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Characterization of Acidic Glycolipids in Porcine Plasma

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Acidic glycosphingolipids including two sulfatides and five gangliosides were isolated from porcine plasma. They were characterized by NMR spectrometry as galactosylceramide-I^3-sulfate and lactosylceramide-II^3-sulfate, gangliosides GM3, GD3, GM1, GD1a and fucosyl GM1.

Glycosphingolipids of extracellular fluids are taken up *in vivo* and *in vitro* by cells. Particular glycolipids in human plasma are adsorbed on erythrocytes to express specific blood group antigenicity [3]. Conversely, release of porcine erythrocyte di- to tetraglycosylceramides into plasma has been suggested [4].

Compared to the abundant knowledge on glycolipids of erythrocytes, few studies have been made on the glycolipid composition of plasma or serum in man [5, 6] and animals [7, 8], partly due to their low concentration. Porcine plasma and serum were demonstrated to contain neutral mono- to tetraglycosylceramides with a predominant globo series core structure [4, 9, 10]. Plasma, like other tissues, of various animal species is considered to have a glycolipid pattern characteristic of the species. The present study was undertaken to characterize the acidic glycolipids of porcine plasma.

Experimental Procedures

Preparation of Glycolipids

Fresh porcine blood was obtained from a local slaughter house. From the pooled, citrated blood, plasma was obtained by centrifugation, dialyzed against water, precipitated by the

The nomenclature and abbreviations used for glycolipids are as recommended [1]. Those by Svennerholm [2] are also used for gangliosides.

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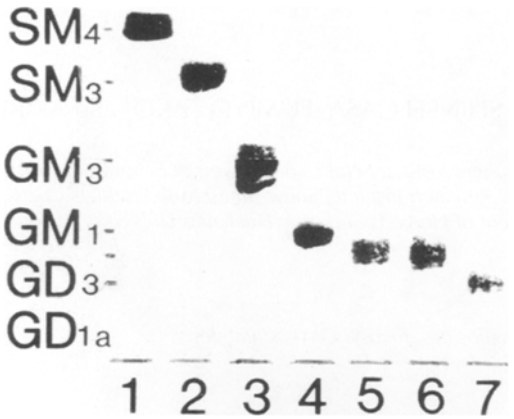

Figure 1. Thin layer chromatogram of acidic glycolipids from porcine plasma. Acidic glycolipids, AG-1 to 7 in lanes 1 to 7, respectively, were developed with solvent 3 and visualized by an orcinol-H_2SO_4 reagent. Migration positions of standard glycolipids of sulfated GalCer (SM4), sulfated LacCer (SM3), GM3, GM1 GD3 and GD1a gangliosides are shown on the left.

addition of ethanol to a final concentration of 70%, and re-centrifuged. The precipitate was extracted with 70% aqueous ethanol, by vol, at 70°C for a few minutes. The first and second extracts were combined and concentrated *in vacuo* to dryness. The dried residue was extracted twice each with acetone and diethylether to remove the bulk of the simple and phospholipids [11], and three times with chloroform/methanol, 2/1 by vol. Chloroform/methanol extracts were combined, and subjected to mild alkaline hydrolysis and to DEAE-Sephadex chromatography, as described previously [11]. The acidic glycolipids were chromatographed on an Iatrobeads (Iatron, Japan) column by stepwise elution with chloroform/methanol/water, 95/5/0, 85/15/0, 80/20/0, 80/20/2, 70/30/3, 60/40/4, 50/50/5, 20/80/5 by vol. Fractions containing heterogeneous glycolipids were re-chromatographed similarly to isolate homogeneous lipids.

Proton NMR Spectrometry

Isolated glycolipids (approx. 1 mg) in hexadeuterodimethyl sulfoxide/^2H_2O, 400/8 by vol, were analysed at 90°C as described previously [12] using a Varian JNM-GX500 in a Fourier transform mode equipped with a JEC-9808 computer at the High Resolution NMR Laboratory, Hokkaido University. The frequency was 500 MHz, and the sweep width was 5 kHz. Chemical shift was indicated by distance (ppm) from tetramethylsilane as an internal standard.

