety of GM1 had the following composition (mole %):- long chain bases: *erythro* C18 sphinganine, 2.5%; *erythro* C18 sphingosine, 50.0%; *erythro* C20 sphinganine, 1.8%; *erythro* C20 sphingosine, 45.7%: fatty acids; stearic acid, 92%; oleic acid, 0.5%; arachidic acid, 4.5%; palmitic acid, 3%.

G_{M1}-, G_{M2}- and G_{M3}-gangliosides used as standards for autoradiography were tritium labelled at C-3 of the long chain bases by the method developed by Ghidoni *et al.* [8] and extended to different gangliosides by Gazzotti *et al.* [9].

Preparation of [^{14}C]-Labelled GM1, Galβ1-3GalNAcβ1-4(Neu [^{14}C]-Acα2-3) Galβ1-4Glcβ 1-1'Cer

GM1 was first *N*-deacetylated at the sialic acid moiety, and then re-*N*-acetylated with [1-^{14}C]-acetic anhydride. The derivative of GM1, de-*N*-acetylated at the sialic acid residue, [Galβ1-3GalNAcβ1-4(Neuα2-3)Galβ1-4Glcβ1-1'Cer, compound 1] was obtained by alkaline hydrolysis of GM1 (20 mg) in the presence of tetramethylammonium hydroxide, according to the method of Sonnino *et al.* [16] as follows. GM1 was dissolved (10 mg/ml) at 100°C in butan-1-ol/10 M aqueous tetramethylammonium hydroxide, 9/1 by vol; the reaction mixture was refluxed at 100°C for 13 h with stirring, evaporated, dialyzed and lyophilized. Compound 1 was purified by chromatography on a silica gel 100 column (1 × 60 cm), previously equilibrated and eluted with chloroform/methanol/water, 60/35/5 by vol. The homogeneity of compound 1, after column chromatography, was over 99%, as as determined by high performance TLC (HPTLC). Seven mg of compound 1 were dissolved in 1 ml of 5% triethylamine in magnesium-dehydrated methanol and 11.1 μl of 5% [1-^{14}C]-acetic anhydride (111 mCi/mmol) in toluene were added. After stirring for 10 min at room temperature, 10 μl of cold acetic anhydride were added, and the mixture stirred for an additional 30 min. The solution was then diluted with 100 ml of distilled water and dialyzed for two days at 4°C against 5 l of distilled water (two changes daily). The dialyzed solution was frozen, lyophilized, and the residue, dissolved in 1 ml of propan-1-ol/water, 7/3 by vol, was stored at 4°C. The optimal conditions for re-*N*-acetylation were established by the use of unlabelled acetic anhydride.

Chemical Analyses of Gangliosides and Ganglioside Derivatives

The carbohydrate, fatty acid and long chain base composition of natural GM1, of the products of its alkaline hydrolysis, and of reconstituted GM1, was determined by GLC and GLC-MS analyses as previously reported [15, 16]. 300 MHz proton NMR spectroscopy of the different compounds was performed on a Bruker CPX 300 spectrometer equipped with an Aspect 2000 computer, operating in the Fourier-transform mode. Each sample (4 mg) was completely solubilized in Me_2SO-$^2H_6$ and placed in a 200 × 5 mm NMR tube. The signals were recorded at 21 ± 2°C. Spectra were obtained with 3000

Hz spectral width (0-10 ppm), 3 s cycle time and 400-600 scans. Signals were assigned putting the central signal of dimethylsulphoxide at 2.52 ppm.

