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Detection of complex gangliosides in human amniotic fluid

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Gangliosides are possibly very potent immunosuppressive molecules. Here we show that human amniotic fluid contains high concentrations of a number of previously unnoted, structurally complex and highly polar gangliosides. These unusual molecules are present early in pregnancy (first trimester), increase in concentration with gestational age, and reach maximum levels (0.8 μM) at term. Since similar gangliosides have been detected in human placenta, trophoblast, and amnion, we suggest that these molecules are shed into the amniotic fluid bathing these tissues.

Shed ganglioside, Amniotic fluid

1. INTRODUCTION

During pregnancy, there is an intense proliferation of the haploidentical fetal allograft, which should result in its rejection. Why this does not occur remains an enigma. However, the maternal–fetal interface may have a role in prevention of fetal rejection [1–6], shedding immunosuppressive molecules which can be found in the amniotic fluid, which itself has immunosuppressive properties [7–10]. One such class of immunosuppressive molecules is the gangliosides, sialic acid-containing glycosphingolipids which are embedded in cell membranes by their ceramide portion.

The shedding of cell surface membrane components such as gangliosides is characteristic of rapidly proliferating cells [11]. Therefore, gangliosides from the maternal–fetal system might be found in the amniotic fluid. Since the shedding of tumor gangliosides inhibits normal cellular immune responses [12], we hypothesize that such shedding could likewise modify the immunological interaction between the fetus and the mother. The object of this study therefore was to characterize the ganglioside content of human amniotic fluid.

We expected the ganglioside composition of human amniotic fluid to reflect the ganglioside composition of the placenta, trophoblast, and amnion, in which the ganglioside G_{M3} is predominant [13–16], because ganglioside shedding generally reflects, qualitatively and quantitatively, ganglioside synthesis [17]. To our surprise, we detected a series of structurally complex and highly polar gangliosides in human amniotic fluid as the major and almost exclusive components.

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Abbreviations. C, chloroform; M, methanol; HPTLC, high-performance thin-layer chromatography; LBSA, lipid-bound sialic acid.

2. MATERIALS AND METHODS

2.1. *Amniotic fluid samples*

Aliquots of amniotic fluid samples which had been obtained from pregnant women at 16–40 weeks of gestation by transabdominal amniocentesis under sterile conditions were studied. The samples were cooled to 4°C immediately and rapidly processed.

After an initial analysis of unfractionated amniotic fluid, all samples were fractionated by centrifugation according to the scheme shown in Fig. 1. Cells (mostly epithelial) present in the amniotic fluid were removed by centrifugation at $800 \times g$ for 15 min at 4°C, and the supernate was recentrifuged at $8{,}000 \times g$ for 15 min at 4°C, to remove cell fragments. In some samples, particularly those obtained at the end of pregnancy, it was also possible to isolate surfactant as a floating white layer after centrifugation. To determine the qualitative and quantitative distribution of gangliosides in human amniotic fluid and its particulate fractions and in surfactant, each component was processed separately under identical conditions.

2.2. *Ganglioside purification*

The total gangliosides of the samples were purified by total lipid extraction, diisopropyl ether (DIPE)/1-butanol/aqueous phase partition and Sephadex G-50 gel filtration as developed for the isolation of gangliosides from fluids [18,19]. Total lipid extracts were prepared using 2 ml chloroform/methanol (C:M) (1:1, v/v) per ml amniotic fluid and 20 ml C/M (1:1, v/v) per gram of cells, cell fragments or surfactant. All samples were extracted twice. The extracts were pooled and clarified by centrifugation ($750 \times g$ for 10 min at 4°C) to remove particulate matter, reduced to approximately 1/4 of their original volume by rotary evaporation, and cooled to −20°C. Additional insoluble material was removed by centrifugation and the final total lipid extracts were taken to dryness.

Partitioning was accomplished by dispersing the dried total lipid extracts in DIPE/1-butanol (60:40, v/v), with several minutes of vortexing and bath sonication. 4 ml of the organic solvent mixture was used for the total lipid extract of each 10 ml of amniotic fluid and 20 ml for the total lipid extract of each gram of cells, cell fragments, or

surfactant Next, distilled water (for amniotic fluid) or 0.1% aqueous NaCl (for cells, cell fragments and surfactant), one half of the volume of the organic solvent, was added with vortexing and sonication. After centrifugation at 750 × *g* for 10 min to separate the two phases, the upper organic phase (containing the neutral lipids and phospholipids) was carefully removed. The ganglioside-containing lower aqueous phase was re-extracted twice with the original volume of fresh organic solvent, and the final aqueous phase was lyophilized. The samples were redissolved in a small volume of distilled water, sonicated, and subjected to Sephadex G-50 gel filtration, using distilled water as the eluting solvent. Gangliosides were recovered in the void volume peak, lyophilized, redissolved in a small volume of C/M (1·1), and centrifuged to remove insoluble residual proteins co-extracted with gangliosides The purified gangliosides were recovered from the clear supernate and stored under N_2 at −20°C

