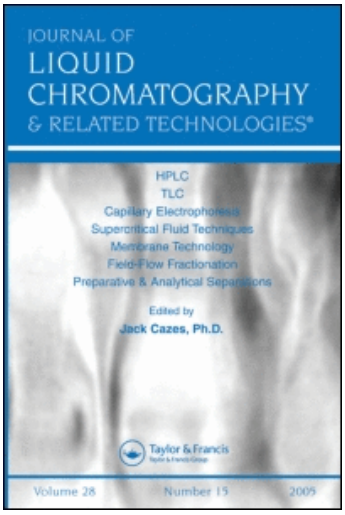

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Increased Levels of Granulocyte-Specific Glycosphingolipids in Preterm Labor Amniotic Fluid

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INCREASED LEVELS OF GRANULOCYTE-SPECIFIC GLYCOSPHINGOLIPIDS IN PRETERM LABOR AMNIOTIC FLUID

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ABSTRACT

In a recent study, Hallman *et al.* reported high levels of lactosyl ceramide in amniotic fluid from preterm labor and postulated that this glycosphingolipid is of leukocytic origin. However, lactosyl ceramide is an ubiquitous glycosphingolipid also present in other cell types. In this study, the concentration of the granulocyte-specific glycosphingolipids, paragloboside and sialyl paragloboside in term and preterm amniotic fluids, was monitored by HPTLC-immunostaining using monoclonal antibodies E5C2 and 534F8, repectively. Amniotic fluid samples were obtained by transabdominal amniocentesis for clinical indications and divided in preterm not in labor (n = 18); preterm labor (n = 20); term not in labor (n = 20); and term labor (n = 16). The gestational age ranged from 25 to 41 weeks and preterm was defined as less than 37 weeks gestation. Diabetic patients were excluded from this study and none of the patients had clinical signs or symptoms of chorioamnionitis. Samples with macroscopic blood contamination were also excluded. The samples from each group were pooled, lyophilized, the residues extracted with chloroform-methanol-water (C-M-W, v/v/v), and the glycosphingolipid fraction isolated by a combination of peracetylation with pyridine-acetic anhydride, Florisil column chromatography, and deacetylation with sodium methoxide. Aliquots of the purified glycosphingolipid fraction were streaked onto either Whatman HP-K or Merck aluminum-backed silica gel plates for high performance thin layer chromatographic analysis (HPTLC), following reaction with the orcinol-ferric chloride reagent; or HPTLC-immunostaining following reaction with anti-paragloboside and anti-sialyl paragloboside monoclonal antibodies E5C2 and 534F8, respectively. Preterm labor amniotic fluid contained significantly higher concentrations of the granulocyte-specific glycosphingolipids lactosyl ceramide, paragloboside and sialyl paragloboside as compared to term labor amniotic fluid. Preterm and term amniotic fluids with no labor had low to undetectable levels of these glycosphingolipids. These results confirm the findings reported by Hallman *et al.* and provide additional evidence that supports the hypothesis that the onset of preterm labor may be associated with subclinical infection.

INTRODUCTION

Preterm labor is one of the leading causes of neonatal morbidity. The factor(s) responsible for the triggering of preterm labor has not yet been elucidated. It is widely accepted that the onset of labor is elicited by the mobilization of arachidonic acid and synthesis of prostaglandins in fetal membranes[2]. The sources of arachidonic acid to support prostaglandin production in human amnion during early labor are phosphatidylethanolamine and phosphatidylinositol[3]. In addition to phopholipase A_2, a phosphoinositoside-specific phospholipase C has been found in human amnion[4]. This phospholipase appears to facilitate the mobilization of arachidonic acid from phosphatidylinositol. Bacterial phospholipases have been implicated in the initiation of preterm labor by releasing arachidonic acid from amnion-associated phospholipids and promoting the synthesis of prostaglandins[5]. Indirect evidence also suggests that products released by bacteria could play a role in the onset of premature labor[6].

Increasing evidence indicates that the onset of premature labor is associated with the presence of granulocyte-derived products in amniotic fluid. Hallman *et al.* reported the presence of high concentrations of lactosyl ceramide in amniotic fluids from women in preterm labor[7]. These authors postulated that lactosyl ceramide is a leukocyte-derived glycosphingolipid and that the presence of leucocytes in the area is secondary to subclinical infection. The question of whether the leucocyte infiltrate was localized in the amnion, and/or in the amniotic fluid or elsewhere, was not addressed in that report. Although lactosyl ceramide is a major granulocyte glycolipid, its isolation from amniotic fluid does not prove its leucocytic origin since this is an ubiquitous glycosphingolipid found in a variety of cell types. Hallman *et al.* also reported that activated human granulocytes release a factor that stimulates prostaglandin E_2 synthesis in human amnion cells[8].

