Photochemical labeling of HL-60 cell membrane proteins with radioiodinated, 4-azidosalicylic acid acylated derivatives of gangliosides[*][○]

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To detect HL-60 human promyelocytic leukemia cell proteins involved in the uptake of gangliosides from the culture medium we used photoreactive, 4-azidosalicylic acid (ASA) acylated and radioiodinated (200 Ci/mmole) derivatives of G_{M3}, G_{D3}, G_{M1}, and $FucG_{M1}$ gangliosides. Gangliosides-ASA, added to the medium at 15–20 nM concentration, followed a similar time course of uptake. After 1 min incubation cell bound gangliosides-ASA could not be removed with trypsin, but only 5–10% remained after incubation with BSA. The proportion of cell bound gangliosides-ASA resistant to BSA treatment increased with time of incubation up to 76% after 20 h. As shown on TLC, G_{M3}- and G_{D3}-ASA were catabolized to LacSph-ASA and ceramide-ASA, while G_{M1}-ASA was hydrolyzed to G_{M2}-ASA. $FucG_{M1}$-ASA was converted to G_{M1}-ASA very slowly. Upon irradiation with UV lamp, cell bound gangliosides-ASA crosslinked to and photolabeled many proteins but the distribution of radioactivity after SDS/PAGE was very uneven and did not correlate with Coomassie staining. In all experiments the 42 kDa protein bands were most intensely photolabeled. Photolabeling of 42 kDa proteins decreased with time of incubation as compared to lower molecular mass pro-

teins. With all gangliosides-ASA used similar but not identical protein photolabeling patterns were obtained. Photolabeling patterns with GM3- and GD3-ASA differed from those with GM1- and FucGM1-ASA.

It has been known for over 20 years [1, 2] that gangliosides added to culture media are taken up by cells (reviewed in [3]). Addition of exogenous gangliosides to cell media is still a procedure commonly used in studies on their effects on various activities in many cell types. Exogenous gangliosides present in the medium bind to and intercalate plasma membrane. Newly incorporated molecules not only can function as receptors for bacterial toxins [4] and viruses [5] but also affect cell growth and differentiation [6, 7]. Exogenous gangliosides in plasma membrane mimic the properties and enter the metabolic pathways of endogenous glycosphingolipids, i.e. those synthesized by cells. They are internalized into endosomes and catabolized in lysosomes [3, 8] while a small proportion sorted into the Golgi apparatus can be used as substrates by glycosyltransferases. Photoreactive gangliosides of high specific radioactivity should prove useful in studies on various aspects of interactions between glycosphingolipids and cell proteins. These include identification of proteins involved in binding and intercalation of exogenous gangliosides from the medium, proteins active in their sorting and further processing as well as those present in glycosphingolipid enriched membrane microdomains [9–11]. Tritium labeled photoreactive derivative of G_{M1} ganglioside was prepared by Sonnino et al. [12] and used for photolabeling of human fibroblast proteins probably involved in ganglioside uptake, and more recently in studies on interaction of this ganglioside with caveolin [13]. We have prepared a photoreactive 4-azidosalicylic acid (ASA) derivative of globoside [14] and of G_{M3}, G_{D3}, G_{M1}, and FucG$_{M1}$ gangliosides [15] which, after radioiodination to about 200 Ci/mmol, were used for photolabeling of human erythrocyte membrane proteins.

The aim of the present study was to characterize the binding of radioiodinated G_{M3}-, G_{D3}-, G_{M1}-, and FucG$_{M1}$-ASA gangliosides to HL-60 cells, to determine whether they are metabolized thus mimicking native glycosphingolipids, and to photolabel membrane proteins possibly involved in binding and intercalation of these compounds into cell membranes. HL-60 cells were chosen as their differentiation can be affected by exogenous gangliosides added to the medium [16, 17].

MATERIALS AND METHODS

Glycosphingolipids. Gangliosides-ASA were prepared by acylation of lysogangliosides with 4-azidosalicylic acid (ASA), purified and radioiodinated as described previously [14, 15]. For purification of gangliosides[125]I-ASA after radioiodination, Sephadex G-25 superfine Pasteur pipette columns were replaced by Sep-Pak C_{18} cartridges. Briefly, the radioiodination mixture was diluted with 0.5 ml of methanol/water (1:3) (all solvent ratios are expressed by volume), and applied to the cartridge previously treated as recommended by Schnaar [18]. Contaminants were removed with 10 ml of methanol/water (1:3) and radioiodinated gangliosides-ASA eluted with methanol. Thereafter the purification step on silicic acid column was performed as described [14, 15]. Radioiodinated gangliosides-ASA were stored in ethanol/water (3:2) at −20°C. When needed, samples were warmed to about 40°C, sonicated briefly in an ultrasonic cleaning bath (Pol Ned) and aliquots added to cell culture media in polystyrene culture tubes (Costar). Before being added to cells, the gangliosides[125]I-ASA containing media were warmed to 40°C, sonicated and left in the cell culture incubator for about 30 min. Fi-

nal concentration of ethanol in cell media did not exceed 0.3 %.

Cells. Human promyelocytic leukemia HL-60 cells were grown at 37°C in a water saturated atmosphere containing 5% CO_2. RPMI 1640 medium (Wytwórnia Surowic i Szczepionek, Lublin, Poland) supplemented with 10% fetal calf serum (Biochrom KG, Berlin, Germany), 50 U/ml of penicillin, and 50 μg/ml of streptomycin was used. Cells were grown to a density of about 1×10^6/ml, collected by centrifugation ($1000 \times g$, 5 min) and washed three times with ice cold PBS. For all experiments cells were incubated in serum-free RPMI 1640 medium supplemented with 10 mM Hepes, pH 7.3 (medium H) unless indicated otherwise.

Uptake of radioiodinated gangliosides-ASA by cells. Cells suspended in serum free medium with gangliosides[125]I-ASA at concentration of about 15 nM were incubated at 37°C under conditions used for cell culture. When needed, aliquots containing about 2×10^5 of cells in 70–100 μl were withdrawn and added to plastic tubes containing ice cold phosphate-buffered saline supplemented with 0.2% gelatin (PBS-G) or, in experiments where the effect of BSA was studied, 1% BSA in PBS. Cells were collected by centrifugation, washed once with cold PBS-G, resuspended in 0.5 ml PBS-G and transferred to new tubes containing 3 ml of cold PBS-G (this step reduces the amount of radioactivity, resulting from adsorption of radioiodinated gangliosides-ASA to plastic, below 0.5% of that originally present in the sample. Addition of gelatin to PBS minimized cell loss during transfer and washes). Finally, cells were pelleted again and their radioactivity determined in the gamma counter (LKB/Wallack, Clinigamma).