2.3 *Ganglioside analysis*

Gangliosides were quantitated as nmol lipid-bound sialic acid (LBSA) by the modified colorimetric resorcinol assay [20]. High-performance thin-layer chromatography (HPTLC) of gangliosides was performed using 10 × 20 cm precoated Silica Gel-60 HPTLC plates (E. Merck, Darmstadt, Germany) Two HPTLC development systems were used to separate the glycophingolipids The first was the solvent system widely used for ganglioside analysis, C/M/0.25% $CaCl_2$ $2H_2O$ (60:40·9, v/v/v) [20] The second, described by Rosner [21], is particularly suitable for visualizing highly polar highly complex gangliosides. In this method gangliosides are applied to the HPTLC plate which is then warmed for 1 h at 50°C. After cooling, the plates are developed by consecutive runs (in the same direction and at 38°C) in two different solvent systems: (a) C/M/12 mM $MgCl_2$/15 N NH_4OH (60·37:7·3, v/v/v/v), and (b) C/M/12 mM $MgCl_2$ (58:40:9, v/v/v). Gangliosides were visualized as purple bands with resorcinaol reagent [22]

3. RESULTS

3.1. *Human amniotic fluid gangliosides*

In initial experiments we analyzed the total gangliosides of four samples of unseparated amniotic fluid obtained at different periods of gestation, using the standard ganglioside HPTLC solvent system of C/M/0.25% $CaCl_2 \cdot H_2O$ (60:40:9, v/v/v) (Fig. 2). Only traces (< 50 pmol LBSA/ml amniotic fluid) or no resorcinol- positive (ganglioside) bands were seen in the region of migration of most of human brain gangliosides in this solvent system (i.e. between G_{M3} and G_{T1b}). However, a very densely staining resorcinol-positive band is visible near the origin of the HPTLC in each of the four amniotic fluid samples (Fig. 2). This band has an exceedingly slight but consistent migration above the origin of the HPTLC, suggesting that these molecules may be very complex gangliosides. To investigate these molecules further, we therefore chose a solvent system which is particularly suitable for resolving slowing migrating gangliosides [21].

In the first experiment in which we applied this method to a sample of pooled amniotic fluid, we detected a series of four highly complex gangliosides which were the main components of this fluid (Fig. 3). For comparison, total human brain and plasma gangliosides are shown, demonstrating the strikingly slower migration of the human amniotic fluid gangliosides, all more polar than G_{T1b}. The ganglioside nature of these resorcinol-positive molecules was further supported by

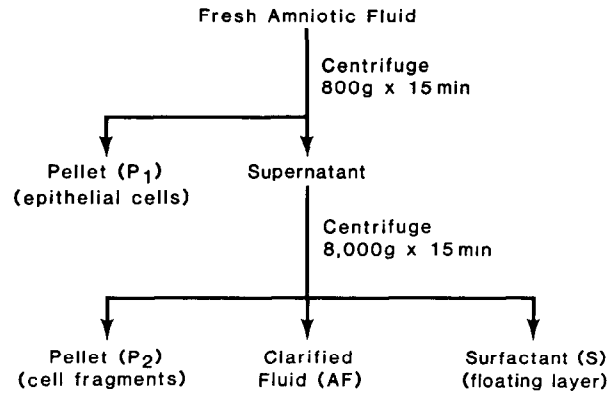

Fig. 1. Separation of amniotic fluid fractions.

the detection of sphingosine by positive ion FAB mass spectrometry after acid hydrolysis [23] of the total ganglioside extract (not shown). Significantly, G_{M3}, the most prevalent extraneural ganglioside, was undetectable in the amniotic fluid. This experiment gave the first suggestion that human amniotic fluid is characterized by an easily visible concentration of highly complex gangliosides, and a paucity of the usually prevalent extraneural ganglioside, G_{M3}.

