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QUARTERLY

Photochemical labeling of human erythrocyte membrane proteins with radioiodinated 4-azidosalicylic acid derivatives of G_{M3}, G_{D3}, G_{M1}, and $FucG_{M1}$ gangliosides

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Photoreactive gangliosides of high specific radioactivity may prove useful for studies on glycosphingolipid functions. We prepared 4-azidosalicylic acid (ASA) acylated derivatives of GM3, GD3, GM1, and FucGM1 gangliosides (gangliosides-ASA). Gangliosides-ASA were characterized by their TLC mobility, UV spectra, carbohydrate composition, and digestion with leech endoceramidase. After radioiodination to about 200 Ci/mmole gangliosides-ASA were used for photochemical labeling of human erythrocytes. Radioiodinated gangliosides-ASA were incorporated into erythrocytes in a time and concentration dependent manner, the kinetics and extent of incorporation being similar for all the gangliosides-ASA used. Radioiodinated gangliosides-ASA incorporated into erythrocytes were resistant to trypsin digestion while treatment with 1% BSA removed about 90% of the label. Incubation with cholera toxin protected radioiodinated GM1-ASA and, to a lesser extent, FucGM1-ASA but not GM3-ASA and GD3-ASA, against removal with BSA. After photolysis about 40-50% of radioactivity was firmly bound to erythrocyte lipids and proteins. The ratio of lipid- to protein-bound radioactivity ranged from 2.2:1 to 3.2:1. Photolabeled proteins were analyzed by SDS/PAGE followed by autoradiography. Band 3 was the most extensively photolabeled protein with all the radioiodinated gangliosides-ASA used. DIDS, an inhibitor of band 3 protein activity, caused reduction in photolabeling of this protein by about 20%.

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Abbreviations: ASA, 4-azidosalicylic acid; NHS-ASA, *N*-hydroxysuccinimidyl-4-azidosalicylic acid; DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulfonate; PMSF, phenylmethanesulfonyl fluoride. Glycosphingolipid nomenclature follows recommendations of IUPAC-IUB (*Eur. J. Bioch.* 1977, **79**, 11–21) and abbreviations of Svennerholm, (*J. Neurochem.* 1963, **10**, 613–623). G_{M3}, $II^3NeuAcLacCer$; G_{D3}, $II^3(NeuAc)_2LacCer$; G_{M1}, $II^3NeuAcGg_4Cer$; $FucG_{M1}$, $IV^2FucII^3NeuAcGg_4Cer$; Gb_4Cer, globoside; Gb_4Sph-ASA, ASA-acylated lysogloboside; Cer, ceramide, Sph, sphingosine.

Gangliosides, sialic acid containing glycosphingolipids, occur probably in all animal cells [1, 2], being localized mostly [3, 4], but not exclusively [5, 6], in the plasma membrane. It is well established that gangliosides added to culture media or present in body fluids are taken up by cells (reviewed in [7]) and may influence cell growth and differentiation [8, 9]. Gangliosides synthesized and shed by tumors can affect the host immune response ([10] and references therein). Photoactivable glycosphingolipid analogs of high specific radioactivity may prove useful in elucidating the mechanisms underlying these phenomena. A photoactivable G_{M1} ganglioside, tritium labeled to about 15 Ci/mmole was synthesized by Sonnino *at al.* [11, 12] and recently used to demonstrate its association with caveolin [13].

We have reported the preparation of a globoside acylated with ASA (Gb_4Sph-ASA) and radioiodinated to about 200 Ci/mmole [14]. Now we extend these studies to ASA-acylated radioiodinated derivatives of gangliosides differing in the structure of their oligosaccharide moieties: G_{M3}, G_{D3}, G_{M1}, and $FucG_{M1}$. Our objective was to characterize the binding of these compounds to, as well as photolabeling of, the human erythrocyte membranes used as a model system.

MATERIALS AND METHODS

Materials. Gangliosides: G_{M3} from canine erythrocytes, G_{D3}, G_{M1}, and $FucG_{M1}$ from bovine retina, brain and thyroid, respectively, were isolated by extraction with chloroform and methanol [15] and purified by DEAE-Sephadex [15] and silicic acid column chromatography [16]. Their identity was confirmed by methylation analysis [17]. Lysogangliosides were prepared from native gangliosides as described in [18]. Ganglioside standards for TLC were the same as previously used [17]. Cholera toxin, DIDS, leupeptin, and pepstatin were from Sigma. Millex LCR4 filters were from Millipore. All the remaining reagents were as specified in the preceding paper [14].

Preparation of ASA-acylated gangliosides and their radioiodination. All work with photoreactive material was done under red light or in the dark. Sphingosine NH_2 group of lysogangliosides was acylated with 4-azidosalicylic acid and the gangliosides derivatized in this way were purified and radioiodinated as before [14] with minor modifications. For preparative TLC solvent A consisting of chloroform/methanol/aqueous 0.25% $CaCl_2$ (60:35:8) was used (all solvent ratios are expressed by volume). After purification of gangliosides-ASA by preparative TLC followed by silicic acid and Sephadex LH-20 column chromatography appropriate fractions were filtered through Millex-LCR4 filter to remove silica particles, dried under a stream of nitrogen, resuspended with sonication in water and stored at -20°C.