Removal with BSA of radioiodinated gangliosides-ASA taken up by cells. To determine the amount of gangliosides[125]I-ASA incorporated into cells in a BSA resistant manner, aliquots withdrawn from incubation mixtures as above were added to cold PBS containing 1% BSA or, as a control, PBS-G.

Cells collected by centrifugation were resuspended in 0.5 ml of medium H containing 1% BSA or only in medium H and incubated at 37°C for 15 min. Thereafter 3 ml of cold PBS-G was added, cells pelleted, transferred to new tubes as above, washed twice with PBS-G and their radioactivity determined.

Effect of energy depletion on uptake of radioiodinated gangliosides-ASA in a BSA stable form. Cells were washed with PBS and incubated on ice for 15 min in medium H containing 10 mM NaN_3 and 20 mM 2-deoxy-D-glucose instead of glucose, warmed up to 37°C and placed in the incubator for additional 15 min. Thereafter gangliosides[125]I-ASA were added, aliquots withdrawn after incubation at 37°C for 15 min and 30 min and the amount of total cell bound radioactivity as well as that remaining after treatment with 1% BSA determined as above.

Removal with trypsin of radioiodinated gangliosides-ASA taken up by cells. Cells at a density of about 2×10^6/ml were incubated for 1 min or 3 h in medium H containing 20 nM of radioiodinated gangliosides-ASA. About 4×10^6 cells in 2 ml were withdrawn, washed once with cold 50 ml PBS and resuspended in 2 ml of medium H containing 0.01% DNase. Aliquots, 100 μl, were withdrawn and mixed with an equal volume of medium H or medium H containing 0.5% trypsin dissolved immediately before use. Cells were incubated for 5 min at 37°C and after addition of 4 ml of cold PBS-G pelleted at $1500 \times g$ for 5 min and their radioactivity determined.

Metabolism of radioiodinated gangliosides-ASA taken up by cells. Cells were suspended at a density of 1.5×10^6/ml in medium H supplemented with 5 μg/ml of insulin and transferrin and incubated at 37°C for 30 min. Gangliosides-ASA were suspended in the same medium and also kept in the incubator for the same time. After their addition to cell suspensions a final cell density of 1×10^6/ml and glycolipid concentration of 20 nM were reached. Aliquots containing about 1×10^6 cells were withdrawn after 1 min, 3 h, and 20

h. Cells were washed twice with 50 ml of cold PBS-G and cell pellets extracted with chloroform and methanol as described by Schnaar [18]. Crude lipid extracts were freed of salts on Sephadex G-25 superfine columns and, after separation on TLC in solvent system A, plates were exposed to X-ray films. Distribution of radioactivity in gangliosides-ASA and their metabolic products was estimated by densitometric scanning of developed films.

Photochemical labeling of cells. After incubation with radioiodinated gangliosides-ASA, followed where indicated by treatment with 1% BSA, cells were washed with cold PBS and resuspended (about $4-7 \times 10^6$ cells/ml) in a solution of NaCl, 124 mM; Tris/HCl, 24 mM; CaCl$_2$, 5 mM; pH 7.4. Cell suspensions in quartz cuvettes were placed at a 10 cm distance from the filter of a UV lamp (Cole Parmer, 30 watts), and subjected to photolysis, i.e. irradiated for 12 min, further treated as previously described [14, 15] and stored at $-80°C$ until used.

Preparation of crude cell membranes. Cells, $1-2 \times 10^7$, were homogenized in 1.5–2.0 ml of a solution containing Tris/HCl, 5 mM; EDTA, 1 mM; MgCl$_2$, 1 mM; pH 7.5; and protease inhibitors: PMSF, 1 mM; chymostatin, leupeptin, antipain, and pepstatin, each at a concentration of 5 μg/ml. Dounce homogenizer, 20 strokes of tightly fitting pestle, was used. Homogenate was centrifuged at 1500 × *g* for 15 min, sediment homogenized again as above and combined supernatants centrifuged at 30 000 × *g* for 45 min. Sediment was suspended in 2% SDS in water containing protease inhibitors as above, an aliquot withdrawn for protein assay, and the remaining part of the sample mixed with an equal volume of a solution containing 60% glycerol, 15% β-mercaptoethanol, 6% SDS and 0.01% bromophenol blue, heated at 80°C for 5 min and stored at $-80°C$.

Electrophoresis. Solubilized cell proteins were analyzed by SDS/PAGE [19] on 7.5–15% polyacrylamide gradient gels. Separated proteins were stained with Coomassie Brilliant Blue. The distribution of radioactivity in proteins photolabeled with radioiodinated gangliosides-ASA was detected by autoradiography of dried gels and evaluated after densitometric scanning of X-ray films with Shimadzu CS 9000 scanning densitometer.

Thin-layer chromatography. Radioiodinated gangliosides-ASA, products of their hydrolysis by glycosidases, and lipids extracted from HL-60 cells, were analyzed with solvent system A (chloroform/methanol/0.25% aqueous CaCl$_2$, 60:35:8) on silica gel precoated plates, Merck article no. 5721.

Estimation of purity of radioiodinated gangliosides-ASA. Radioiodinated gangliosides-ASA prepared and used in the present study were subjected to TLC in solvent system A and distribution of radioactivity on lanes was measured with Phosphor-Imager and Image Quant 3 program of Molecular Dynamics.

Preparation of radioiodinated Sph-ASA, GlcSph-ASA, LacSph-ASA and G$_{M2}$-ASA. These compounds, needed as TLC standards to evaluate the catabolism of gangliosides-ASA by HL-60 cells, were prepared from radioiodinated G$_{M3}$-ASA and G$_{M1}$-ASA with exoglycosidases and ceramide glycanase. Briefly, to prepare Sph-ASA radioiodinated G$_{M3}$-ASA was treated with leech ceramide glycanase (Boehringer) [20]; LacSph-ASA was obtained after its desialylation by *Vibrio cholerae* neuraminidase (Sigma) [21] in the presence of 0.1% Triton X-100, and GlcSph-ASA after removal of both sialic acid and galactose by *Vibrio cholerae* neuraminidase and jack bean β-galactosidase [21, 22], respectively. Jack bean β-galactosidase was also used for the preparation of G$_{M2}$-ASA from G$_{M1}$-ASA. Reactions with glycosidases were terminated by the addition of chloroform and methanol (2:1) and the sphingolipid reaction products freed from salts on Sephadex G-25 Pasteur pipette columns prepared and eluted with chloroform/methanol/water (120:60:9). The glycosidase reaction products prepared in this way were analyzed on TLC and detected by autoradiography.