In this study we monitored the concentration of the granulocyte-specific glycosphingolipids paragloboside and sialyl paragloboside in amniotic fluid from term and preterm pregnant women with and without labor, by HPTLC-immunostaining using monoclonal antibodies E5C2 and 534F8.

MATERIALS AND METHODS

Chemicals and Reagents

The glycolipid standards including, galactosyl ceramide (GalCer), lactosyl ceramide (LacCer), Gb_3, Gb_4, GM_1, GD_{1a}; the orcinol ferric chloride spray reagent; and type VI neuraminidase were purchased from Sigma Chemical Co (St. Louis, MO). A glycolipid mixture from HL-60 granulocyte cell line (1μg/μL); and the monoclonal antibodies 534F8 and E5C2 were a generous gift of Dr. Steven Spitalnik from the University of Pennsylvania. Poly(iso-butyl methacrylate) was obtained from Polysciences Inc. (Warrington, PA). Precoated silica gel HP-K high performance plates (10 x 10 cm, 250 μm thickness) were obtained from Whatman Inc. (Clifton, N.J.). Precoated aluminum-back silica gel plates (20 x 20 cm, 250 μm thickness) were obtained from Merck. Silica gel (60A pore, 35-75 μm particle size) for column chromatography, was purchased from Analtech Inc. (Newark, DE, U.S.A.). DEAE-Sephadex A-25 was obtained from Pharmacia Fine Chemicals (New Jersey). Solvents were EM Science chromatographic grade. Inorganic salts were from J.T. Baker (Phillisburg, N.J.) and of the highest purity available.

Sample preparation

Amniotic fluid samples were obtained by transabdominal amniocentesis for clinical indications and divided in preterm not in labor (n = 18); preterm labor (n = 20); term not in labor (n = 20); and term labor (n = 16). The gestational age ranged from 25 to 41 weeks and preterm was defined as less than 37 weeks gestation. Diabetic patients were excluded from this study and none of the patients had clinical signs or symptoms of chorioamnionitis. All fluid cultures were negative for bacteria and mycoplasma. Following collection, the samples were stored at -4°C until the indicated number, n, was reached.

Glycosphingolipid Extraction and Purification

Samples were thawed at 37°C, pooled, lyophilized, and the resulting residue extracted with 20 volumes of chloroform-methanol-water (C:MW) (4:8:3, v/v/v)[9].

The C-M-W extracts corresponding to each group were evaporated to dryness, and the residue hydrolyzed with 1 mL of 0.1N sodium methoxide for 1 hr at 40°C[10]. The hydrolysate was cooled to room temperature, the alkali-stable glycolipids extracted with 6 volumes of C-M 2:1, evaporated to dryness and peracetylated with a mixture of pyridine-acetic anhydride. The peracetylated glycosphingolipid fraction was purified by Florisil column chromatography and deacetylated with 0.1N sodium methoxide according to the procedure of Saito and Hakomori[11].

High Performance Thin Layer Chromatography (HPTLC)

Aliquots of 4μL of the glycosphingolipid fraction dissolved in 1 mL of C-M were applied to Whatman HP-K silica gel plates, the plates predeveloped in C-M (1:1, v/v) to ca. 8 mm from the lower edge of the plate, thoroughly dried, and developed in C-M-W (60:30:8, v/v/v) to 5 cm. The plates were sprayed with the orcinol-ferric chloride reagent and placed in an oven with initial and final temperatures of 24°C and 100°C, respectively for the visualization of sugar hemiketal-containing compounds including saccharides and/or glycolipids. The resulting bands were scanned at 550 nm with a Shimadzu CS-9000 spectrodensitometer in the transmittance mode[9].

Analysis of glycosphingolipid-bound sphingoid bases by HPTLC-fluorescence spectrodensitometry

Aliquots of 50 μg of the glycosphingolipid fraction isolated from preterm amniotic fluids were hydrolyzed with 1 mL of 1N HCl at 100°C for 14 hr. and the glycosphingolipid-bound sphingoid bases analyzed by HPTLC-fluorescence spectrodensitometry, as described by Alvarez *et al* [12]. The resulting fluorescent bands corresponding to the sphingoid bases from the glycosphingolips isolated from preterm amniotic fluids were scanned with a Shimadzu CS-9000 spectrodesitometer in the fluorescence mode using 254 nm for excitation and a cut-off filter at 400 nm for emission.