Radio-GLC Analysis of the Sialic Acid Residue of Labelled GM1

A sample of [^{14}C]-labelled GM1 (about 10^5 dpm) mixed with a known amount of the corresponding unlabelled compound (1 mg) was hydrolyzed in 0.5 ml of 0.05 N methanolic HCl at 80°C for 1 h [17]. After extraction of fatty acid methyl esters with *n*-hexane (3 × 1.5 ml), the methanolic phase was dried under a gentle stream of nitrogen and the residue was submitted to trimethylsilylation (15 min, 60°C) by reaction with 20 μl of trimethylsilylimidazole. Radio-GLC analyses of the trimethylsilyl derivative of sialic acid was performed at 240°C using a model 9GV (C. Erba, Italy) chromatograph, equipped with a glass column (200 × 0.3 cm) packed with 3% SE-30 on Chromosorb W, and connected with a Nuclear Chicago flow counter, model 4998.

Radiochemical Purity and Specific Radioactivity of Labelled Ganglioside

The homogeneity of labelled ganglioside was determined by HPTLC using chloroform/methanol/0.2% aqueous $CaCl_2$, 50/42/11 by vol, as solvent system. A sample (10^5 dpm) was mixed with a known amount of unlabelled GM1, used as carrier. After TLC, the plate was dried and analyzed for distribution of radioactivity as specified below. The specific radioactivity was determined assaying radioactivity in a liquid scintillation counter (Packard TriCarb 2425) using 5 ml of emulsifier (Instagel®, Packard) and by measuring sialic acid by the colorimetric procedure of Warren [18].

Verification of the Distribution of Radioactivity in the Ganglioside Molecules

The original [^{14}C]-GM1 and the radiolabelled gangliosides extracted from cultured fibroblasts and from the solutions used for different treatments (10% FCS-EMEM, PBS solution containing 0.1% trypsin) were submitted to mild acid hydrolysis, according to the method of Sonnino *et al.* [16]. In order to obtain the different neutral glycosphingolipids, these were separated [16] and counted for associated radioactivity (see below).

Cell Culture Conditions

Skin biopsies were obtained by the punch technique from normal young indiviudals and fibroblast cultures were initiated and maintained as described by Leroy *et al.* [19] using 75 cm^2 Corning plastic flasks. Subcultures were made on 28 cm^2 culture dishes using 5 ml of EMEM, containing 10% fetal calf serum (10% FCS-EMEM). Fibroblast cultures were used at confluence (130-150 μg cell protein/dish).

Treatment of Human Fibroblasts in Monolayer Culture with GM1

A sample of [^{14}C]-GM1 was pipetted into a sterile tube and dried in a stream of nitrogen. The residue was resuspended in few μl of chloroform/methanol, 2/1 by vol, dried, and dissolved in an appropriate volume of EMEM with or without 10% FCS, to obtain the desired ganglioside concentration (5×10^{-6} M, 10^{-7} M and 5×10^{-9} M). The preparation of the 10^{-4} M GM1 solution was carried out by previous dilution (1:10) of labelled with unla-

belled GM1. A separate solution was prepared for each of the different ganglioside concentrations employed. All the solutions were maintained at 37°C and used within 1 h.

Two ml of the ganglioside-containing medium were added to each culture dish, from which the original medium had previously been carefully removed. The mode of association of GM1 to cultured fibroblasts was studied as already reported [2, 3], with the following sequence of treatments: 1) [^{14}C]-GM1-containing medium was removed after the incubation, and the cells washed with 3 ml of Hank's solution (3 times), in order to eliminate unbound GM1; 2) after discarding the Hank's solution, cells were maintained at 37°C for 30 min in 2 ml of 10% FCS-EMEM (removal of the amount of GM1 weakly attached to fibroblasts - "serum removable" associated radioactivity); 3) after removal of previous medium, cells were treated with 2 ml of PBS solution containing 0.1% trypsin (removal of the amount of GM1 interacting with membrane proteins - "trypsin-removable" associated radioactivity); 4) after removal of the trypsin solution, cells were processed as described below ("trypsin stable" associated radioactivity). In some experiments, the medium, which contained labelled GM1, was removed after the incubation and replaced with medium containing unlabelled GM1.