After radioiodination followed by desalting on Sephadex G-25 Superfine and purification on silicic acid columns [14], fractions containing gangliosides ^{125}I-ASA were filtered as above, dried under nitrogen, redissolved with sonication in methanol/water (1:1) and stored at -20°C. Aliquots were withdrawn as needed, added to phosphate-buffered saline (PBS) in a polypropylene tube (Costar), the mixture was heated briefly at 40°C with sonication and left for 15 min at 37°C for equilibration before its addition to erythrocyte suspension.

Photochemical labeling of erythrocytes. Erythrocytes collected from a healthy blood donor were washed 3 times with PBS, buffy coat discarded and the remaining leukocytes removed on a cellulose column [19]. For experiments in which the effects of DIDS were studied, erythrocytes were prepared as a 20% suspension in PBS containing 10 μM DIDS and incubated in the dark for 30 min at 37°C. Unbound DIDS was removed by washing once with 1% bovine serum albumin (BSA) in PBS and twice with PBS. Erythrocytes used as a control were treated in the same way except

that DIDS was not added to the incubation mixture. Thus prepared erythrocytes as well as erythrocytes in experiments in which DIDS treatment was not included, were suspended in PBS (1×10^6 cells in 1 μl) and incubated at 37°C with gangliosides-ASA as specified in the legend to Fig. 2. At the end of incubation erythrocytes were washed twice with ice cold solution of NaCl, 124 mM; Tris/HCl, 25 mM, pH 7.4 (buffer 1).

For photolysis the erythrocytes prepared as a 3% suspension in buffer 1 containing 5 mM $CaCl_2$ were stirred at room temperature in a quartz cuvette placed 10 cm from the filter of a long wavelength UV lamp (30 watt, Cole Parmer 9815-23). Alternatively, the erythrocyte suspension in a rectangular quartz cuvette was placed in the path of a laser beam covering the whole sample, and irradiated for 1.5 s with 100 pulses of 10 ns duration and about 36 mJ energy each. A Lumonics TE861 M XeCl (Lumonics, Ottawa, Canada) excimer laser generating light at 308 nm was used. To remove unbound radioactive material immediately after photolysis the erythrocyte suspension was mixed with an equal volume of PBS containing 2% BSA (for laser photolysed samples also 2% β-mercaptoethanol) and further treated as described previously [14].

The distribution of radioactivity bound to erythrocyte lipids and proteins at the end of this treatment was determined after extraction with chloroform and isopropanol according to Rose & Oklander [20].

Preparation of erythrocyte membranes. After photolysis followed by removal of unbound radioactive material with BSA, erythrocytes were washed with PBS and used for preparation of membranes. The procedure of Dodge *et al.* [21] was followed with 5 mM phosphate, pH 8.0, hemolysis buffer modified by addition of PMSF, 1 mM; leupeptin, 2 μg/ml; and pepstatin, 1 μg/ml. Hemoglobin free membranes were dissolved in 0.5% SDS, an aliquot withdrawn for the determination of protein and the rest of the sample mixed with an equal volume of a solution consisting of 60% glycerol; 15% β-mercaptoethanol; 6% SDS and 0.01% bromophenol blue, and stored at -20°C.

Solubilized membrane proteins were analyzed by SDS/PAGE [22] in 10% polyacrylamide gels.

Other procedures

Thin-layer chromatography. For purification of ASA-acylated gangliosides by preparative TLC, solvent system A consisting of chloroform/methanol/0.25 % aqueous $CaCl_2$ (60:35:8), and silica precoated glass plates (Merck, article No. 5721) were used. Gangliosides-ASA, after separation on silica precoated aluminum plates (Merck article No. 5553) with solvent B consisting of chloroform/methanol/0.2% aqueous $CaCl_2$ (50: 40: 10), were detected with radioiodinated cholera toxin [23]. Oligosaccharides released from gangliosides-ASA by leech ceramide glycanase [24] were separated in solvent C, i.e. n-propanol/0.2% aqueous $CaCl_2$ (8:2).

Gangliosides-ASA separated by TLC were analyzed at 272 nm with Shimadzu CS 9000 scanning densitometer. UV spectra of gangliosides-ASA dissolved in methanol/water (1:1) were recorded between 200 nm and 400 nm with Shimadzu UV 2102 spectrophotometer.

The protective effect of cholera toxin against removal of gangliosides ^{125}I-ASA from erythrocytes with 1% BSA was studied as follows. Erythrocytes were incubated with gangliosides ^{125}I-ASA for 60 min as specified in the legend to Fig. 2, aliquots containing approximately 1×10^7 erythrocytes were withdrawn, washed twice with buffer 1 and their radioactivity determined in a gamma counter (LKB/Wallack 1272 Clinigamma). Erythrocytes were resuspended in buffer 1 containing 5–100 nM of cholera toxin and incubated for 45 min at 4°C. Unbound toxin was removed by two washes with cold PBS and the erythrocytes further treated with 1% BSA at 37°C [14]. The amount of gangliosides ^{125}I-ASA remaining bound to erythrocytes at the end of

this procedure was again determined in a gamma counter.

