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Glycosphingolipid expression in spontaneously aborted fetuses and placenta from blood group p women. Evidence for placenta being the primary target for anti-Tj^a-antibodies

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A 12-week-old fetus and one 17-week-old fetus + placenta were obtained after spontaneous abortions from two women of blood group p. The 17-week-old fetus was dissected into intestine, liver, brain and residual tissue. Nonacid glycosphingolipid fractions were prepared from the tissues. Glycolipid characterization was carried out using thin layer chromatography immunostained with monoclonal antibodies and bacteria and by ^{1}H NMR spectroscopy and mass spectrometry. In the placental fraction substantial amounts of globotetraosylceramide (P-antigen) and globotriaosylceramide (P^k-antigen) were identified. In contrast, the fetuses contained only trace amounts of these structures, as revealed by immunostaining. These results indicate that the primary target for the antibodies of the anti-Tj^a serum is the placenta tissue, resulting in termination of the pregnancy.

Keywords: Blood group p, glycosphingolipids, spontaneous abortions, mass spectrometry, NMR spectroscopy

The human blood group P-system contains three antigens, P^k, P and P$_1$ (Table 1) which have been identified as glycosphingolipids [1, 2]. Individuals belonging to the rare blood group p phenotype lack all these antigens on their red cells and have preformed or natural antibodies against them, defined as anti-Tj^a serum, which is believed to be a mixture of anti-P, anti-P^k and anti-P$_1$ antibodies [3]. Women belonging to the p blood group have a high incidence of early spontaneous abortions believed to be caused by anti-Tj^a antibodies [4, 5] since the father (statistically) always belongs to another group within the P blood group system (P$_1$, P$_2$). The mechanisms for these abortions, however, are not fully understood. IgG antibodies produced by Rh immunizations are known to pass the placental barrier and bind to the red blood cells of the fetus. Mature placentas have been shown to contain the P- and P^k-antigens [4, 6]. Spontaneously aborted fetuses of p women are usually macroscopically intact, in contrast to fetuses aborted due to Rh incompatibility (B. Cedergren, unpublished observations). In order to answer the question whether the fetus or the placenta or both is/are a target for the anti-Tj^a antibodies we have analysed two cases of spontaneously aborted fetuses from p women.

Materials and methods

Glycosphingolipid preparation

A 17-week-old fetus and placenta were obtained after spontaneous abortion from a woman of blood group A$_1$ p Le(a − b+) and stored at −80 °C. The father belonged to blood group P$_2$ (having the P^k- and P-antigens but lacking

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Table 1. Structures of antigens mentioned in the text.

P^k-Antigen	Galα1-4Galβ1-4Glcβ1-1Ceramide
P-Antigen	GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Ceramide
P_1-Antigen	Galα1-4Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Ceramide
A-6-1[a]	GalNAcα1-3(Fucα1-2)Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ1-1Ceramide
A-7-1	GalNAcα1-3(Fucα1-2)Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glcβ1-1Ceramide
A-6-2	GalNAcα1-3(Fucα1-2)Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Ceramide
A type 3	GalNAcα1-3(Fucα1-2)Galβ1-3GalNAcα1-3(Fucα1-2)Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Ceramide
A type 4	GalNAcα1-3(Fucα1-2)Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glc1-1Ceramide

[a] In the short-hand designation for blood group glycolipids, the letter indicates blood group determinant, the first numeral is the number of sugar residues, and the second numeral the type of carbohydrate chain. Thus A-6-1 means a hexaglycosylceramide with a blood group A determinant based on a type 1 chain.

the P_1-antigen). After thawing, the placenta was separated from the fetus which was further macroscopically dissected into intestine, liver, brain and residual tissue. An additional 12-week-old fetus spontaneously aborted from a p woman was obtained. After lyophilizaton, total nonacid glycosphingolipid fractions were prepared from the different tissues as described [7].