Determination of protein. Protein content was estimated by bicinchoninic acid assay according to the manufacturer (Pierce, Rockford, U.S.A.)

RESULTS AND DISCUSSION

Gangliosides-ASA

Radioiodinated gangliosides-ASA used in the present study migrated on TLC in solvent system A as shown in Fig 1. Their purity was somewhat higher than previously reported [15]. As determined by the Phosphor Imager, major bands separated by TLC contained: for G_{M3}-ASA, 93.2%; for G_{D3}-ASA, 87.7%; for G_{M1}-ASA, 92.8%; and for FucG$_{M1}$-ASA 93.2% of the total radioactivity present on the respective lane including origin and solvent front areas. The major contaminant of G_{D3}-ASA migrated similarly to G_{M3}-ASA and accounted for 5.4% of total radioactivity.

Uptake of radioiodinated gangliosides-ASA by HL-60 cells

Gangliosides-ASA were taken up by HL-60 cells in a time and concentration dependent manner. Time course of their incorporation was similar to that for unmodified ganglioside G_{M1} [23] being initially very rapid but levelling off after 15–30 min (Fig. 2). In view of the lack of accuracy in determining specific radioactivity of our preparations we consider it similar for all gangliosides-ASA used. This observation apparently differs from that of Nakamura *et al.* [23] who reported that G_{M3} ganglioside was taken up by HL-60 cells more efficiently than G_{M1} ganglioside. However, in the experiments of Nakamura *et al.* [23, 24] the difference between these two gangliosides was detectable at concentrations above 5 μM, that is about 300 times higher than used by us. We did not study in detail the effect of ganglioside-ASA concentration on their incorporation into HL-60 cells. In a single experiment, a fourfold increase (up to 80 nM) in the concentration of radioiodinated gangliosides-ASA resulted in a 3.0–3.4-fold increase in their amount taken up by the cells (not shown).

Removal of radioiodinated gangliosides-ASA with BSA and trypsin

As discussed by Saqr *et al.* [3] exogenous gangliosides bind to cells in three forms: as
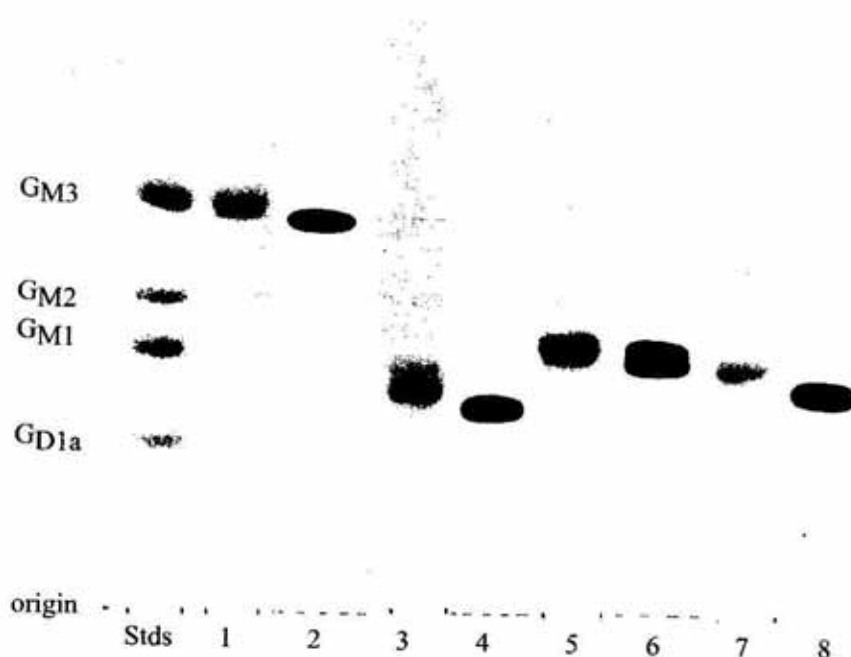
Figure 1. TLC analysis of radioiodinated gangliosides-ASA.

Ganglioside standards, (Stds) as indicated. Lanes 1, 3, 5, and 7, unmodified gangliosides G_{M3}, G_{D3}, G_{M1}, and FucG$_{M1}$, respectively. Lane 2, G_{M3} ^{125}I-ASA; lane 4, G_{D3} ^{125}I-ASA; lane 6, G_{M1} ^{125}I-ASA; lane 8, FucG$_{M1}$ ^{125}I-ASA. Solvent system of chloroform/methanol/0.25% aqueous CaCl$_2$ was used. Unmodified gangliosides were stained with orcinol. Radioiodinated gangliosides-ASA were detected after exposure to X-ray film. Purity of gangliosides-ASA was estimated with Phosphor Imager.
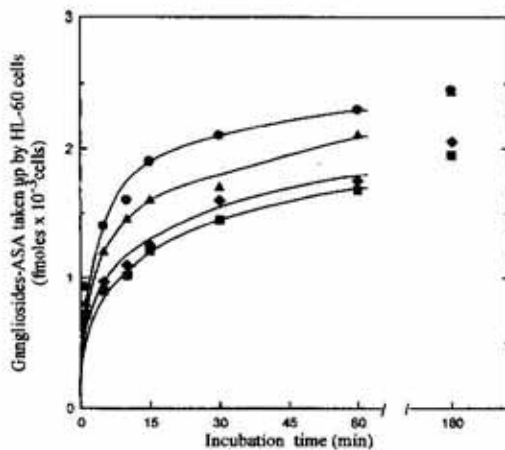
G_{M3}

G_{M2}
G_{M1}

G_{D1a}

origin

Stds   1   2   3   4   5   6   7   8

Figure 2. Effect of time on uptake of radioiodinated gangliosides by HL-60 cells.