To quantitate the effect of DIDS on photolabeling of erythrocyte membrane proteins two procedures were followed. In both, membranes containing the same amount of protein prepared from DIDS treated and control erythrocytes and photolabeled with gangliosides ^{125}I-ASA, were subjected to SDS/PAGE. In the first procedure electrophoregrams were stained with Coomassie Brilliant Blue and the amount of band 3 protein was estimated by densitometric scanning (Shimadzu CS 9000 scanning densitometer). Subsequently the gels were dried, exposed to X-ray films, the band 3 containing regions (area I in Fig. 5) cut out and their radioactivity determined. In this way the radioactivity in band 3 protein was compared for DIDS treated and control erythrocytes. In the second procedure the gels were dried, exposed to X-ray films, cut into 1 mm slices and their radioactivity determined. In this way the extent of photolabeling of different protein bands was determined for DIDS-treated and control erythrocytes.

Cholera toxin was radioiodinated with chloramine T [25], then it was used for detection of G_{M1}- and $FucG_{M1}$-ASA gangliosides on TLC plates as well as for characterization of binding sites formed after incorporation into erythrocytes of G_{M1}-ASA and unmodified G_{M1} gangliosides. Conditions of ^{125}I cholera toxin binding were adapted after Miller-Podraza *et al.* [4]. Briefly, erythrocytes prepared as a 10% suspension were incubated in PBS containing 0.1 μM G_{M1}-ASA or 0.2 μM G_{M1} for 60 min at 37°C, washed twice with PBS and suspended in buffer 1. G_{M1}-ASA treated erythrocytes were irradiated with a UV lamp for 10 min and washed with 1% BSA like the samples photolabeled with radioiodinated gangliosides-ASA. Thereafter erythrocytes preincubated with G_{M1}-ASA or G_{M1} were prepared as a 10% suspension in buffer 1 and added as 10 μl aliquots (1×10^7 erythrocytes) to a solution containing BSA, 0.1%; NaCl, 128 mM; Tris/Cl, 25 mM; EDTA, 1 mM, pH 7.4; ^{125}I-cholera toxin, 0.35 nM; and increasing concentration of unlabeled toxin in a final volume of 100 μl. After 60 min incubation at room temperature the erythrocytes were washed twice with a solution containing NaCl, 147 mM; Tris/Cl, 3 mM; and EDTA, 1 mM, pH 7.4; and their radioactivity determined.

Concentration of cholera toxin was determined at 280 nm, absorption of a 1% solution being equal to 11.41 cm^{-1} [26]. DIDS dilutions were based on its molar extinction coefficient at 340 nm of 36000 M^{-1} cm^{-1} [27].

Carbohydrate composition of gangliosides-ASA was determined by the alditol acetate procedure of Yang & Hakomori [28] as recommended by Merkle & Poppe [29]. Sialic acid was determined with resorcinol [15], hexoses with phenol/sulphuric acid [30].

Protein was determined by the bicinchoninic acid procedure according to the instructions of the manufacturer (Pierce Chemical Co. Rockford, U.S.A.)

RESULTS AND DISCUSSION

Characterization of gangliosides-ASA

Lysogangliosides migrated on TLC as shown in Fig. 1 and could be stained with orcinol for hexoses, with resorcinol for sialic acid as well as ninhydrin for the presence of NH_2 group of sphingosine. Based on the sialic acid content they were prepared from native gangliosides in about 25–30% final yield, slightly lower than reported by other workers [18]. As described earlier for lysogloboside [14], lysogangliosides readily reacted with NHS-ASA. After purification by preparative TLC, silicic acid and Sephadex LH-20 column chromatography, final yields based on sialic acid assay were about 50% for G_{M3}-ASA, 27% for G_{D3}-ASA, 25% for G_{M1}-ASA and 40% for $FucG_{M1}$-ASA. The ASA-acylated gangliosides migrated on TLC in solvent system A faster than lysogangliosides, did not react with ninhydrin