Thin layer chromatography with immunostaining and bacterial overlay

This layer chromatograms were run on glass plates (Merck, Darmstadt, Germany) for chemical detection and on glass plates (Whatman International Ltd, Maidstone, UK) or aluminium sheets (Merck, Darmstadt, Germany) for immunostaining and bacteria overlay. The solvent was chloroform–methanol–water (60:35:8, by vol.). Chemical detection was achieved with anisaldehyde reagent [7]. The immunostaining and bacterial overlay were performed as described [8–10]. Antibodies used were anti-P [4], anti-P^k [11] and the following blood group A monoclonal antibodies: Dakopatts A581 reactive with terminal A trisaccharide irrespective of core chain type [12], AH-21, reactive with monofucosyl A type 1 determinant [13], HH-3, reactive with difucosyl A type 1 determinant [14], TH-1, reactive with A type 3 determinant [14] and HH-5, reactive with A type 3 and 4 determinants [14]. Serum from the p woman who aborted the 17-week-old fetus + placenta was tested against the prepared glycosphingolipid fractions and the detection of bound antibodies was accomplished using anti-IgG3 antibodies (BIO-Zac) [15]. ^{125}I labelled anti-mouse immunoglobulins (Dakopatts, Denmark) were used as sandwich antibodies. ^{35}S labelled P-fimbriated *Escherichia coli* bacteria (HB101/pPIL2GI-15) recognizing the Galα-4Gal sequence were kindly provided by Dr T. Korhonen.

Proton NMR spectroscopy

400 MHz proton NMR spectroscopy of the native glycolipids in 0.5 ml [$^2H_6$]dimethylsulfoxide containing 2% ^2H_2O at a probe temperature of 30 °C was performed on a Varian XL400 apparatus (Varian, USA). Chemical shifts are given relative to tetramethylsilane.

Mass spectrometry

Mass spectrometry of the permethylated-reduced glycolipids [16–18] was performed on a high-field ZAB-2F mass spectrometer (VG Analytical Ltd, Manchester, UK) equipped with a PDP 11/250 data system. The sample was analysed with electron ionization (EI) using the 'in beam' technique [19].

Results

Thin layer chromatography

The results from analysis with thin layer chromatography are shown in Fig. 1. As can be seen in Fig. 1, plate II, the Galα1-4Gal specific *E. Coli* strain binds the reference globoside (lane 9) and two compounds in the placenta fraction (lane 3), one in the four sugar region and one in the three sugar region. Faint staining with the bacteria is also seen of compounds in the fractions from the 12-week-old fetus (plate II, lane 7) and the residual tissue of the 17-week-old fetus (lane 8). The P-antibody (Fig. 1, plate III) has affinity for one compound in the placenta fraction (lane 3) and for one in the fraction from the 17-week-old fetus (lane 8). One band in the placenta fraction is also stained by P^k-antibody (Fig. 1, plate IV, lane 3) and weak staining can be seen in the fraction from the residual fetus tissue (lane 8). Note that no binding can be detected to the fraction from A_1 p Le(a−b+) plasma (lane 1) on plates I, II and III while the fraction from A_1 P_1 Le(a−b+) plasma (lane 2) is positive with the reagents used.

The results from the analyses with the different blood group A antibodies are not shown. Earlier studies have shown that placenta tissue probably does not express blood group A and B antigens [20, 21], and the positive results we obtained with the anti-A antibodies are most likely due to remaining maternal red blood cells, as the staining of the placenta fraction was very similar to the staining of red blood cells (not shown). Both the intestine and the residual tissue fractions were stained with all A-antibodies used, while the liver and the brain were negative.