Cells were prepared as specified under Methods and incubated at a density of 3×10^6/ml in medium H containing 15 nM, respectively, of G_{M3}-ASA, (●); G_{D3}-ASA, (▲); G_{M1}-ASA, (◆); and FucG$_{M1}$-ASA (■). Aliquots (70 μl, 2.1×10^5 cells) were withdrawn as indicated and added to tubes containing 4 ml of ice-cold PBS-G. Cells were washed with PBS-G and their radioactivity determined in a gamma counter (Methods).

loosely adhering micelles, easily removable with BSA, as micelles or monomers bound to membrane proteins and released after digestion with trypsin, and as monomers with ceramide moieties intercalated with membrane lipids. In this latter form gangliosides are resistant to trypsin. As already shown and discussed, gangliosides-ASA incorporated into human erythrocytes are resistant to trypsin and seem not to adhere to but rather intercalate plasma membrane even though they are easily removed with BSA [15]. Likewise, radioiodinated gangliosides-ASA bound to HL-60 cells are resistant to trypsin but susceptible to BSA treatment. However, while for human erythrocytes the proportion of gangliosides-ASA resistant to BSA was not changed, for HL-60 cells it increased from about 5–10% after 1 min to 38–42% after 3 h (Table 1 and Fig. 3). The increase in the proportion of different radioiodinated gangliosides-ASA bound to HL-60 cells in a BSA-stable form followed similar time course (Fig. 3). Moreover, when cells at a density of 1×10^6/ml were in-

cubated with gangliosides-ASA for 20 h, the proportion of radioiodinated G_{M3}-, G_{D3}-, G_{M1}-, and FucG$_{M1}$-ASA resistant to BSA increased to 53%, 54%, 59% and 62%, respectively, in one, and to 76%, 68%, 61% and 65% in the second independent experiment. BSA resistant association of radioiodinated gangliosides-ASA with cells could have resulted from at least two phenomena. Firstly, it could reflect active endocytosis which would direct gangliosides-ASA into endosomes [8]. Secondly, it could involve ganglioside lateral movement and association with specific proteins and lipids within membrane microdomains different from those in which initial binding and intercalation took place. Our preliminary experiments indicate that both these processes seem to occur with gangliosides-ASA. Incubation of cells under conditions limiting energy supplies had no effect on the amount or kinetics of gangliosides-ASA incorporated (not shown) yet it decreased up to one
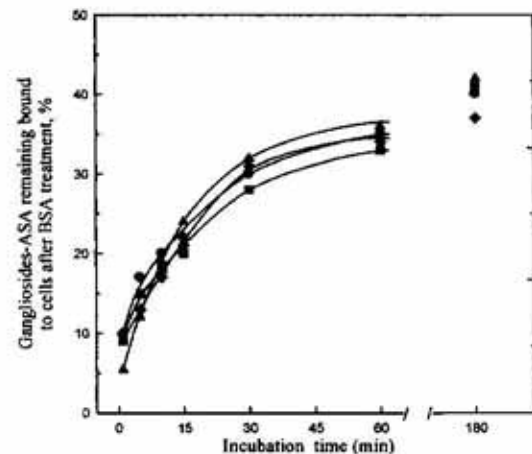

Figure 3. Effect of time of incubation on removal by BSA treatment of the radioiodinated gangliosides-ASA taken up by HL-60 cells.

Cells were incubated at 37°C with G_{M3}^{125}I-ASA, (●); G_{D3}^{125}I-ASA, (▲); G_{M1}^{125}I-ASA (◆), and FucG$_{M1}$^{125}I-ASA (■), as specified in the legend to Fig. 2. Aliquots (70 μl, 2.1×10^5 cells) were added to tubes containing ice-cold PBS-G or PBS with 1% BSA. Cells were pelleted by centrifugation and further treated as specified under Methods. Cell bound radioactivity at the end of the procedure was determined in a gamma counter.

Table 1. Effect of incubation time of HL-60 cells with radioiodinated gangliosides-ASA on their removal with BSA or trypsin

Treatment	Incubation time	G_{M3}	G_{D3}	G_{M1}	Fuc-G_{M1}
		gangliosides ^{125}I-ASA remaining bound to cells after treatment (%) ±S.D.			
BSA, (1%)	1 min	9.8±1.2	5.4±2.8	10.0±1.6	9.1±0.7
	3 h	40.4±3.1	42.1±2.5	37.6±4.0	41.2±2.3
Trypsin,	1 min	92.4±4.0	96.4±3.2	96.2±2.9	88.9±4.8
(0.25%)	3 h	93.9±3.7	103.8±2.5	95.5±3.8	92.2±5.6

Cells were incubated with radioiodinated gangliosides-ASA for 1 min or 3 h and treated with 1% BSA or 0.25% trypsin as specified under Methods

third the percentage of these compounds remaining cell bound after BSA treatment.

Metabolism of radioiodinated gangliosides-ASA

Distribution of radioactivity in lipids extracted from HL-60 cells after incubation with radioiodinated gangliosides-ASA changed with time (Fig. 4). Lipid extracts from cells after 1 min incubation contained radioactive material migrating on TLC as radioiodinated G_{M3}-, G_{D3}-, G_{M1}-, and FucG_{M1}-ASA before they were added to media. When G_{M3}-, G_{D3}- and G_{M1}-ASA were incubated for 3 h, new radioactive lipid bands appeared with TLC mobility similar to that of Lac-Sph-ASA and G_{M2}-ASA „standards" prepared from G_{M3}- and G_{M1}-ASA with *Vibrio cholerae* neuraminidase and jack bean β-galactosidase, respectively. After additional 17 h, in the extract from the cells incubated with G_{M3}-ASA a new radioactive lipid was detected, migrating on TLC like Sph-ASA (ceramide-ASA) (Fig. 4A). A radioactive lipid of similar mobility was also found in the extract from the cells incubated with G_{D3}-ASA (Fig. 4B) but not when G_{M1}-ASA was used, even though a substantial formation of a lipid with TLC mobility characteristic of G_{M2}-ASA was detected (Fig. 4C). Our results can not be directly compared with those of Nakamura *et al.* [24] who used over a thousand fold higher concentration of tritium labeled G_{M3} and G_{M1} gangliosides; however, in their experiments G_{M1} ganglioside was also con-