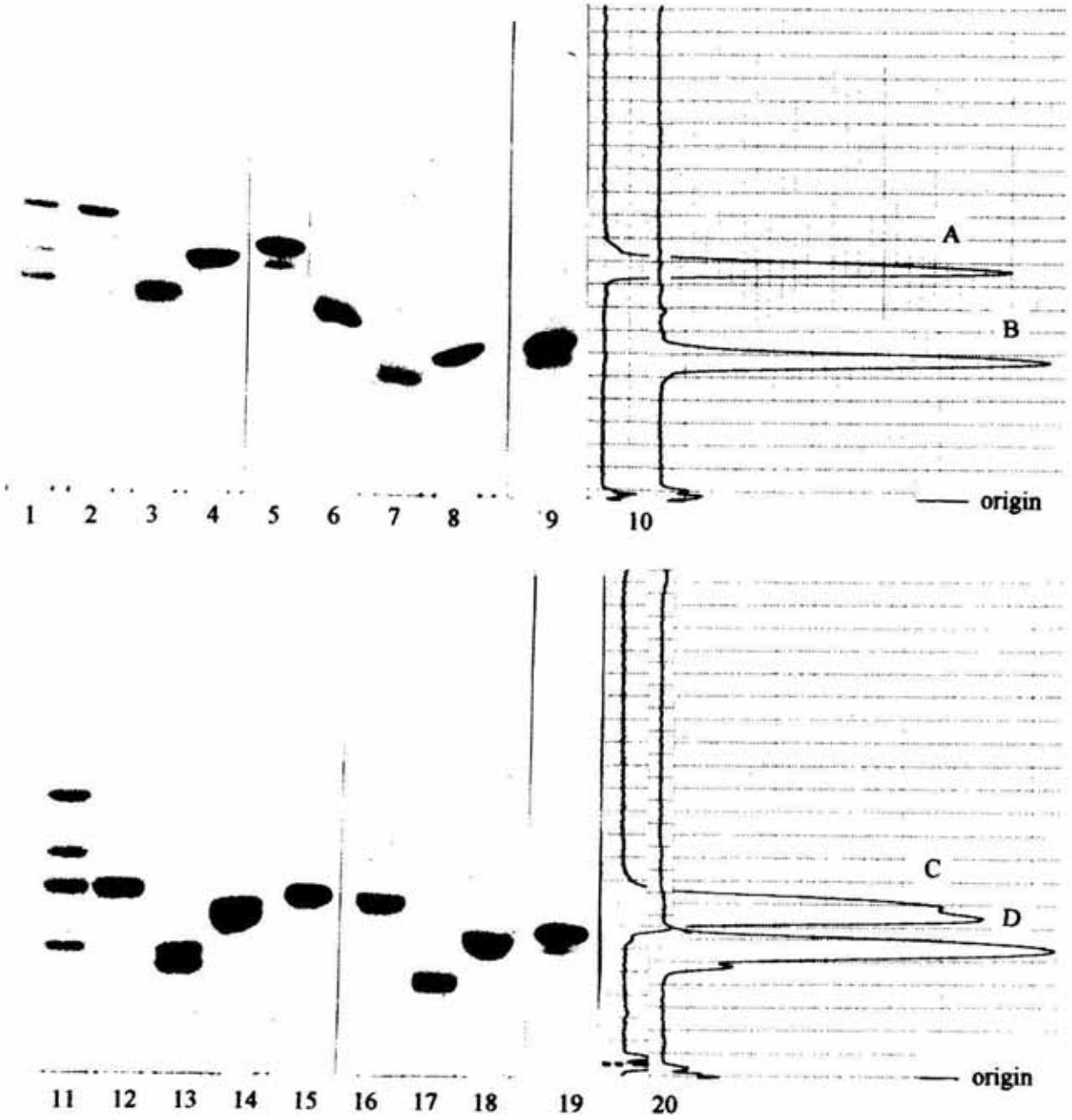

Figure 1. Thin-layer chromatography of ASA-acylated gangliosides.

Gangliosides were separated by TLC on silica-gel precoated plates with chloroform/methanol/0.25% aqueous $CaCl_2$ (60:35:8) and stained with orcinol. Radioactive material on lanes 5, 9, 15 and 19 was detected by autoradiography with X-ray film. Lanes 4, 8, 14, and 18 were analyzed by densitometric scanning at 272 nm before detection with orcinol. Lanes 1 and 11, G_{M3}, G_{M2}, G_{M1} and G_{D1a} ganglioside standards, top to bottom; lane 2, G_{M3} used in this study; lane 3, lyso G_{M3}; lane 4, G_{M3}-ASA; lane 5, G_{M3} ^{125}I-ASA; lane 6, G_{D3}; lane 7, lyso G_{D3}; lane 8, G_{D3}-ASA; lane 9, G_{D3} ^{125}I-ASA; lane 12, G_{M1}; lane 13, lyso G_{M1}; lane 14, G_{M1}-ASA; lane 15, G_{M1} ^{125}I-ASA; lane 16, $FucG_{M1}$; lane 17, lyso $FucG_{M1}$; lane 18, $FucG_{M1}$-ASA and lane 19, $FucG_{M1}$ ^{125}I-ASA. Lane 10 shows densitometric scanning of lane 4 (scan A) and of lane 8 (scan B). Lane 20 shows densitometric scanning of lane 14 (scan C) and of lane 18 (scan D). C scan has its origin moved upward to avoid overlapping.

but could be detected by densitometric scanning at 272 nm (Fig. 1).

The UV spectra of purified gangliosides-ASA dissolved in methanol/water (1:1) and

Table1. Analysis of carbohydrate composition of gangliosides-ASA

	Molar ratio of carbohydrates (Gal = 1.00)				
	Glc	Gal	GalNAc	Fuc	NeuAc
G_{M3}-ASA	1.12	1.00	n.d.	n.d.	0.95
G_{D3}-ASA	0.95	1.00	n.d.	n.d.	1.87
G_{M1}-ASA	1.03	2.00	0.98	n.d.	0.96
$FucG_{M1}$-ASA	1.04	2.00	1.02	1.00	0.94

nd, not detected; NeuAc was determined colorimetrically [15] and its ratio calculated on the basis of hexose contents [30] in the same sample.

scanned between 200 nm and 400 nm are characterized by the presence of an absorption peak at about 272 nm (272 nm for G_{M3}-, G_{D3}-, and G_{M1}-ASA, and 273 nm for $FucG_{M1}$-ASA) typical of ASA-containing compounds, as well as a smaller peak at 308 nm for the first three. For $FucG_{M1}$-ASA this peak was broader with a maximum at 332 nm. Extinction coefficients at 272 nm, calculated on the basis of sialic acid content, were for G_{M3}-ASA, $2.11 \times 10^4\ M^{-1}\ cm^{-1}$; for G_{D3}-ASA, $2.13 \times 10^4\ M^{-1}\ cm^{-1}$; for G_{M1}-ASA, $1.96 \times 10^4\ M^{-1}\ cm^{-1}$; and at 273 nm for $FucG_{M1}$-ASA, $1.84 \times 10^4\ M^{-1}\ cm^{-1}$.