HPTLC-Immunostaining

When indicated, 20cm x 20cm aluminum-backed Merck silica gel HPTLC plates were cut to the appropiate size and 4μL aliquots of the standards and test

samples applied 5mm from the lower edge of the plate. The plates were predeveloped in C-M, developed in C-M-W (50:45:10, v/v/v) and thoroughly dried at room temperature. Following development the lane corresponding to the standards was cut off, sprayed with the orcinol-ferric chloride reagent, and placed in an oven as indicated above. The other portion of the plates was then dipped in a 0.1% poly(iso-butyl methacrylate) (Polysciences, Inc. Warrington, PA) solution in hexane for 90 sec.and processed for HPTLC immunostaining of glycolipids according to the procedure described by Magnani *et al.* [13] as modified by Dubois *et al.* [14]. The plates were sequencially overlayed with 60 μL/cm^2 of the appropiately diluted solution of the primary monoclonal antibodies E5C2 and/or 534F8, specific for paragloboside , and for sialyl paragloboside[14], respectively, followed by ^{125}I-labelled secondary antibody (mouse IgM and rabbit IgM, respectively) (2 x 10^6 cpm/mL). The immunoreacted glycolipids were visualized by autorradiographic detection using a Kodak X-omat autorradiography film for 16-24 hr.

In a another set of experiments and following development, the plates were washed x 5 with a buffer solution consisting of 0.05M sodium acetate, 0.15M NaCl, 0.009M $CaCl_2$, pH 5.5.(neuroaminidase buffer) and the plates overlayed with 60 μL/cm2 of neraminidase buffer alone or 0.2 units/mL of *Clostridium perfringes* in neuraminidase buffer. The plates were then treated with mAb E5C2 and ^{125}I-labelled secondary antibody, as indicated above. In situ removal of sialic acid from sialyl paragloboside by neuroaminidase treatment following separation of the glicolipids allowed probing of this glycosphingolipid using the monoclonal antibody, E5C2.

RESULTS AND DISCUSSION

The orcinol-stained HPTLC chromatogram corresponding to the glycosphingolipids isolated from term and preterm amniotic fluids, is shown in Fig. 1. Based on comigration with authentic standards, resistance to alkaline hydrolysis, and on the sphingoid base content, the orcinol-reactive components were tentatively identified as the glycosphingolipids, monohexosyl ceramide, dihexosyl ceramide, trihexosyl ceramide, and tetrahexosylceramide ceramide. The concentration of these glycosphingolipids in amniotic fluid from women in preterm labor was significantly higher when compared to the other three groups (Table1).

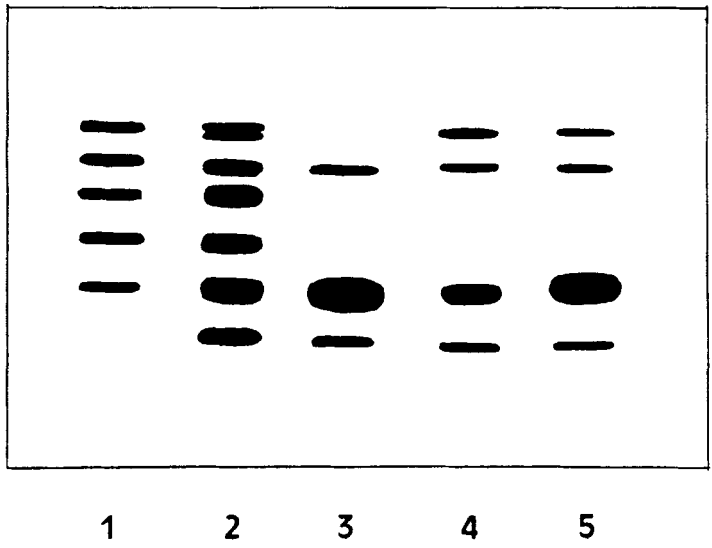

FIG. 1: Orcinol-Stained Chromatogram of Glycosphingolipids from Amniotic Fluid. 1: glycosphingolipid standards including from top to bottom, galactosyl ceramide, lactosyl ceramide, globotriaosylceramide, globotetraosylceramide, and GD1a; 2: preterm labor glycolipids;3: term labor glycolipids;4: preterm not in labor glycolipids; 5: term not in labor glycolipids.