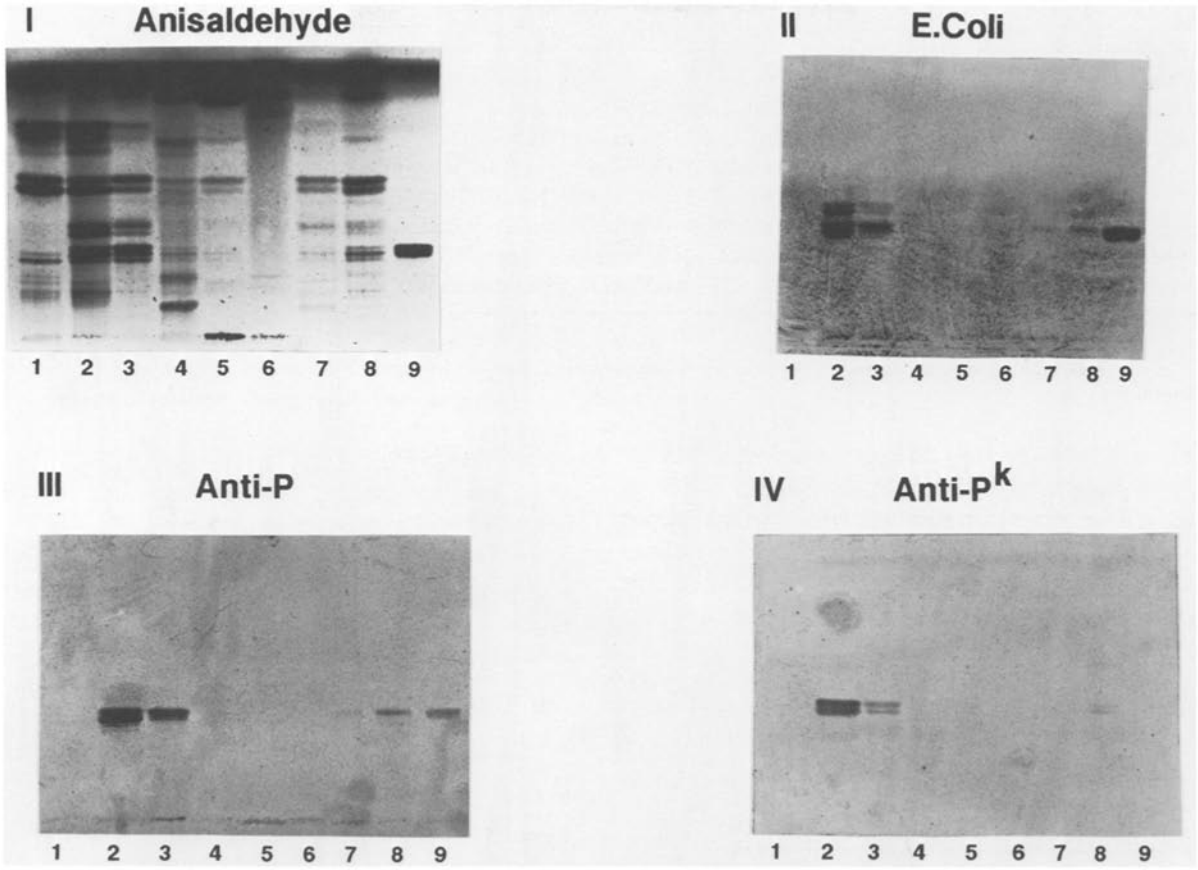

Figure 1. Thin layer chromatograms of total nonacid glycosphingolipid fractions from blood group A_1 p Le(a−b+) plasma (lane 1), blood group A_1 P_1 Le(a−b+) plasma (lane 2), aborted placenta of a p woman (lane 3), intestine from 17-week-old aborted fetus (lane 4), liver from 17-week-old aborted fetus (lane 5), brain from 17-week-old aborted fetus (lane 6), 12-week-old aborted fetus (lane 7), residual tissue from 17-week-old aborted fetus (lane 8). Globoside was applied as reference (lane 9). Plate I was visualized with anisaldehyde reagent. Plate II was submitted to bacteria overlay and plates III and IV are autoradiograms after immunostaining with antibodies as indicated. About 40 μg glycolipids of the total fractions and 2 μg of the reference samples were applied for chemical detection, and about 20 μg and 0.2 μg, respectively, for immunostaining and bacteria overlay.

The serum of the p mother was found to contain IgG3 antibodies that bound strongly to placenta glycolipids containing three and four sugars, whereas no or very faint binding was seen to the glycolipid fractions from the fetus.

NMR spectroscopy

The anomeric region of the proton NMR spectrum of the total placenta glycosphingolipid fraction is shown in Fig. 2. Two signals are indicated: one α signal at 4.81 ppm and one β signal at 4.52 ppm. These two signals are in accordance with -3Galα1-4 and GalNAcβ1-3 of globotetraosylceramide [22]. The triplet of the α signal is due to an additional signal from terminal Galα1-4 of globotriaosylceramide [22]. The signals from the anomeric protons from the two proximal hexoses cannot be distinguished in the large group of signals in the 4.0–4.4 ppm region as this group contains several signals from other structures in the mixture. Other signals originating from these structures are present in the spectrum but not identified.