Analysis of carbohydrate composition of gangliosides-ASA yielded data confirming their identity (Table 1).

As already reported for Gb_4Sph-ASA [14], gangliosides-ASA were susceptible to hydrolysis by leech ceramide glycanase which released oligosaccharides migrating on TLC in solvent C like those of parent glycosphingolipids (not shown). We conclude that a single ASA residue was coupled to sphingosine NH_2 group of lysogangliosides.

Since G_{M1} [31] and $FucG_{M1}$ [32], the parent gangliosides of G_{M1}-ASA and $FucG_{M1}$-ASA, bind cholera toxin we used radioiodinated toxin for characterization of gangliosides-ASA on TLC. As expected, G_{M1}-ASA (both components of the doublet) and $FucG_{M1}$-ASA, but not G_{M3}- and G_{D3}-ASA, bound radioiodinated toxin and could be detected on TLC by autoradiography (not shown).

Gangliosides-ASA were radioiodinated by the chloramine T procedure [25] to a specific activity of about 200 Ci/mmole. The final yield of radioiodination based on the amount of ^{125}I in gangliosides-ASA containing fractions from silicic acid column was about 50%, comparable to that obtained with Gb_4Sph-ASA [14]. Radioiodinated gangliosides-ASA migrated on TLC slightly faster than unlabeled material (Fig. 1). As already discussed for $Gb_4{}^{125}I$-ASA, all gangliosides-ASA upon radioiodination yielded not only major reaction products but also minor ones, slower migrating on TLC. The amount of radioactivity in these latter products as compared with that in the major ones ranged from about 5% for $G_{M1}{}^{125}I$-ASA (Fig. 1, lane 15, barely visible) to 15% for G_{D3}-^{125}I-ASA (Fig. 1, lane 9). We used this material without further purification.

Incorporation of radioiodinated gangliosides-ASA into human erythrocytes

Incorporation of radioiodinated gangliosides-ASA into human erythrocytes was time (Fig. 2) and concentration dependent (not shown). After about 1 h of incubation a plateau was reached corresponding to approximately 7.2×10^4; 7.8×10^4; 5.3×10^4; and 6.0×10^4 molecules of G_{M3}-, G_{D3}-, G_{M1}-, and $FucG_{M1}$-ASA, respectively, incorporated per a single erythrocyte. These numbers, quite similar in view of difficulties in estimating high specific radioactivity of gangliosides ^{125}I-ASA, imply lack of selectivity as well as a common mechanism of their binding to erythrocytes. Systematic studies on the incorporation of

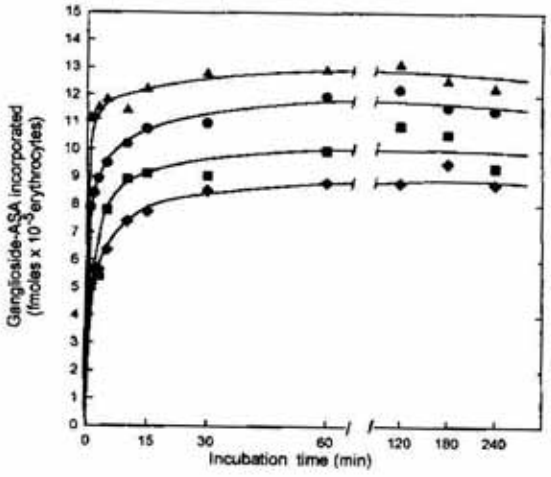

Figure 2. Effect of time on incorporation of G_{M3} ^{125}I-ASA, ●; G_{D3} ^{125}I-ASA, ▲; G_{M1} ^{125}I-ASA, ◆; and $FucG_{M1}$ ^{125}I-ASA, ■; into human erythrocytes.

Erythrocytes were incubated as a 10% suspension (1×10^6 cells per 1 μl) in 500 μl of PBS containing about 100 pmoles, 2.5×10^7 c.p.m., of different radioiodinated gangliosides-ASA. When indicated, 10 μl aliquots were withdrawn, washed twice with cold PBS and their radioactivity determined. When needed, to reduce below 0.5% the background radioactivity resulting from adsorption of radioiodinated gangliosides-ASA to plastic, erythrocytes were transferred to new tubes after the first wash.

gangliosides and their derivatives differing in oligosaccharide structures into cells and membranes was studied in only a few reports [7]. Thus far only in the case of HL-60 cells G_{M3} was found to bind several fold more efficiently than did G_{M1} [33].

Before photolysis radioiodinated gangliosides-ASA could be removed to a varying degree by treatment with different agents (Table 2). It is noteworthy that digestion with trypsin removed not more (actually slightly less) of the label than incubation in buffer 1 while most of the radioiodinated gangliosides-ASA were removed with 1% BSA. Similar susceptibility to trypsin and BSA has been reported for ASA-acylated globoside [14]. As reviewed by Saqr *et al.* [7], membrane inserted gangliosides should be resistant not only to trypsin as shown in Table 2, but also to BSA treatment. Clearly this latter criterion can not be applied to ASA-acylated glycolipids (Table 2).