verted into ceramide very slowly. Even though characterization of the radioiodinated ganglioside-ASA metabolic products by TLC mobility alone should be considered tentative yet our observations indicate that these compounds are catabolized by HL-60 cells. Formation of catabolic products from exogenous glycosphingolipids reflects not only the rate of their internalization and sorting into lysosomes but also relative activities of relevant exoglycosidases. We assume that slow conversion of FucG_{M1}-ASA into G_{M1}-ASA, barely visible in Fig. 4D, could be due to low activity of α-fucosidase in HL-60 cells rather than to a different rate of internalization. The observation that gangliosides-ASA are catabolized by HL-60 cells is not surprising. Replacement of ganglioside fatty acids with ASA is a substantial modification, yet not greater than substitution of ceramide by alkyl amine, cholesterol, or phosphatidylcholine in G_{M1}-oligosaccharide based neoglycolipids which could enter both catabolic and anabolic pathways in C6 rat glioma cells [25]. However, so far we have not demonstrated the formation of anabolic reaction products from gangliosides-ASA. Considering the endogenous ganglioside content of HL-60 cells [17], it is possible that G_{M3}-, G_{D3}-, and G_{M1}-ASA are not the substrates for the relevant glycosyltransferases. On the other hand, it should be noticed that lipid extracts from cells incubated for 20 h with radioiodinated FucG_{M1}-ASA contained a small amount of radioactive material slowly migrating on TLC, as could be expected of an
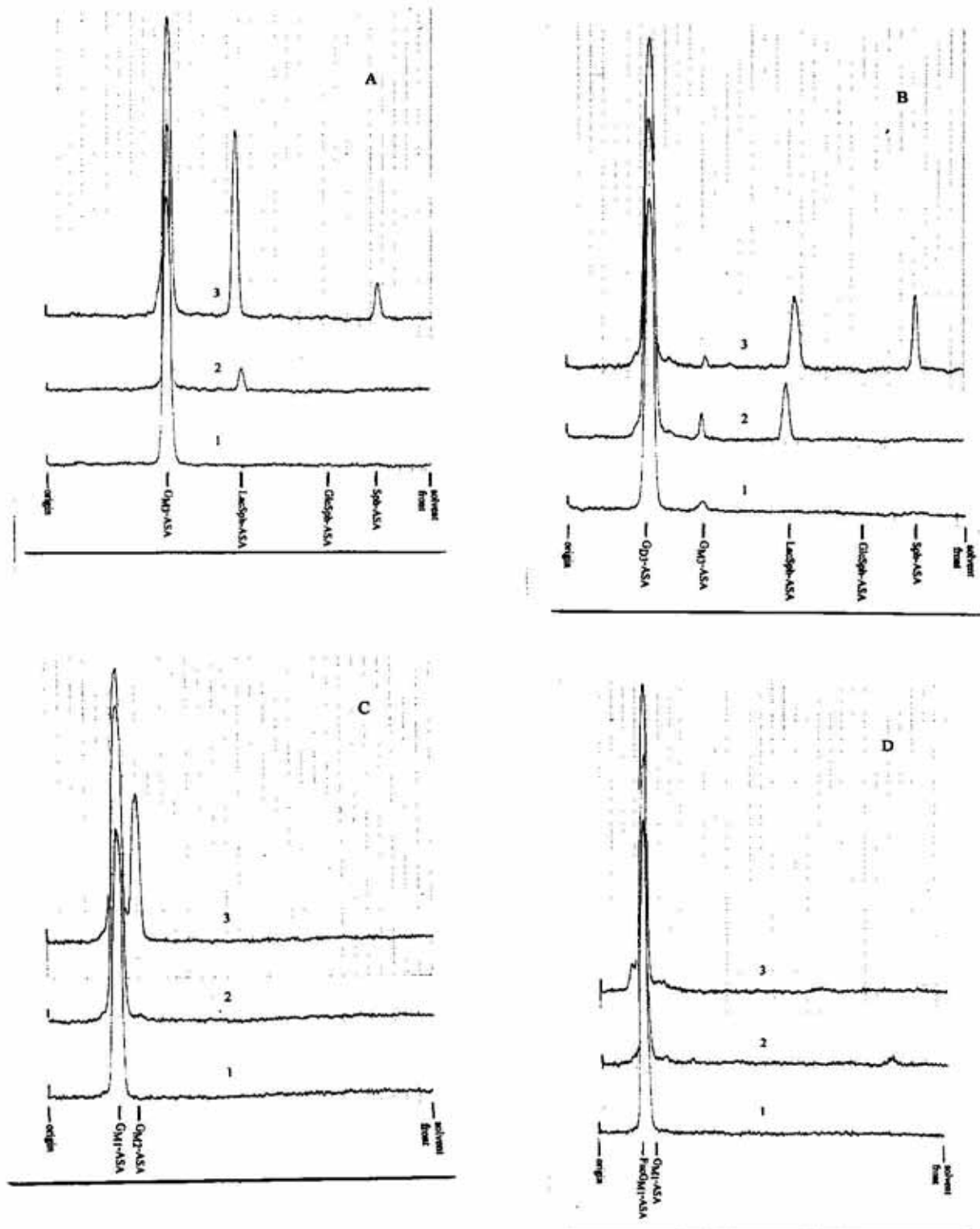
Figure 4. Metabolism of radioiodinated gangliosides-ASA by HL-60 cells.

Lipids were extracted from cells incubated with radioiodinated gangliosides-ASA for 1 min, 3 h, and 20 h and analyzed by TLC in solvent system of chloroform/methanol/0.25% aqueous $CaCl_2$ (60:35:8) followed by autoradiography. Distribution of radioactivity was evaluated by densitometric scanning of X-ray films. A. Lipids extracted from cells after incubation with G_{M3} ^{125}I-ASA for 1 min, tracing 1; 3 h, tracing 2; and 20 h, tracing 3. B. Same as in A, but cells incubated with G_{D3} ^{125}I-ASA. C. Same as in A, the cells incubated with G_{M1} ^{125}I-ASA. D. Same as in A, the cells incubated with $FucG_{M1}$ ^{125}I-ASA. TLC mobility of radioiodinated Sph-ASA, GlcSph-ASA, LacSph-ASA, and G_{M2}-ASA, prepared as specified under Methods, is indicated.

anabolic product of this ganglioside-ASA (Fig. 4D).