Determination of Cell-associated Radioactivity

Cell-associated radioactivity was measured on detached cells after centrifugation (1 000 × *g*, 10 min) and solubilization of the pellet by overnight treatment with 1M NaOH (3 ml/mg cell protein) as alreadyreported [3]. Samples were counted in a liquid scintillation counter [3].

Analysis of Associated Radioactivity

The chemical nature of removable and of cell-associated radioactivity was established as follows. The solutions were dialyzed against distilled water to remove low molecular weight molecules and lyophilized. The residues and the cells were submitted to ganglioside extraction according to the method of Tettamanti *et al.* [20]. The extracts were separated by TLC [16] and the distribution of radiolabelled gangliosides was determined by radiochromatoscanning [6].

Colorimetric Methods

Gangliosides were assayed as bound sialic acid by the resorcinol method [21, 22], pure NeuAc being used as the reference standard. Protein was determined in solubilized cell pellets, according to Peterson's modification [23] of the method of Lowry *et al.* [24], BSA being used as the reference standard.

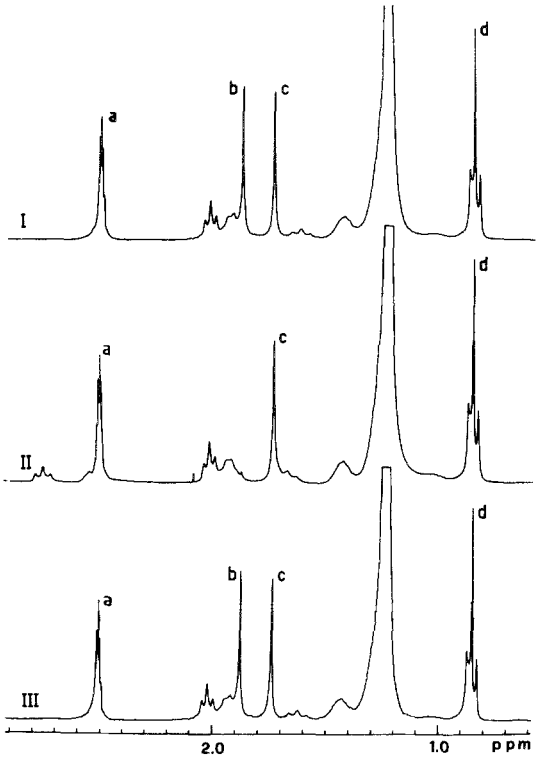

Figure 1. 300 MHz proton NMR spectra of the starting material, natural GM1 (I); GM1 after de-*N*-acetylation (compound 1) (II); reconstituted GM1 (compound 1 after re-*N*-acetylation) (III). Peak a, dimethylsulphoxide; peak b, acetyl protons of *N*-acetylneuraminic acid; peak c, acetyl protons of *N*-acetylgalactosamine; peak d, methyl protons of the alkyl region.

Results and Discussion

Preparation and Characterization of [^{14}C]-GM1

Alkaline treatment of natural GM1 by tetramethylammonium hydroxide provides two main products; a derivative of GM1 de-*N*-acetylated in the sialic acid (compound 1) and a derivative of GM1 de-*N*-acetylated in the sialic acid and also de-*N*-acylated in the long chain base moiety [16]. Compound 1 was purified by silica gel column chromatography, to 99% homogeneity, in a final yield of 42%.

The NMR spectrum of purified compound 1 was compared to that of natural GM1 (Fig. 1) and clearly indicated the absence of the sialic acid acetyl group. In natural GM1 the acetyl protons of *N*-acetylneuraminic acid and *N*-acetylgalactosamine were found at 1.89 and 1.75 ppm, respectively, and the methyl protons of the alkyl regions at 0.87 ppm [16, 25]. The NMR spectrum of compound 1 completely lacked the peak at 1.89 ppm.