We assume that gangliosides-ASA incorporated into erythrocytes resemble native glycolipids in that they reside in the membranes with their lipid residues inserted into the bilayer and oligosaccharide chains directed towards the environment free to interact with protein ligands, such as cholera toxin, when added to the medium. ASA residues would be close to the surface of the membrane and accessible to BSA. This assumption is supported by the following experiments. First, incorporation into erythrocyte membranes of G_{M1}-ASA followed by photolysis results in the formation of a single class of cholera toxin binding sites with an affinity comparable to that characteristic of unmodified G_{M1} ganglioside

Table 2. Removal of different radioiodinated gangliosides-ASA incorporated into human erythrocytes by various treatments

Treatment	G_{M3}-	G_{D3}-	G_{M1}-	$FucG_{M1}$-
			^{125}I-ASA	
	remaining bound to erythrocytes (%) ±S.D.			
Buffer 1	87.0 ± 0.5	85.0 ± 5.1	87.5 ± 1.1	85.5 ± 1.2
Trypsin, 0.25%	88.9 ± 1.3	87.2 ± 0.8	87.7 ± 3.2	83.6 ± 2.5
BSA, 1%	6.5 ± 0.4	4.9 ± 0.2	4.9 ± 0.3	4.4 ± 0.2
Cholera toxin and BSA, 1%	5.7 ± 0.2	4.2 ± 0.1	86.7 ± 0.4	26.4 ± 1.7

After incorporation of different radioiodinated gangliosides-ASA, erythrocytes were preincubated with 50 nM cholera toxin before treatment with 1% BSA. See the text for details.

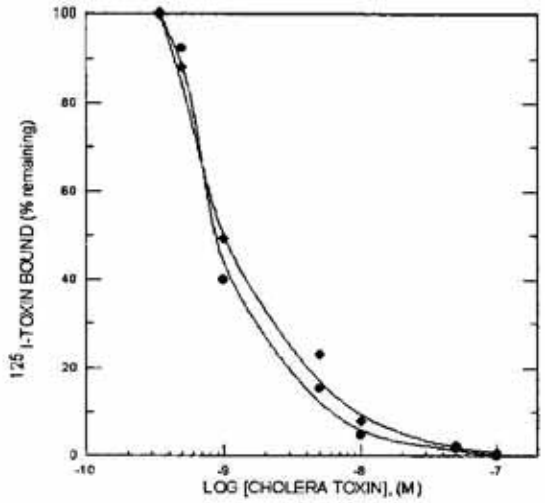

Figure 3. Competition binding of cholera toxin to erythrocytes treated with G_{M1}-ASA (◆) or unmodified G_{M1} (●).

Erythrocytes were treated with non radioactive glycosphingolipids, washed, subjected to photolysis, and aliquots of their suspension incubated with 0.35 nM ^{125}I-labeled cholera toxin in the presence of increasing concentration of unlabeled toxin which competes with radioiodinated toxin molecules in binding to erythrocyte membrane receptors. Details under Methods.

(Fig. 3). Second, the cholera toxin pretreatment before photolysis protects G_{M1} ^{125}I-ASA and also, but to a lesser extent, $FucG_{M1}$ ^{125}I-ASA, but not G_{M3}- and G_{D3} ^{125}I-ASA, against removal from erythrocytes by BSA (Table 2). We assume that cholera toxin binding to the oligosaccharide moieties of of G_{M1}- and $FucG_{M1}$-ASA prevents their contact with BSA. The stability of cholera toxin association with membranes results from several factors including the multiple character of binding in which toxin's B subunit binds to five molecules of G_{M1} ganglioside [31]. The difference in the effectiveness with which the cholera toxin protects radioiodinated G_{M1}- and $FucG_{M1}$-ASA against removal by BSA is not quite clear. It may reflect a difference in affinity with which the toxin binds to G_{M1} and $FucG_{M1}$ structures. Third, as shown below, gangliosides-ASA photolabel predominantly erythrocyte membrane lipids.

Photochemical labeling of erythrocytes

After 20 min photolysis with a UV lamp the proportion of the gangliosides ^{125}I-ASA derived radioactivity firmly bound to erythrocytes, i.e. not removed by 1% BSA, increased several fold amounting for G_{M3}-ASA to 47.6% (46.8–48.5%, n = 4), for G_{D3}-ASA to 39.0% (36.8–40.5%, n = 4), for G_{M1}-ASA to 46.6% (45.5–47.4%, n = 4), and for $FucG_{M1}$-ASA to 39.6% (37.9–40.9%, n = 4) (Fig. 4). Lipid and protein fractions obtained after photolysis and erythrocyte extraction with chloroform and isopropanol contained different amounts of radioactivity. For all gangliosides ^{125}I-ASA used in this study, the amount of radioactivity recovered in lipid fractions was about 2–3-fold higher than that remaining in protein residues. This ratio seems to depend not on the charge but rather on the bulk of an oligosaccharide of gangliosides-ASA corresponding for G_{M3}- and G_{D3}-ASA to 2.2:1; for G_{M1}-ASA to 3.0:1; and for $FucG_{M1}$-ASA to 3.2 :1. A similar ratio of lipid to protein associated radioactivity was reported for experiments in which human erythrocytes were photolabeled with Gb_4Sph-ASA [14] or glucosamine derivatized photoactivable fatty acids [34]. Evaluation of photolabeling of membrane lipids requires special studies which we did not perform in the present work. It should take into account several factors including the occurrence of double bonds in phospholipids [34].