3.2. *Changes in human amniotic fluid ganglioside content with gestational age*

Seventeen human amniotic samples, freshly obtained and immediately clarified by centrifugation, were studied to determine the range of ganglioside concentration in human amniotic fluid, and the effect of gestational age. Table I shows that the ganglioside concentration in amniotic fluid increases at least fourfold from the first trimester of pregnancy until term, with the median concentration rising from 0.2 μM LBSA at 16 weeks to 0.8 μM LBSA at the end of pregnancy.

To determine whether the total ganglioside composition undergoes qualitative change with increasing gestational age, the total gangliosides isolated from amniotic

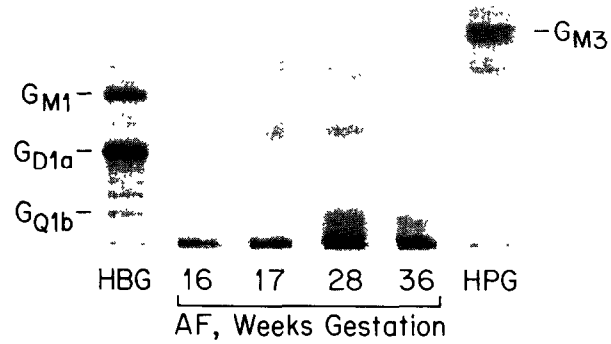

Fig. 2. Amniotic fluid gangliosides. Total purified amniotic fluid gangliosides (AF) were separated by HPTLC using the solvent system, C/M/0.25% $CaCl_2 \cdot 2H_2O$ (60.40.9, v/v/v). 2–5 nmol were spotted per lane. HBG, human brain standard gangliosides; HPG, human plasma gangliosides. All bands were resorcinol-positive.

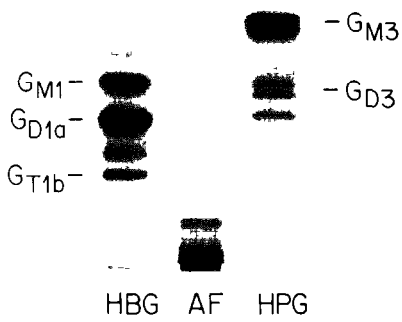

Fig. 3. HPTLC separation of highly polar amniotic fluid gangliosides. 4 nmol total human amniotic fluid gangliosides were separated by development of the plate in C/M/12 mM $MgCl_2$/15 N $NH_3 \cdot H_2O$ (60:37:7:3, v/v/v/v), followed by C/M/12 mM $MgCl_2$ (58:40·9, v/v/v). Abbreviations as in Fig. 2

fluid obtained at different periods of pregnancy were analyzed by the HPTLC method of Rosner [21]. As shown in Fig. 4, over the period of gestation studied (16–40 weeks of pregnancy), the pattern of amniotic fluid gangliosides is constant. Furthermore, there are not more than traces of simple ganglioside such as G_{M3} in amniotic fluid. The striking constancy of individual species from the first sample (16 weeks) to the final sample (end of pregnancy) suggest that the production of these highly complex gangliosides continues, without qualitative change, throughout pregnancy.

3.3. *Gangliosides of amniotic fluid cellular components and surfactant*

The above studies detecting complex gangliosides were performed on amniotic fluid which had been clarified by centrifugation to assure that gangliosides isolated were not associated with cellular or other particulate matter contained in amniotic fluid. Removed from the amniotic fluid in this process are centrifugation pellets (which include epithelial cells and cell fragments) and sometimes a floating lower density fraction, probably surfactant, especially visible in the case of samples obtained at term. Gangliosides from these two minor fractions, comprised at most only a few percent of the total amniotic fluid gangliosides. However, these fractions yielded a surprising pattern (Fig. 5); in contrast to the amniotic fluid gangliosides (complex), the predominant ganglioside in each of these fractions was G_{M3}.

Table I

Ganglioside concentration of amniotic fluid: Effect of gestational age

Gestational Age (weeks)	Gangliosides (μM)	
	Mean	Range
16–17 (n = 4)	0.2	0.1–0.3
27–29 (n = 4)	0.5	< 0.1–1.0
34–35 (n = 2)	0.4	0.3, 0.4
36–40 (n = 7)	0.8	0.4–1.3

Only traces of the complex gangliosides predominant in the clarified fluid were detected in the cell pellets and the surfactant. Therefore, the major complex gangliosides of amniotic fluid are not related to those of surfactant or of epithelial cells found in the fluid.