Photolabeling of HL-60 cell proteins

Radioiodinated gangliosides-ASA taken up by cells photolabel many proteins (Fig. 5). As detected after autoradiography of the SDS-PAGE separated proteins, distribution of radioactivity is very uneven and does not correlate with the intensity of Coomassie Blue staining (Fig. 5, lane 0 *versus* the remaining lanes). In all experiments protein bands with electrophoretic mobility corresponding to molecular mass of about 42 kDa were most intensely photolabeled. As tentatively estimated by densitometric scanning, for cells incubated with radioiodinated gangliosides-ASA for 1

min without subsequent BSA treatment, these bands contained about 40% (33.1-47.4%, n = 2) of radioactivity present on the electrophoregram between the origin of the separating gel and the 14 kDa standard (most of the radioactive material crosslinked to lipids migrated with the tracking dye, not shown and not analyzed in the present study). In all experiments photolabeling patterns were affected by incubation time; when this was prolonged by additional 20 h, the percentage of radioactivity associated with 42 kDa proteins decreased slightly to about 36% (33.6-39.6%, n = 2). At the same time photolabeling of lower molecular mass proteins migrating between the 42 kDa band and the 14 kDa standard increased from about 8% (4.1-13.3%, n = 2) after 1 min to 20.4% (16.5-23.2%, n = 2).
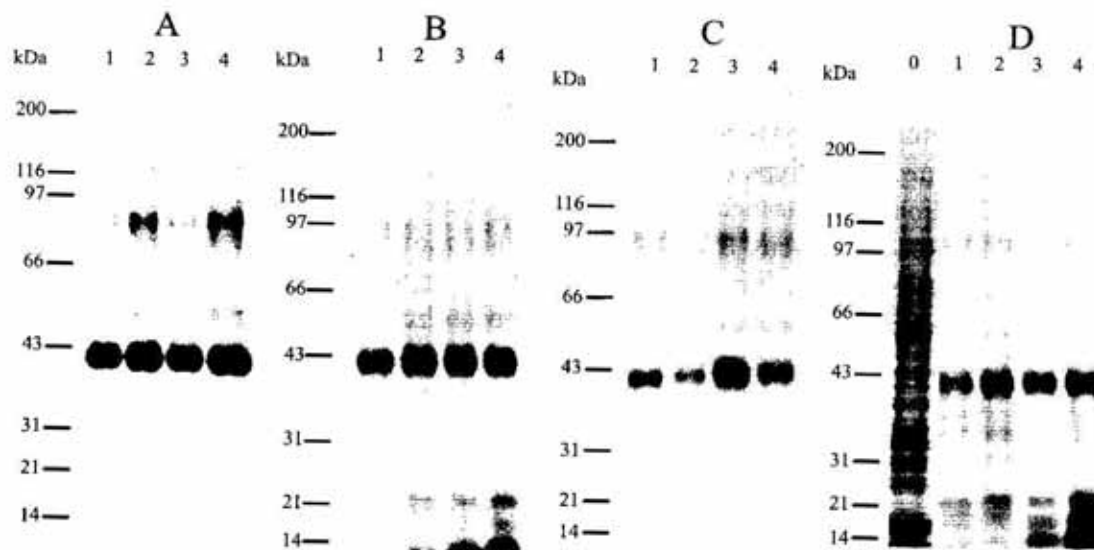

Figure 5. Effect of time and BSA treatment on photolabeling patterns of HL-60 cell membrane proteins with different radioiodinated gangliosides-ASA.

A. Cells at a density of 1×10^6/ml were incubated with radioiodinated gangliosides-ASA for 1 min or 20 h and, where indicated, treated with 1% BSA before photolysis. Crude membrane preparations were prepared and solubilized proteins separated by SDS/PAGE on 7.5-15% gradient gels. Electrophoregrams were stained with Coomassie Brilliant Blue, dried and exposed to X-ray films. All lanes except lane 0 in D are autoradiograms of the separated proteins. Lane 0 in panel D shows proteins stained with Coomassie Brilliant Blue. A similar protein staining pattern was obtained for all membrane preparations (not shown). Most of the radioactivity (lipids) migrated with the solvent front (not shown). A. Cells incubated for 1 min. B. Cells incubated for 20 h. C. Cells incubated for 1 min and treated with 1% BSA before photolysis. D. Cells incubated for 20 h and treated with 1% BSA before photolysis. All lanes 1, cells incubated with G_{M3} ^{125}I-ASA; lanes 2, cells incubated with G_{D3} ^{125}I-ASA; lanes 3, cells incubated with G_{M1} ^{125}I-ASA; lanes 4, cells incubated with $FucG_{M1}$ ^{125}I-ASA. Solubilized membrane preparations contained 40-60 μg protein and for A, 120 000 c.p.m.; for B, 250 000 c.p.m.; for C, 10 000 c.p.m.; and for D, 180 000 c.p.m. Electrophoreograms A, B, and D were exposed to X ray films overnight while C was exposed for 4 days.

As already mentioned, a percentage of cell bound radioiodinated gangliosides-ASA, initially small but increasing upon incubation, could not be removed with 1% BSA. This could have resulted from their association with membrane proteins and lipids other than those at the beginning of incubation. To check this possibility, cells incubated with radioiodinated gangliosides-ASA for 1 min and 20 h were treated with 1% BSA before photolysis. As could be expected, for cells incubated for 1 min, BSA treatment strongly reduced the extent of photolabeling, but this had little effect on the photolabeling patterns (Figs. 5 and 6). Again the most intensely photolabeled were the 42 kDa proteins containing about 45% (38.8–53.7%, n = 2) of the radioactivity, while 8% (5.95–9.7%, n = 2) was detected in the less than 42 kDa to 14 kDa electrophoregram region. After incubation with radioiodinated gangliosides-ASA for additional 20 h, BSA treatment preceding photolysis affected photolabeling of cell proteins (Figs. 5 and 6). The proportion of radioactivity associated with 42 kDa band decreased to about 30.9% (29.0–33.3%, n = 2) while that in lower molecular mass proteins increased fourfold to 32.1% (29.1–36.1%, n = 2). An increase with time of incubation in photolabeling of lower molecular mass proteins was detectable even without BSA treatment which, however, enhanced this effect (Figs. 5 and 6). To explain these observations two processes, already discussed in relation to BSA-resistant binding of gangliosides-ASA, should be considered. It can be assumed that, during incubation, the radioiodinated gangliosides-ASA initially bound to or intercalating plasma membrane in the vicinity of the 42 kDa proteins move by lateral diffusion to different membrane microdomains enriched in lower molecular mass proteins migrating on electrophoregrams between 42 kDa and 14 kDa proteins. Interactions with proteins and lipids in these microdomains not only make radioiodinated gangliosides-ASA more resistant to BSA extraction but also increase photolabeling of