Photochemical labeling of erythrocyte proteins

Photolabeling of erythrocyte membrane proteins was studied by SDS/PAGE followed by autoradiography and densitometric scanning [14, 34]. Photolabeling patterns obtained after laser flash photolysis or irradiation with a UV lamp for 10 min (not shown) were similar for different gangliosides ^{125}I-ASA used (Fig.

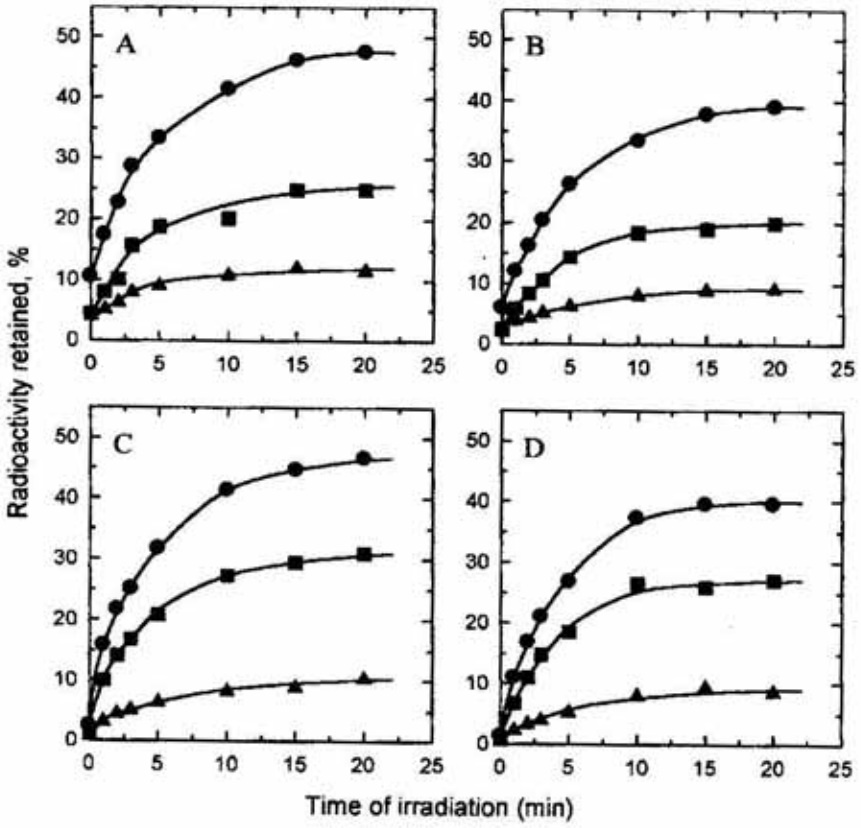

Figure 4. Time course of the photochemical labeling of erythrocytes with G_{M3} ^{125}I-ASA, (A); G_{D3} ^{125}I-ASA (B); G_{M1} ^{125}I-ASA (C), and FucG_{M1} ^{125}I-ASA (D).

Erythrocytes were incubated with radioiodinated gangliosides-ASA for 60 min as described in the legend to Fig. 2, washed with PBS, prepared as a 3% suspension in buffer 1 containing 5 mM $CaCl_2$ and irradiated with a UV lamp. Aliquots were withdrawn as indicated, treated with 1% BSA and fractionated with chloroform/isopropanol as specified in [14]. The amount of radioactivity bound to erythrocytes (●), recovered in lipid extracts (■), and protein residues (▲) was determined in a gamma counter.

5). In all experiments band 3 corresponded to the most heavily photolabeled protein. This result can be easily explained since band 3, an anion transporter and a multifunctional intrinsic protein, occurs in erythrocyte membrane in greatest abundance and spans the bilayer twelve times [36]. The degree of photolabeling of the remaining erythrocyte proteins was difficult to evaluate due to the presence of band 3 proteolytic products previously detected by immunostaining with an anti-band 3 antibody [14] and found on electrophoregrams in regions II and III (Fig. 5). Particularly interesting are the proteins located in region II: our preliminary experiments indicate, that the ratio of radioactivity to protein in this region is at least as high as for the undegraded band 3 in region I.

It has been also reported that band 3 strongly interacts with phosphoinositides [37] and perhaps can function as a flippase for anionic phospholipids, this latter activity being influenced by several inhibitors [38, 39]. To investigate if the extent of photolabeling with gangliosides-ASA can be affected by inhibitors of band 3 activity, we used DIDS, a well known irreversible inhibitor of this protein [40].

Preincubation with 10 μM DIDS had no effect either on kinetics or on the amount of radioiodinated ASA-gangliosides incorporated into erythrocytes (not shown). However, after