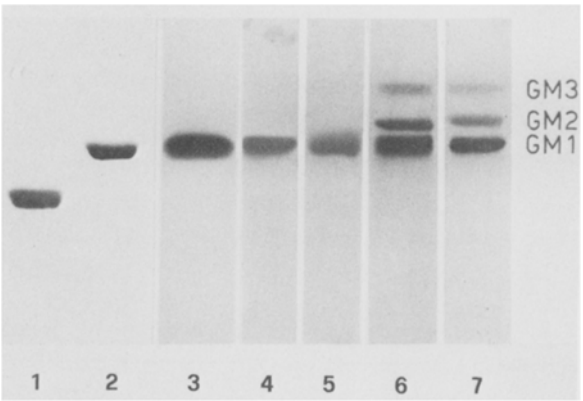

Figure 2. TLC of [^{14}C]-GM1 and of its metabolic derivatives, after incubation with cultured fibroblasts. Lane 1; purified compound 1: lane 2; compound 1 after re-*N*-acetylation: lane 3; compound 1 after re-*N*-[^{14}C]-acetylation: lane 4; "serum-removable" form: lane 5; "trypsin-removable" form: lane 6; "trypsin-stable" form, incubation performed in the presence of FCS: lane 7; "trypsin-stable" form, incubation performed in the absence of FCS. Lanes 1 and 2; colorimetric visualization with Erlich reagent: lanes 3-7; autoradiographic visualization. Added GM1 concentration, 5×10^{-6} M; incubation time, 24 h. Solvent system; chloroform/methanol/0.2% aqueous $CaCl_2$, 50/42/11 by vol.

Peak intensity measurements showed a ratio of about two between the methyl protons of the alkyl region and the acetyl protons of *N*-acetygalactosamine in both natural GM1 and in compound 1. Compositional analyses indicated that the long chain base and fatty acid content of compound 1 were exactly the same as those of starting GM1. Re-*N*-acetylation, in the presence of unlabelled or [^{14}C]-acetic anhydride, yielded an unlabelled and a [^{14}C]-labelled compound, respectively (Fig. 2, lanes 2 and 3), displaying the same chromatographic behaviour as the GM1 standard. The NMR spectrum of the unlabelled re-*N*-acetylated compound (Fig. 1) was indistinguishable from that provided by natural GM1.

GLC-MS analyses of the trimethylsilyl derivatives of the sialic acid residues released by mild acid methanolysis from compound 1, before and after re-*N*-acetylation are shown in Figs. 3 and 4. The mass spectra of the two sialic acid residues, in good agreement with those already reported [26, 27], clearly show the $[M\text{-}15]^+$ fragments at 568 m/e for the neu-

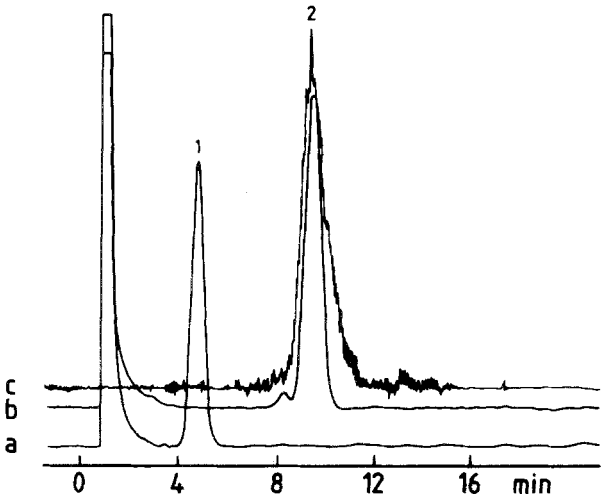

Figure 3. GLC (a,b) and radio-GLC (c) of trimethylsilyl derivatives of sialic acid residues released by mild acid methanolysis from compound 1 (a), compound 1 after re-*N*-acetylation (b) and compound 1 after re-*N*-[^{14}C]-acetylation (c). Peak 1; 4,7,8,9-tetra-*O*-trimethylsilyl-neuraminic acid methyl ester methyl glycoside: peak 2; 4,7,8,9-tetra-*O*-trimethylsilyl-*N*-acetylneuraminic acid methyl ester methyl glycoside.