4. DISCUSSION

Tissue gangliosides of the fetal-maternal system, including of placenta [13–15], placental syncytiotrophoblast [24] and meconium [25,26], have been characterized. Several glycoconjugates have been identified in amniotic fluid (e.g. lactosylceramide [27], blood group I and i antigens [28] and galactosyloligosaccharides [29]), but little is known about the gangliosides, which are difficult to purify when present only at low concentrations. The major finding of this study was that the major gangliosides of human amniotic fluid are a series of highly polar and complex molecules. This is surprising because the major ganglioside of placenta is G_{Me} [13–15] and no other normal human tissue or fluid is known to have these highly complex gangliosides as *major* components.

The origin of complex gangliosides in human amniotic fluid is unknown. Amniotic fluid cells (epithelial cells) are excluded as a likely source by our studies because the major ganglioside of these cells is G_{M3} [30], and the complex gangliosides found in amniotic fluid were absent in the cell fraction. The same is true of surfactant. Human placenta does contain trace amounts of several highly polar neolacto series gangliosides [15,31,32], migrating near the origin by standard HPTLC. Syncytiotrophoblast and amnion also contain such complex molecules [16,24,33]. Therefore, these tissues could be the origin of the highly complex gangliosides in amniotic fluid. By the process of shedding, these molecules could accumulate and concentrate in amniotic fluid during gestation.

What is the potential significance of the finding of

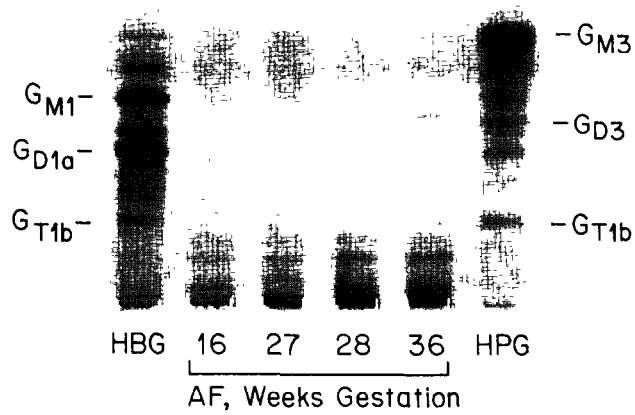

Fig. 4. Effect of gestational age upon human amniotic fluid ganglioside Samples obtained in each trimester were purified and compared. Abbreviations as in Fig. 2. Solvent system: as in Fig. 3.

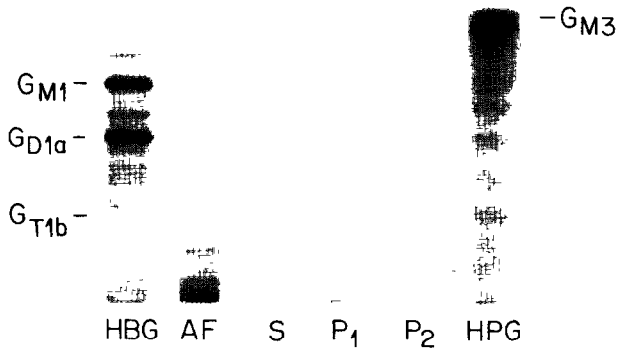

Fig 5. Gangliosides of amniotic fluid fractions. Gangliosides of the cellular components (P_1 and P_2) and surfactant (S) are compared to those of the clarified amniotic fluid. Solvent system: as in Fig. 3.

these highly polar gangliosides in human amniotic fluid? Amniotic fluid has a strategy disposition near the maternal-fetal interface and is continuously circulating through the fetus; soluble factors secreted or shed at this interface could therefore be concentrated in the fluid. In fact, several soluble immunologically active proteins or glycoproteins secreted by the tissues of this interface have been isolated [34,35], and both murine [6,7,9,10] and human [8,36] amniotic fluid have immunosuppressive properties. The total lipid extract of crude human amniotic fluid (which would contain any gangliosides) also inhibits [^{3}H]thymidine uptake by PHA-activated human peripheral blood mononuclear leukocytes [36], as do the lipid components of the syncytiotrophoblast plasma membrane [37]. We speculate that the complex gangliosides we detected contribute to immunosuppressive activity of human amniotic fluid. Proof of this hypothesis will now entail the preparative purification of these molecules for determination of their structures and immunoregulatory activity.