their protein and lipid neighbors. The other possibility involves the use of crude membrane preparations for SDS/PAGE. The $30000 \times g$ membrane pellets contained 78% to 97% of radioactivity originally present in the "post nuclear" i.e. $1500 \times g$ supernatant and did not represent only plasma membranes. Thus an apparently increased photolabeling of lower molecular mass proteins could have resulted from degradation of 42 kDa proteins in the lysosomes. Detailed subcellular fractionation experiments should answer this question. Also puzzling are spatial relations between 42 kDa proteins and radiolabeled gangliosides-ASA which seem to reside in the membrane in their vicinity both in a form susceptible as well as resistant to BSA treatment, the amount of the latter increasing with time.

Photolabeling patterns of membrane proteins obtained with various radioiodinated gangliosides-ASA were similar but not identical (Figs. 5 and 6). Differences dependent on the structure of oligosaccharide chains of gangliosides-ASA were most evident in the experiments in which cells were treated with 1% BSA after 20 h incubation, and pertained to proteins detected on SDS/PAGE in the less than 42–14 kDa region (Figs. 5 and 6). Interestingly, structurally related $G_{M3}{}^{125}$I-ASA and $G_{D3}{}^{125}$I-ASA gave similar photolabeling patterns which differed, however, from those obtained with $G_{M1}{}^{125}$I-ASA and FucG$_{M1}{}^{125}$I-ASA. Likewise, incubation with $G_{M1}{}^{125}$I-ASA and FucG$_{M1}{}^{125}$I-ASA, again resulted in similar photolabeling patterns (Figs. 5 and 6). Limited resolving power of one dimensional electrophoresis, complexity of photolabeling patterns and use of crude membrane preparations limit the conclusions which can be drawn from the results obtained in our studies.

Possible functions of 42 kDa proteins in the uptake and further processing of gangliosides are at present unclear. They seem to be involved in the initial contact of radioiodinated gangliosides-ASA with HL-60 cell membrane. This contact could consist in binding of
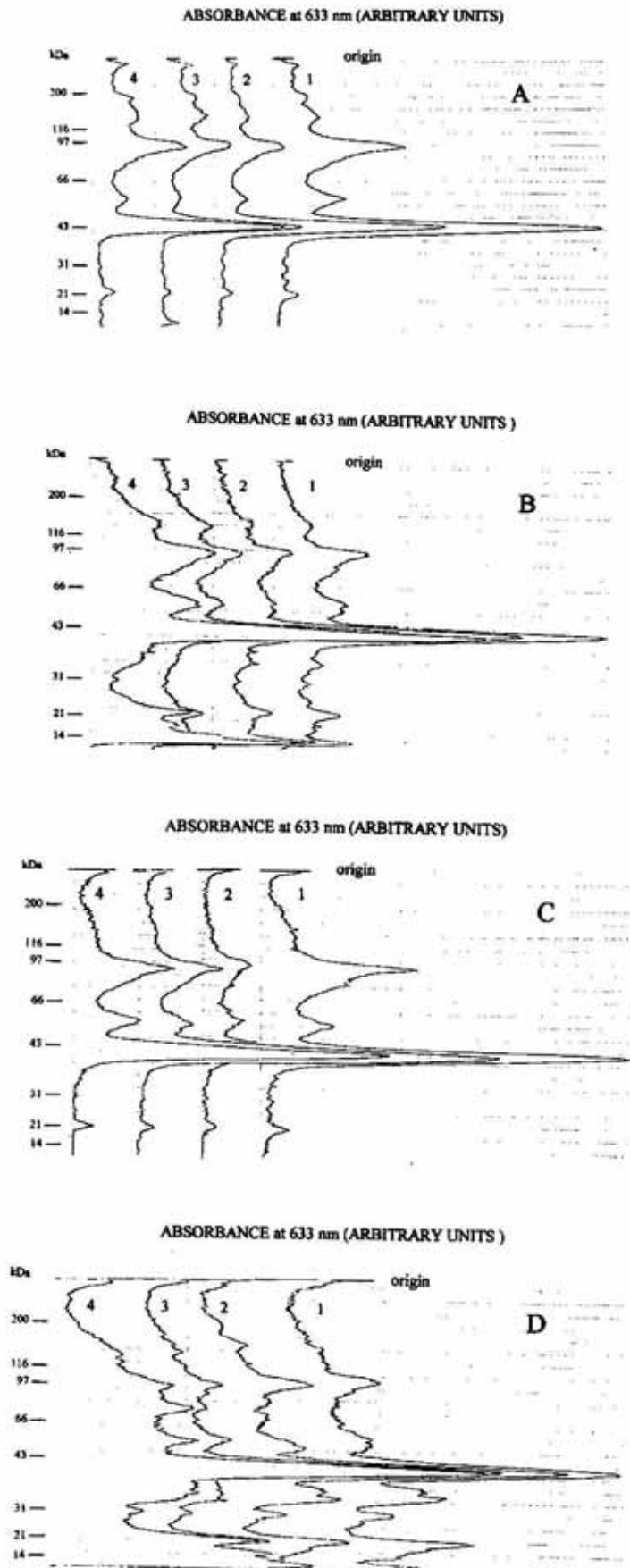
ABSORBANCE at 633 nm (ARBITRARY UNITS)

ABSORBANCE at 633 nm (ARBITRARY UNITS)

ABSORBANCE at 633 nm (ARBITRARY UNITS)

ABSORBANCE at 633 nm (ARBITRARY UNITS)

Figure 6. Densitometric scanning of HL-60 cell proteins photolabeled with radioiodinated gangliosides-ASA.