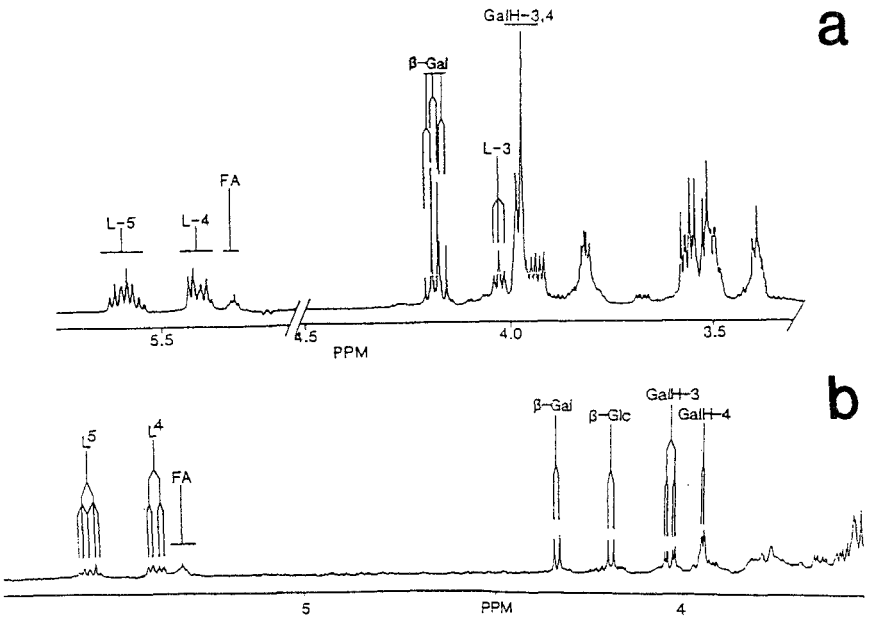

Figure 2. Proton NMR spectra of sulfatides from porcine plasma: a, AG-1; b, AG-2. FA, fatty acid; L, long chain base.

Other Methods

Thin layer chromatograms were developed with chloroform/methanol/2.5 M aqueous ammonia, 50/40/10 by vol, (solvent 1), chloroform/methanol/water, 65/25/4 by vol, (solvent 2) or chloroform/methanol/2% $CaCl_2$, 60/40/9 by vol, (solvent 3), and visualized by a resorcinol-HCl reagent or an orcinol-H_2SO_4 reagent. Desulfation of sulfatides was performed as follows. An incubation mixture contained 100 µg of a sulfatide, 1 mg sodium taurodeoxycholate and 0.5 IU of arylsulfatase A (galactose-3-sulfatase), which was partially purified from human placenta (at step 6 in [13]) in 200 µl of 50 mM sodium citrate buffer, pH 5.0. The mixture was incubated at 37°C for one day and subjected to Folch's partition. The lower phase was concentrated, and the glycolipid was subjected to TLC. Species of sialic acids in gangliosides was determined by GLC as trimethylsilyl derivatives [14]. Standard glycolipids were prepared in this laboratory except for GD3-ganglioside which was kindly provided by Dr. Eiki Deya, Technical Research Institute, Snow Brand Milk Products Co., Japan.

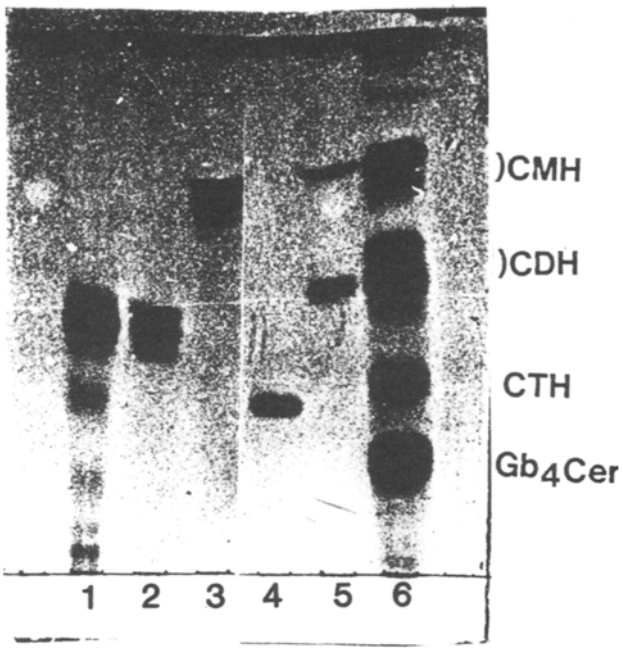

Figure.3. Thin layer chromatogram of porcine plasma sulfatides treated with a sulfatase. Lane 1, standard I^3SO_3-GalCer (upper spot); lane 2, intact porcine AG-1; lane 3, AG-1 after treatment with arylsulfatase A; lane 4, intact porcine AG-2; lane 5, AG-2 after enzyme treatment; lane 6, standard GalCer (CMH), LacCer (CDH), Gb_3Cer (CTH) and Gb_4Cer. The chromatogram was developed with solvent 2 and visualized by an orcinol-H_2SO_4 reagent.