Mass spectrometry

Extracts of the results from the mass spectrometric analysis of the permethylated-reduced glycosphingolipid fraction from the aborted placenta are shown in Fig. 3. The average spectrum contains peaks representing immonium ions from two different carbohydrate compounds containing the whole carbohydrate chain (three hexoses and three hexoses plus one hexosamine, respectively) and the fatty acids of several species. Peaks at *m/z* 925, 955, 1009, 1037 and 1067 are derived from the trisaccharide with 16:0, h16:0, 22:0, 24:0 and h24:0 fatty acids. Peaks at *m/z* 1156, 1186, 1212, 1240, 1268 and 1298 are derived from the tetrasaccharide with 16:0, h16:0, 20:0, 22:0, 24:0 and h24:0 fatty acids. The nonhydroxy fatty acids dominate. The tetrasaccharide seems to be present in a larger amount than the trisaccharide, which is in accordance with the ion curves for the traces for these two compounds with 16:0 fatty acids (*m/z* 925 and 1156). The trace for the trisaccharide is normalized against the trace for the tetrasaccharide. Also found in the spectrum

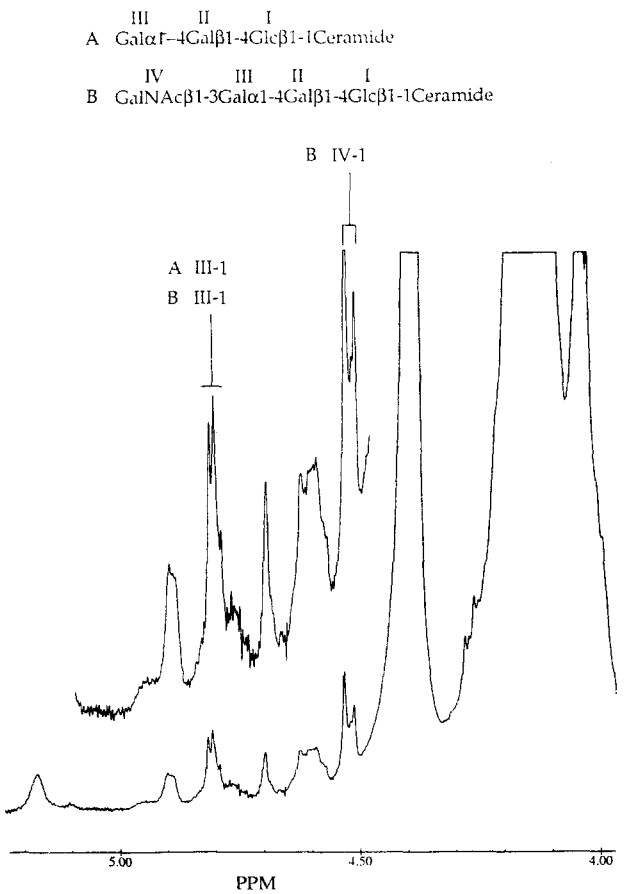

Figure 2. The anomeric region of the proton NMR spectrum recorded from the total glycolipid fraction isolated from the aborted placenta. Inserted in the spectrum is an amplification of part of the anomeric region. Formulae of the P- and the P^k-antigens suggested to be present in the fraction are shown. 3400 pulses were recorded at a temperature of 30 °C.