Electrophoregrams presented in Fig. 5 were scanned with Shimadzu scanning densitometer. Numbering of panels and lanes are the same as in Fig. 5. Lanes in panel C were scanned at higher sensitivity than in the remaining panels.

gangliosides-ASA to, or inserting into the membrane in the vicinity of 42 kDa proteins. Understanding of this interaction, as well as identification of 42 kDa proteins and definition of their role in the uptake of native gangliosides by HL-60 cells requires further experiments. Our results on photolabeling of HL-60 cell proteins can not be directly compared with those of Sonnino et al. [12] who used human fibroblasts, photoreactive G_{M1} ganglioside with aryl azide placed at the end of the fatty acid, and a final ganglioside concentration of about $100 \mu M$, that is 5 thousand fold higher than used by us. However, their observations and ours seem to support the possibility, discussed by Saqr et al. [3], that binding of the bulk of exogenous gangliosides seems to be mediated by a few proteins.

REFERENCES

1. Cuatrecasas, P. (1973) Gangliosides and membrane receptors for cholera toxin. *Biochemistry* **12**, 3558–3566.

2. Fishman, P.H., Moss, J. & Manganiello, V.C. (1977) Synthesis and uptake of gangliosides by choleragen-responsive human fibroblasts. *Biochemistry* **16**, 1871–1875.

3. Saqr, H.E., Pearl, D.K. & Yates, A.J. (1993) A review and predictive models of ganglioside uptake by biological membranes. *J. Neurochem.* **61**, 395–411.

4. Fishman, P.H., Moss, J. & Vaughan, M. (1976) Uptake and metabolism of gangliosides in transformed mice cells. *J. Biol. Chem.* **251**, 4490–4494.

5. Markwell, M.A.K., Moss, J., Hom, B.E., Fishman, P.H. & Svennerholm, L. (1986) Expression of gangliosides at the cell surface controls infection of NCTC 2071 cells by Sendai virus. *Virology* **155**, 356–364.

6. Nagai, Y. (1995) Functional roles of gangliosides in bio-signalling. *Behav. Brain Res.* **66**, 99–104.

7. Hakomori, S. & Igarashi, Y. (1995) Functional role of glycosphingolipids in cell recognition and signaling. *J. Biochem.* (Tokyo) **118**, 1091–1103.

8. Sandhoff, K. & Kolter, T. (1996) Topology of glycosphingolipid degradation. *Trends Cell Biol.* **6**, 98–103.

9. Simons, K. & Ikonen, E. (1997) Functional rafts in cell membranes. *Nature* (London) **387**, 569–572.

10. Harder, T. & Simons, K. (1997) Caveolae, DIGs, and the dynamics of sphingolipid-cholesterol microdomains. *Curr. Opin. Cell Biol.* **9**, 534–542.

11. Yamamura, S., Handa, K. & Hakomori, S. (1997) A close association of G_{M3} with c-Src and Rho in G_{M3}-enriched microdomains at the B16 melanoma cell surface membrane: A preliminary note. *Biochem. Biophys. Res. Commun.* **236**, 218–222.

12. Sonnino, S., Chigorno, V., Aquotti, D., Pitto, M., Kirschner, G. & Tettamanti, G. (1989) A photoreactive derivative of G_{M1} ganglioside: Preparation and use to establish the involvement of specific proteins in G_{M1} uptake by human fibroblasts in culture. *Biochemistry* **28**, 77–84.

13. Fra, A.M., Masserini, M., Palestini, P., Sonnino, S. & Simons, K. (1995) A photoreactive derivative of ganglioside G_{M1} specifically cross-links VIP21-caveolin on the cell surface. *FEBS Lett.* **375**, 11–14.

14. Pacuszka, T. & Panasiewicz, M. (1995) Photochemical labeling of human erythrocyte membranes with radioiodinatable azidosalicylic

acid derivative of globoside. *Biochim. Biophys. Acta* **1257**, 265–273.

15. Pacuszka, T. & Panasiewicz, M. (1998) Photochemical labeling of human erythrocyte membrane proteins with radioiodinated 4-azido-salicylic acid derivatives of G_{M3}, G_{D3}, G_{M1}, and $FucG_{M1}$ gangliosides. *Acta Biochim. Polon.* **45**, 509–522.

16. Nojiri, H., Takaku, F., Terui, Y., Miura, Y. & Saito, Y. (1986) Ganglioside G_{M3}: An acidic membrane component that increases during macrophage-like cell differentiation can induce monocytic differentiation of human myeloid and monocytoid leukemia cell lines HL-60 and U 937. *Proc. Natl. Acad. Sci. U.S.A.* **83**, 782–786.

17. Nojiri, H., Kitagawa, S., Nakamura, M., Kirito, K., Enomoto, Y. & Saito, M. (1988) Neolacto-series gangliosides induce granulocytic differentiation of human promyelocytic leukemia cell line HL-60. *J. Biol. Chem.* **263**, 7443–7446.

18. Schnaar, R.L. (1994) Isolation of glycosphingolipids. *Methods Enzymol.* **230**, 348–370.

19. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* (London) **227**, 680–685.

20. Zhou, B., Li, S.C., Laine, R., Huang, R.T.C. & Li, Y.T. (1989) Isolation and characterization of ceramide glycanase from the leech *Macrobdella decora*. *J. Biol. Chem.* **264**, 12272–12277.

21. Bartoszewicz, Z., Koscielak, J. & Pacuszka, T. (1986) Structure of a new disialoganglioside G_{D1c} from spontaneous murine thymoma. *Carbohydr. Res.* **151**, 77–88.

22. Li, S.C., Mazotta, M.Y., Chien, S.F. & Li, Y.T. (1975) Isolation and characterization of jack bean β-galactosidase. *J. Biol. Chem.* **250**, 6786–6791.

23. Nakamura, M., Ogino, H., Nojiri, H., Kitagawa, S. & Saito, M. (1989) Characteristic incorporation of ganglioside G_{M3} which induces monocytic differentiation in human myelogenous leukemia HL-60 cells. *Biochem. Biophys. Res. Commun.* **161**, 782–789.

24. Nakamura, M., Tsunoda, A., Furukawa, Y., Sakai, T. & Saito, M. (1997) Rapid internalization of exogenous ganglioside G_{M3} and its metabolism to ceramide in human myelogenous leukemia HL-60 cells compared with control ganglioside G_{M1}. *FEBS Lett.* **400**, 350–354.

25. Pacuszka, T. & Fishman, P.H. (1991) Metabolism of cholesterol, phosphatidylethanolamine and stearylamine analogues of G_{M1} ganglioside by rat glioma C6 cells. *Biochim. Biophys. Acta* **1083**, 153–160.