Results

From porcine plasma, seven acidic glycolipids (AG-1 - AG-7) were isolated, two (AG-1 and 2) of which were not stained with a resorcinol reagent. They were homogeneous when examined on TLC with solvents 1, 2 and 3 (Fig. 1) and from their NMR spectra (see below).

Characterization of Plasma Sulfatides

The two resorcinol-negative, acidic glycolipids (AG-1 and 2) co-migrated with authentic sulfated GalCer and sulfated LacCer, respectively, on a thin layer plate (Fig. 1). In the proton NMR spectrum of AG-1 (Fig. 2), a triplet signal at 4.02 ppm due to a proton at C-3 of the long chain base, a double-triplet at 5.39 ppm and a double-doublet at 5.57 ppm due to *trans*-double bond protons on C-4 and C-5 of the long chain base, respectively, three doublets at 4.18, 4.19 and 4.20 ppm due to anomeric proton of β-galactose, and signals at 3.97 ppm due to the H-3 and H-4 of galactose were assigned by a spin decoupling technique, being consistent with that of sulfated GalCer of kidney [15]. In AG-2 (Fig. 2b), two doublets (4.19 and 4.33 ppm) due to anomeric protons of β-glucose and β-galactose, respectively, were observed. A double-doublet at 4.04 ppm was assigned to H-3 of galactose, which was