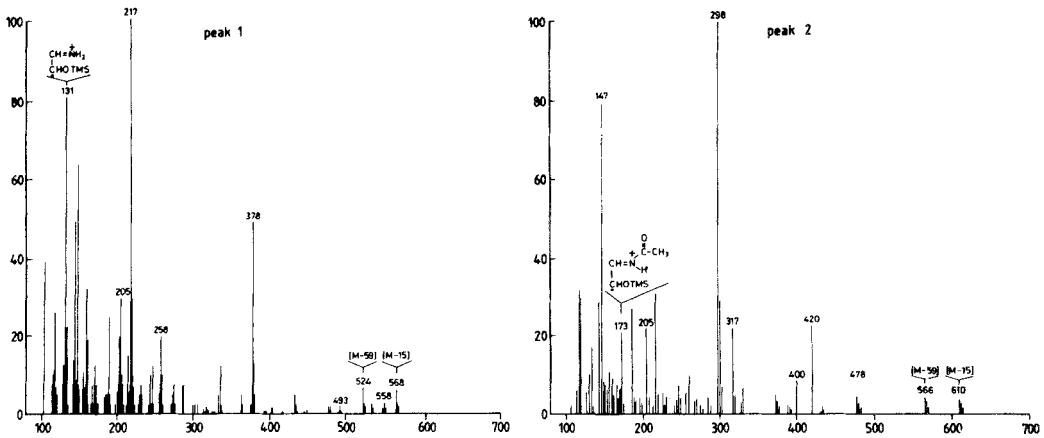

Figure 4. Mass spectra of peak 1 and peak 2 (see Fig. 3). TMS, trimethylsilyl.

raminic acid derivative and at 610 m/e for the *N*-acetylneuraminic acid derivative. Furthermore, the presence of a fragment at 131 m/e in peak 1, suggested to be $[H_2N{=}CH\text{-}CHO.TMS]^+$, and the fragment at 173 m/e in peak 2 strongly indicated the structural assignment of peak 1 to the neuraminic acid derivative and of peak 2 to the *N*-acetylneuraminic acid derivative. In the radio-GLC analysis of re-*N*-[^{14}C]-acetylated compound 1 the only radioactive peak observed corresponded to the *N*-acetylneuraminic acid derivative.

Partial acid hydrolysis of [^{14}C]-GM1, followed by dialysis, gave rise to a series of unlabelled neutral glycosphingolipids, the radioactivity present in the original compound 1 being completely recovered in the dialysis water. These data are consistent with the conversion of compound 1 into GM1 by acetylation. As assessed by TLC and radiochromatoscanning, the [^{14}C]-GM1 was 99% pure, with a specific radioactivity of 53 mCi/mmol. Since the [1-^{14}C]acetic anhydride used had a specific radioactivity of 111 mCi/mmol and recalling that only half of the starting radioactivity can be incorporated into the GM1 molecule, the radioactivity yield (95.5%) was very close to theoretical.

After purification, 6.4 mg of radiolabelled GM1 were obtained from 7 mg of compound 1. Therefore the yield of [^{14}C]-GM1 was greater than 90%.

Association of GM1 to Fibroblast Cells in Monolayer Culture

Binding of GM1 to fibroblasts depended on the cell culture conditions (presence or absence of FCS), ganglioside concentration (from 5×10^{-9} M to 10^{-4}) and incubation time (up to 24 h). Under these conditions, cell viability was 95%, as determined by the exclusion test with trypan blue.