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REFERENCES

[1] McIntyre, J.A. and Faulk, P (1979) Proc. Natl. Acad. Sci. USA 76, 4029–4032.
[2] Chaouat, G., Chaffaux, S., Duchet-Suschaux, M. and Voisin, G.A. (1980) J. Reprod Immunol. 2, 127–139.
[3] Lala, P.K., Chatterjee-Hasrouni, S., Kearns, M., Montgomery, B. and Colavincenzo, V (1983) Immunol Rev 75, 87–116.
[4] Daya, S., Clark, D.A., Devlin, C., Jarrell, J. and Chaput, A. (1985) Am. J. Obstet. Gynecol. 151, 267–270
[5] Nakayama, E., Asano, S., Kodo, H. and Miwa, S. (1985) J. Reprod. Immunol 8, 25–31.
[6] Clark, D.A., Chaput, A., Walker, C. and Rosenthal, K.L. (1985) J. Immunol. 134, 1659–1664.
[7] Suzuki, K. and Tomasi Jr., T.B. (1980) J. Immunol 125, 1806–1810.
[8] Wajner, M., Papiha, S S. and Wagstaff, T.I (1986) Eur. J. Obstet. Gynecol. Reprod. Biol. 21, 225–232.
[9] Reilly, B.D., Goldberg, E.H. and Tomasi, T B (1988) Transplantation 45, 996–998
[10] Yoshimura, N., Matsui, S., Hamashima, T. and Lee, C.J. et al. (1991) Transplantation 52, 540–545.
[11] Black, P H. (1980) Adv Cancer Res. 32, 75–199.
[12] Ladisch, S., Gillard, B., Wong, C. and Ulsh, L (1983) Cancer Res. 43, 3808–3813.
[13] Svennerholm, L (1965) Acta Chem. Scand. 19, 1506–1507.
[14] Portoukalian, J and Zwingelstein, G. (1981) Int. J Tiss. Reac 3, 147–150.
[15] Taki, T., Matsuo, K.-i., Yamamoto, K., Matsubara, T., Hayshi, A., Abe, T. and Matsumoto, M (1988) Lipids 23, 192–198.
[16] Asano, K., Suzuki, M., Handa, S. and Oshima, M. (1991) J Biochem. 109, 106–112.
[17] Li, R and Ladisch, S. (1991) Biomed. Biochim Acta 1083, 57–64.
[18] Ladisch, S and Gillard, B. (1985) Anal. Biochem. 146, 220–231.
[19] Ladisch, S and Gillard, B. (1987) Methods Enzymol. 138, 300–306.
[20] Ledeen, R. and Yu, R. (1982) Methods Enzymol. 83, 139–191
[21] Rosner, H. (1980) Anal. Biochem 109, 437–442.
[22] Svennerholm, L. (1957) Biochim. Biophys. Acta 24, 604–611.
[23] Kryukova, E.V., Lyubushkina, S A and Dyatlovitskata, E.V. (1990) Biokhimiya 55, 836–838.
[24] Cheng, H M and Johnson, P.M. (1985) Placenta 6, 229–238.
[25] Karlsson, K.-A and Larson, G. (1978) FEBS Lett 87, 283–287
[26] Karlsson, K.-A. and Larson, G. (1981) J. Biol. Chem. 256, 3512–3524
[27] Hallman, M., Bry, K., Il and Pitkanen, O. (1989) Am. J Obstet. Gynecol 161, 313–318.
[28] Feizi, T., Cederqvist, L.L. and Childs, R (1975) Br. J. Haematol. 30, 489–497.
[29] Warner, T.G., Robertson, A.D., Mock, A.K., Johnson, W.G. and O'Brien, J S. (1983) Am. J. Hum. Genet 35, 1034–1041.
[30] Bach, G., Zeigler, M. and Kohn, G. (1980) Clin Chim. Acta 106, 121–128
[31] Nudelman, E.D., Mandel, U., Levery, S.B., Kaizu, T. and Hakomori, S. (1989) J. Biol. Chem. 264, 18719–18725.
[32] Levery, S B., Nudelman, E.D., Salyan, M.E.K. and Hakomori, S.-i. (1989) Biochemistry 28, 7772–7781.
[33] Friedman, S.J., Cheng, S. and Skehan, P. (1983) FEBS Lett. 152, 175–179.
[34] Hamaoka, T., Itoh, K., Izumi, Y and Ono, S. (1987) in: Immunoregulation and Fetal Survival, pp. 169–180.
[35] Matsui, S., Yoshimura, N and Oka, T. (1989) Transplantation 47, 678–683
[36] Rueda, R., Vargas, M.L., Garcia-Pacheco, M and Garcia-Olivares, E. (1990) Am J. Reprod. Immunol. 24, 40–44.
[37] Lalmanach-Girard, A.C., Thibault, G., Lacord, M., Degenne, D. and Bardos, P (1992) Immunol. Lett. 32, 131–138.