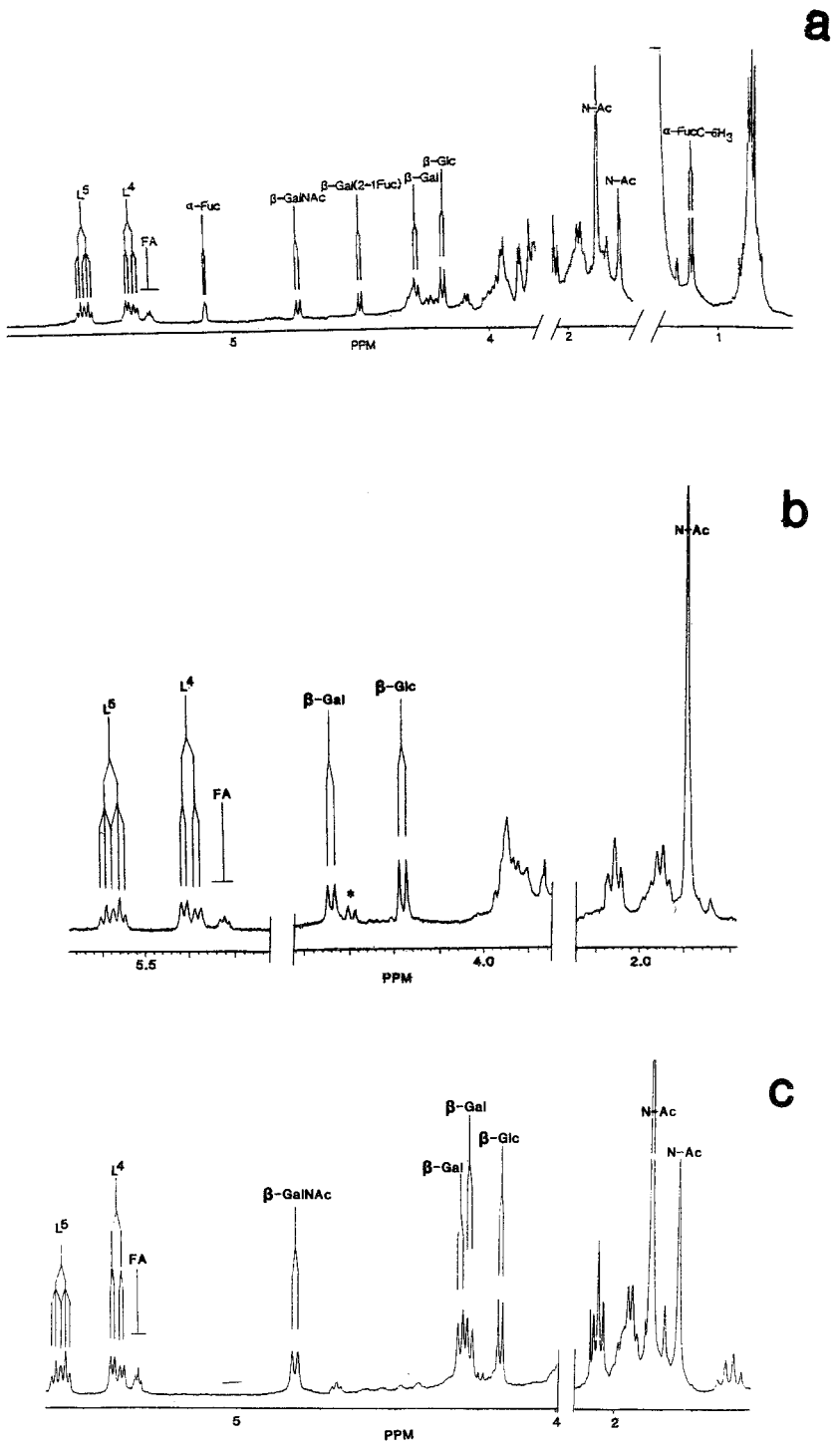

Figure.4. NMR spectra of porcine plasma gangliosides: a, AG-6; b, AG-5; c, AG-7.

Table 1. Relative contents of porcine acidic glycolipids, and their sialic acid composition.

	Relative content (%)	NeuAc (%)	NeuGc (%)
I^3SO_3-GalCer	10	-	-
II^3SO_3-LacCer	8	-	-
GM3	46	58	42
GM1	3	59	41
GD3	24	77	23
GD1a	5	73	27
Fuc-GM1	4	56	44

shifted downfield by sulfate bonding, and a doublet at 3.95 ppm to H-4 of galactose. The spectrum of AG-2 was consistent with that of sulfated LacCer from human renal cell carcinoma [16]. Upon treatment with arylsulfatase A, S-1 was converted into a glycolipid corresponding to GalCer, and AG-2 into LacCer with a small amount of monohexosylceramide which was probably produced by a β-galactosidase activity present in the enzyme preparation (Fig. 3). On the basis of the above results, AG-1 and AG-2 from porcine plasma were identified as I^3SO_3-GalCer and II^3SO_3-LacCer, respectively.

Characterization of Plasma Gangliosides

Fig. 4 shows the proton NMR spectra of resorcinol-positive, acidic glycolipids from porcine plasma. Assigned signals in one (AG-6, Fig. 4a) of them are: Methyl protons of fucose at 1.11 ppm; methyl protons of *N*-acetyl groups in *N*-acetylneuraminic acid and in *N*-acetylgalactosamine at 1.80 and 1.89 ppm, respectively; H-1 of β-glucose at 4.18 ppm; H-1 of β-galactose at 4.29 ppm; H-1 of β-galactose, to which a fucose is bound, at 4.50 ppm; H-1 of β-*N*-acetylgalactosamine at 4.74 ppm; H-1 of α-fucose at 5.10 ppm; *cis* double bond protons of fatty acids at 5.32 ppm; *trans* double bond protons of C-4 and C-5 in the long chain base at 5.38 and 5.57 ppm, respectively. These signals suggest the structure $IV^2\alpha Fuc,II^3NeuAc\text{-}Gg_4Cer$, and were essentially identical with those of equine erythrocyte fucosyl GM1 (Fuc-GM1), which contains only *N*-glycolylneuraminic acid [17], except for the *N*-acyl group in the sialic acid. Likewise, AG-3, AG-4, AG-5 (Fig. 4b) and AG-7 (Fig. 3c) were properly characterized as GM3, GM1, GD3 and GD1a, respectively. The porcine plasma gangliosides contained both *N*-acetyl- and *N*-glycolylneuraminic acids (Table 1). The relative amounts of porcine acidic glycolipids are shown in Table 1, with GM3 being predominant.

Discussion

Although sulfatides had not been demonstrated in plasma, Hara and Taketomi [18] recently characterized sulfated GalCer in rabbit serum, and a considerable increase of the sulfatide as well as other glycolipids in an inherited hyperlipidemic rabbit. On the basis of analysis of the lipid moiety in the sulfatide, they presumed that the serum sulfatide associated with lipoproteins is derived from liver rather than the small intestine. On the other hand, it has been demonstrated that sulfatides activate human blood coagulation Factor XII [19], and bind specifically to Factor VIII/ von Willebrand factor [20]. Thus it seems likely that plasma sulfatides, besides sulfatides in erythrocytes [20, 21] and platelets [20], play an essential role in blood coagulation. To our knowledge, gangliosides other than GM3 have not been completely characterized in plasma. In the present study, GD3, GM1, GD1a and Fuc-GM1, in addition to GM3 were characterized in porcine plasma. Thus, in pig, plasma has a more complex distribution pattern of gangliosides than those (GM3 and GD3) in the erythrocytes [22].

Acknowledgements

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