Experiments in the absence of FCS. The amount of exogenous GM1 that remained associated to cells after 4 h incubation was 30 nmol, 1.3 nmol, 23 pmol and 2 pmol/mg cell protein after incubation with GM1 concentrations of 10^{-4}, 5×10^{-6}, 10^{-7} and 5×10^{-9} M, respectively. Since 1 mg protein corresponded to about 6×10^6 cells,the average number of GM1 molecules that were associated to a single cell was 2.8×10^9, 1.2×10^8, 7.2×10^5 and 6.2×10^4, at each of the four concentrations investigated, respectively. Repeated washings with Hank's solution or with cold GM1 had no effect on the release of associated ganglioside. Associated radioactivity could be fractionated, by means of cell washings with 10% FCS-EMEM or with PBS solution containing 0.1% trypsin (see Methods) into three different portions, that were defined as "serum-removable", "trypsin-removable" and "trypsin-stable" forms of associated radioactivity. The "serum-removable" form of association predominated at high GM1 concentrations and short incubation times, while a decrease in the GM1 concentration and prolonged times of incubation resulted in an enhancement of the "trypsin-stable" form of associated radioactivity. The maximum (80% of the total bound radioactivity) was reached at a concentration of 5×10^{-9} M GM1, after 4 h incubation (Fig. 5).

GM1 is an amphiphilic molecule able to form micelles in aqueous media; its critical micellar concentration (CMC) has been recently determined to be as low as 2×10^{-8} M [28], with the consequence that at the lowest concentration investigated (5×10^{-9} M) the monomeric state is the main form in which GM1 is present in the incubation medium, with the micellar state becoming more predominant at higher concentrations of added

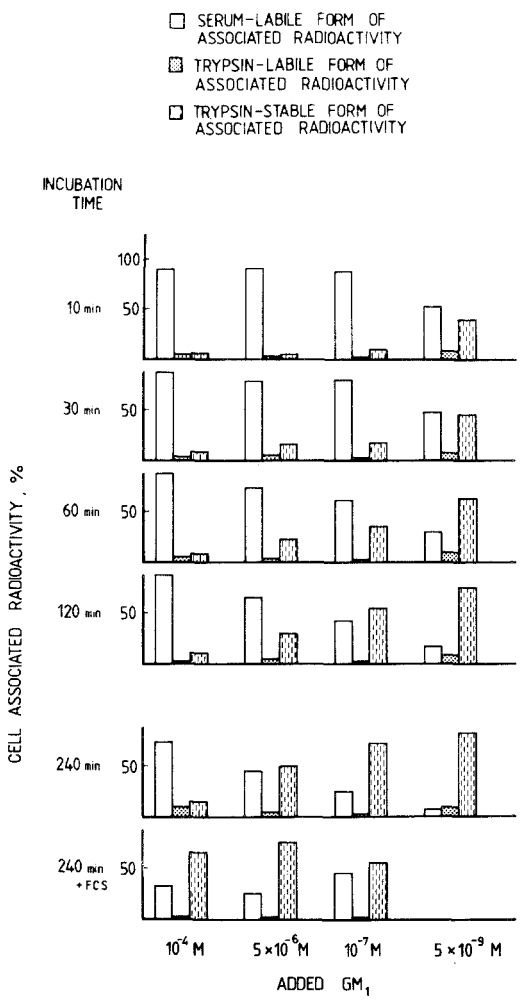

Figure 5. Distribution of the different forms of associated radioactivity as a function of added GM1 concentration, incubation time and presence of FCS.

GM1. This could explain the dependence of the relative percentage of each form of association on GM1 concentration.

The "serum-removable" radioactivity can be assumed to correspond to micelles that are superficially associated to the cell surface and can be easily removed by exchange with some components present in the fetal calf serum. It is known that bovine serum albumin can form very stable complexes with GM1 micelles [29] and that FCS can bind GM1, forming complexes with the albumin fraction [30].