TABLE 1: Levels of Glycosphingolipids in Amniotic Fluid

glycosphingolipid	PL	TL	PNL	TNL
ceramide dihexoside	4.0[a]	1.3	0.6	0.6
ceramide trihexoside	4.5	ND	ND	ND
ceramide tetrahexoside	5.2	ND	ND	ND

PL: preterm labor
TL: term labor
PNL: preterm no labor
TNL: term no labor

a: values are expressed in μg/20mL of amniotic fluid

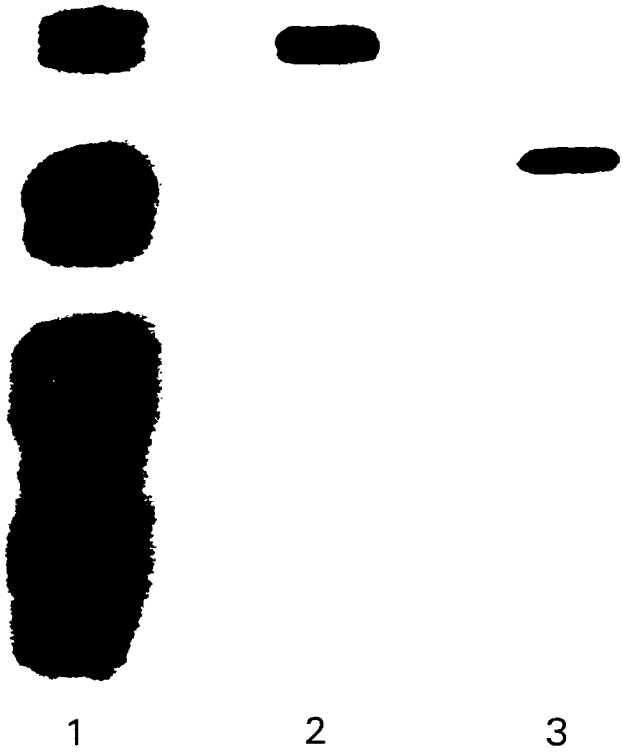

FIG. 2: Autoradiogram of Preterm Labor Amniotic Fluid Glycolipids Following HPTLC-Immunostaining Monoclonal Antibodies E5C2 and 534F8. 1: HL-60 cell line glycolipid mixture; 2: monoclonal antibody E5C2; 3: monoclonal antibody 534F8.

Amniotic fluid glycolipids isolated from the four selected groups, were probed with the granulocyte-specific anti-glycosphingolipid monoclonal antibodies E5C2 (anti-paragloboside) and 534F8 (anti-sialylparagloboside). Two immnoreactive bands that comigrated with paragloboside and sialyl paragloboside from the granulocytic cell line HL-60, were only detected in amniotic fluids from preterm labor (Fig. 2). Amniotic fluids from the other three groups had no detectable levels of these glycosphingolipids. Treatment of the developed chromatogram with neuroaminidase followed by immunostaining with monoclonal

antibody E5C2 resulted in an additional immunoreactive component that comigrated with sialyl paragloboside, indicating that indeed this glycosphingolipid is sialylated paragloboside.

The predominant glycosphingolipid-bound sphingoid base, as analyzed by HPTLC-fluorescence spectrodensitometry, was sphingenine (95%) with trace amounts of sphinganine (2%), icosasphinganine (2%) and icosasphingenine (1%). All orcinol-reactive bands released sphingoid bases following acid hydrolysis, indicating that these components are glycosphingolipids.

These results indicate that only amniotic fluid from preterm labor contained significant amounts of the glycosphingolipids lactosyl ceramide, paragloboside, and sialyl paragloboside. This study confirms the results reported by Hallman *et al.* where high concentrations of lactosyl ceramide were found in amniotic fluid from preterm labor. In addition, the presence of the granulocyte-specific glycosphingolipids, paragloboside and sialyl paragloboside in amniotic fluid from preterm labor provides additional evidence that supports the hypothesis that the onset of premature labor may be associated with subclinical infection.

Abbreviations[1]: GalCer, Gal(β1-1)Cer; LacCer, Gal(β1-4)Glc(β1-1)Cer; Gb_3, Gal(α1-4)Gal(β1-4)Glc(β1-1)Cer; Gb_4, GalNAc(β1-3)Gal(α1-4)Gal(β1-4)Glc(β1-1)Cer; GM_1, $II^3\alpha NeuAcGgOse_4Cer$; paragloboside, Gal(β1-4)GlcNac(β1-3)Gal(β1-4)Glc(β1-1)Cer; sialyl paragloboside, NeuAc(α2-3)Gal(β1-4)GlcNac(β1-3)Gal(β1-4)Glc(β1-1)Cer; GD1a, $IV^3NeuAc,II^3NeuAc-GgOse_4Cer$; GD1b, $II^3\alpha(NeuAc)_2GgOse_4Cer$.

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