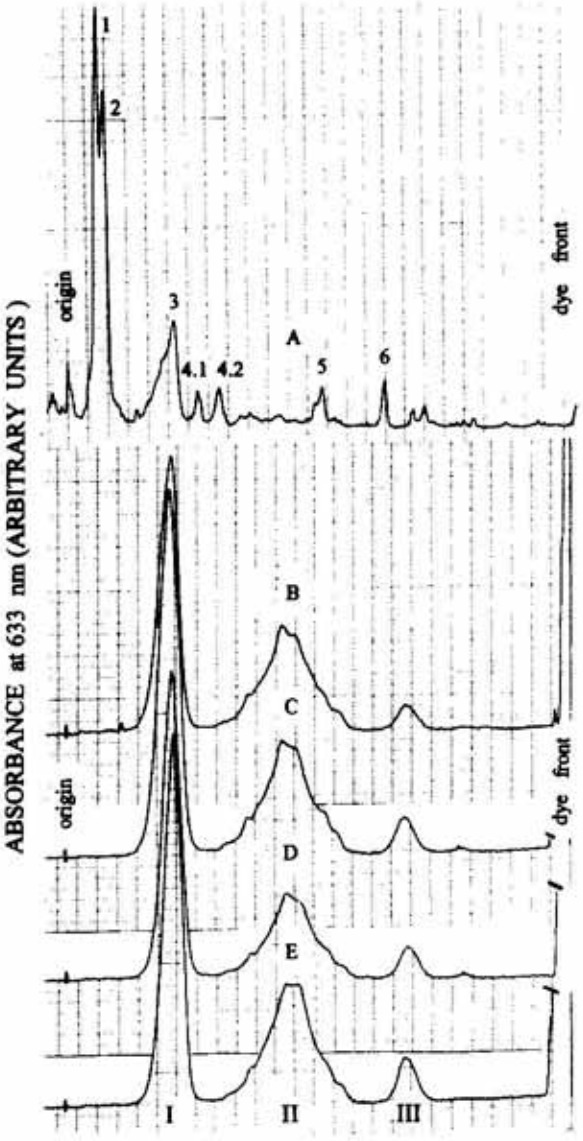

Figure 5. Photolabeling of erythrocyte membrane proteins with radioiodinated gangliosides-ASA.

Erythrocytes were incubated with gangliosides^{125}I-ASA for 60 min as specified in the legend to Fig. 2, subjected to laser flash photolysis and used for preparation of membranes. Solubilized samples containing the same amount of radioactivity (150000 c.p.m.) were separated in 10% gels by SDS/PAGE. Gels were stained with Coomassie Brilliant Blue and scanned at 633 nm. Thereafter gels were dried, exposed to X-ray films at -80°C and autoradiograms also scanned at 633 nm. A, densitometric tracing of membrane proteins stained with Coomassie Brilliant Blue and numbered after Fairbanks *et al.* [35]. B, C, D and E are densitometric tracings of autoradiograms of membrane proteins photolabeled with G_{M3}^{125}I-ASA, G_{D3}^{125}I-ASA, G_{M1}^{125}I-ASA and $FucG_{M1}$^{125}I-ASA, respectively. Undegraded band 3 in region I and its proteolytic fragments in regions II and III were previously identified with a specific anti-band 3 antibody [14].

electrophoresis of photolabeled membrane proteins the amount of radioactivity detected in band 3 was in DIDS treated erythrocytes always lower than in controls for all the gangliosides^{125}I-ASA used. When calculations were based on the densitometric estimation of band 3 protein, DIDS treatment reduced its photolabeling by 22% (14–26%, n = 3) for G_{M3}^{125}I-ASA; 23% (19–32%, n = 3) for G_{D3}^{125}I-ASA; 24% (15–30%, n = 3) for G_{M1}^{125}I-ASA; and 17% (16–18%, n = 3) for $FucG_{M1}$^{125}I-ASA. The second procedure, based on cutting the dried electrophoregrams into slices and determination of their radioactivity, gave similar results. Now DIDS caused a reduction in band 3 photolabeling by 18% (13–25%, n = 3), 25% (23–27%, n = 3), 22% (17–29%, n = 3), and 23% (15–30%, n = 3) when radioiodinated G_{M3}-ASA, G_{D3}-ASA, G_{M1}-ASA, and $FucG_{M1}$-ASA gangliosides, respectively, were used for photolabeling. DIDS treatment had little effect on photolabeling of erythrocyte proteins other than band 3, the only changes being a slight increase in the amount of radioactivity close to the origin of the electrophoregram, perhaps due to band 3 dimerization, and in the region III in Fig. 5 where band 3 proteolysis products migrate. These changes were accounted for in evaluation of the decrease of band 3 photolabeling. The increase in DIDS concentration to 50 μM did not influence its effect upon photolabeling of band 3 protein. Also the results obtained with laser flash photolysis which may increase specificity of photolabeling ([41] and references therein), fell within the range recorded when erythrocytes were irradiated with a UV lamp (not included).

The mechanism of DIDS binding to band 3 has been extensively studied ([42] and references therein). Our preliminary results do not justify excessive speculations, however, they agree well with the possibility that, in human erythrocytes, band 3 resides in glycosphingolipid enriched microdomains. Rodgers & Glaser [43] using the fluorescence imaging microscopy technique, detected in erythrocyte membranes microdomains enriched in

band 3 and phosphatidylcholine. These results as well as our observations support the assumption that one of the band 3 protein functions consists in membrane stabilization achieved through interactions with lipids [44].

This report demonstrates that acylation of lysoglycosphingolipids with ASA is a simple procedure for preparation of their photoreactive, radioiodinatable derivatives. The advantage of gangliosides-ASA is their high specific radioactivity which after separation from unlabeled material can be increased to over 2000 Ci/mmole. After incorporation of these compounds into membranes ASA residues placed close to oligosaccharide chains may facilitate photolabeling not only of membrane proteins but perhaps also of ligands binding to cells.

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