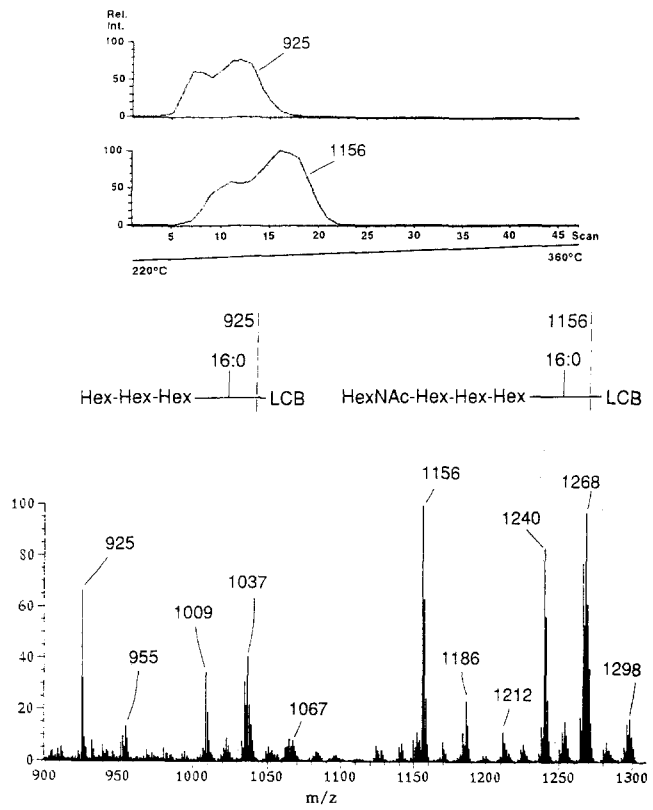

Figure 3. Results from the mass spectrometric analysis of the total nonacid glycosphingolipid fraction from the placenta. About 10 μg of the permethylated-reduced sample was evaporated at a temperature gradient of 6 °C min^{-1} from 220 °C to 360 °C. At the top are shown selected ion curves that indicate fragment ions as a function of evaporation temperature. The curve for *m/z* 925 is normalized against the trace for *m/z* 1156. Simplified formulae of proposed structures present are shown below. At the bottom is shown an average spectrum (scans 6–22) from the same analysis. Conditions of analysis were: electron energy 70 eV, trap current 200 μA, acceleration voltage 8 kV. The sample was introduced into the mass spectrometer using the 'in beam' technique [19]. The sugars are abbreviated to Hex for hexose, HexNAc for *N*-acetylhexosamine, and long chain base to LCB.

were peaks representing immonium ions derived from a monoglycosylceramide containing one hexose and fatty acids 16:0, h16:0, h20:0, h22:0 and h24:0 (*m/z* 516, 546, 603, 631 and 659, respectively). A diglycosylceramide was also identified by peaks at *m/z* 721, 777, 805, 833, 751, 835 and 863, representing immonium ions containing two hexoses with 16:0, 20:0, 22:0, 24:0, h16:0, h22:0 and h24:0 fatty acid species, respectively.

From the combined results of thin layer chromatography with immunostaining, NMR spectroscopy and mass spectrometry presented above, it is concluded that the aborted 17-week-old placenta from the p woman expresses the P- and the P^k-antigens in large amounts, while the aborted 17- and 12-week-old fetuses only express these substances in small amounts.

Discussion

The fact that the two fetuses were macroscopically intact after the spontaneous abortions, and since the placental tissue contained large amounts of globotriaosylceramide and globoside and only small amounts of these antigens were found in the fetuses, it is postulated that the placenta was the main target for the anti-Tj^a antibodies of the mother's serum, thus causing the abortions. The presence of the P-antigen has been demonstrated previously in mature placenta tissue [4, 6] and has been located in the trophoblasts [4], which are the cells exposed to the maternal circulation. The abortions are most likely caused by antibodies against the antigens of the blood group P-system. That these antibodies are involved in the abortion mechanism is supported by the successful pregnancies after plasmapheresis therapy [5] and adsorption of plasma antibodies with erythrocytes containing the P-antigen [23]. It is also supported by the fact that serum from a p woman

bound to glycolipids of her own aborted placenta and that this binding was lost after passing the serum through a column of Synsorb® containing saccharides of the blood group P-system [15]. An additional indication is that the trophoblasts have been found to lack ABH-antigens [20, 21], and it is well known that ABO-incompatible pregnancies are well tolerated. The trophoblasts also seem to lack the HLA-antigens [24] which, if they were expressed by the placenta, could be expected to cause abortions.

While it is established that the immunoglobulins of the IgG class pass the placenta barrier and attack the fetus in Rh immunizations, it is difficult to draw any conclusions about which immunoglobulin class is mostly responsible for the abortion mechanisms in P incompatible pregnancies, since the P- and P^k-antigens are expressed by the trophoblasts and no placenta passage is necessary. That PP$_1$P^k-antibodies should belong mainly to the IgG3 class has been suggested [25], but recent studies have shown a wider distribution of these antibodies within the immunoglobulin subclasses [15]. Since not every P-incompatible pregnancy ends in an abortion, a comparison of anti-Tj^a immunoglobulins between women with several abortions and women who have borne healthy children might give an answer to the question. Of interest also would be a study of the possible expression of the P$_1$-antigen of placenta, as this antigen is based on a core structure different from that of the P- and P^k-antigens.

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