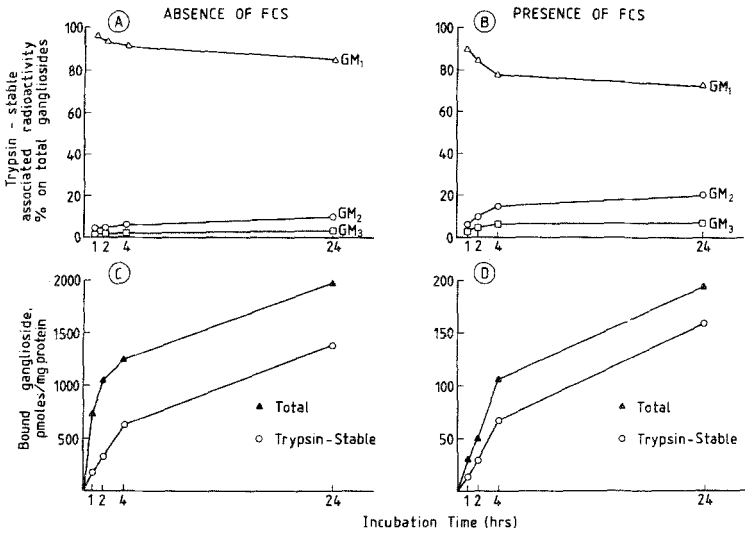

Figure 6. Time course of ganglioside association to cultured fibroblasts. A, B; distribution of gangliosides in the "trypsin-stable" form: C, D; difference between total and "trypsin-stable" bound gangliosides. Added GM1 concentration, 5×10^{-6} M.

The "trypsin-removable" form probably corresponds to simple monomers or micelles of GM1 interacting with some proteins protruding from the external layer of the cell membrane. Its relative percentage was rather low under all the conditions investigated. This can be explained by assuming that this form represents a dynamic state, intermediate between the binding of ganglioside micelles to the cell surface and the insertion of ganglioside into the membrane lipid layer.

The "trypsin-stable" form of associated GM1 probably represents GM1 molecules inserted into the membrane layer *via* a monomer-mediated mechanism. To support this hypothesis it has recently been shown [4] that paramagnetic gangliosides can be inserted into the external membrane layer of fibroblast cells. The dependence on incubation time can be explained assuming a progressive, time-dependent micelle-monomer transition, mediated by some surface proteins, that could correspond to those implicated in the "trypsin-removable" form of associated radioactivity. The radioactivity present in the "serum-removable" and "trypsin-removable" forms was found to be carried almost exclusively by GM1 (Fig. 2, lanes 4 and 5), whereas radioactivity present in the "trypsin-stable" form of association was found to be shared between GM1 and its catabolic derivatives, G_{M3}- and G_{M2}-gangliosides (Fig. 2, lane 7).

The distribution of different radiolabelled gangliosides with time is given in Fig. 6A. GM1 predominated throughout the investigation and after 24 h of incubation the radioactivity in G_{M2}- and G_{M3}-gangliosides did not exceed 13% of the total. This probably means that the catabolic process was slow and/or that the cell membrane requires a considerable incorporation of GM1, before any process of internalization can be initiated.

Experiments in the presence of FCS. GM1 bound to cells to a lower extent than in the parallel experiments in the absence of FCS (Fig. 6C and 6D). The radioactivity that remained associated to cells, after 4 h incubation, corresponded to 105 pmoles of ganglioside/mg cell protein, when 5×10^{-6} M of GM1 was initially added (Fig. 6D). This may be explained by the presence in the medium of a lipoprotein complex between GM1 and some components of the FCS, which reduced the availability of GM1 in a form suitable for the binding to cell membranes. In addition, the presence of such a complex could alter the equilibrium between GM1 free monomers and micelles and, as shown in Fig. 5, the relative percentage of the different forms of association seems to be independent of the concentration of added GM1. Analyzing the radioactivity present in the "trypsin-stable" form of association, a sizeable percentage (27%, after 24 h of incubation) of GM1 catabolites (G_{M3}- and G_{M2}-gangliosides) was observed (Fig. 2 lane 6, and Fig. 6B). This may be interpreted as a significant stimulation of GM1 endocytosis, due to the action of some components present in the FCS, with the consequence of an increased catabolic activity of the cell.

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