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Blood group glycosphingolipid expression in kidney of an individual with the rare blood group A_1 Le(a−b+) p phenotype: absence of blood group structures based on the globoseries

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Total neutral glycolipid fractions were isolated from kidney and ureter tissue obtained at autopsy of an individual of the rare blood group A_1 Le(a−b+) p. The amount of glycolipids isolated were 3.7 and 2.5 mg g^{-1} dry tissue weight for the kidney and ureter tissue, which is in the range of reference blood group P kidneys. Part of the kidney glycolipid fraction was subfractionated by HPLC. Glycolipid compounds were structurally characterized by thin-layer chromatography (chemical detection and immunostaining with monoclonal antibodies), proton NMR spectroscopy and mass spectrometry. Globotriaosyl- and globotetraosyl-ceramides, which are the major compounds in kidneys of P individuals, were absent in the p kidney, and a comparatively increased amount of monoglycosyl- and lactosylceramides was found. A shift to longer fatty acyl chains in the ceramide part of lactosylceramides was noted. Elongated globoseries compounds with five to seven sugar residues, including the blood group A type 4 chain structure, were lacking. A slight increase in neolactotetraosyl- and blood group X pentaglycosyl-ceramides was noticed. The study confirms an enzymatic block in the conversion of lactosylceramide to elongated globoseries compounds in the kidney tissue similar to that of erythrocytes of p individuals.

Keywords: glycolipids, blood group p, human kidney, mass spectrometry, proton NMR spectroscopy

Abbreviations: for blood group glycolipid antigens the short hand designation stands for: blood group – number of sugar residues – type of carbohydrate chain. Thus A-7-4 means a blood group A heptaglycoconjugate on a type 4 chain. The sugar types are abbreviated for mass spectrometry to Hex for hexose, HexNAc for *N*-acetylhexosamine and dHex for deoxyhexose.

HPLC, high-performance liquid chromatography; HPTLC, high performance thin layer chromatography; EI, electron impact ionisation; LSI, liquid secondary ion; MS, mass spectrometry; NMR, nuclear magnetic resonance.

Introduction

The P blood group system consists of three antigens: globotriaosylceramide (the P^k antigen), globotetraosylceramide (the P antigen) and Galα1-4 neolactotetraosylceramide (the P_1 antigen) [1, 2]. The p phenotype individuals are characterized by an inability to express the P^k, P and P_1 antigens on their red blood cells [3]. These individuals are very rare, but can be found at a higher frequency within restricted geographic areas [4]. For obvious reasons, most of the glycolipid structural work has been performed on erythrocytes and plasma from p individuals. The P and P^k antigens are major glycolipid components in erythrocyte membranes, together with the more sparsely

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occurring polymorphic P_1 antigen. These antigens are lacking in erythrocytes [3, 5, 6] and in normal gastric tissue [7, 8] from individuals of the p phenotype, resulting in an accumulation of the precursor lactosylceramide. An aberrant expression of the bloodgroup P antigens in gastric cancer tissue of p individuals has been demonstrated [8, 9]. Fibroblasts of the p phenotype were negative for the blood group P antigen, but polykaryon cells obtained by fusion of P^k and p fibroblasts express the P antigen [10]. Spontaneously aborted fetuses and placenta tissue from p women express the P and P^k antigens [11]. However, these tissues have a genotype of both maternal and paternal origin.

In kidney tissue from P individuals globotetraosylceramide is elongated by a terminal Galβ1-3 residue forming globopentaosylceramide, which is further converted into blood group H and A structures [12–14]. Kidney is the human tissue which contains the largest amount of globoseries based A antigen (A type 4), and the A antigens based on type 1, 2 and 3 core saccharides are minor components [14]. This work describes the blood group type glycolipid expression in a unique kidney tissue specimen obtained from a blood group A_1 Le(a−b+) p individual. The results show the complete absence of P^k and P antigens, as well as elongated globoseries compounds. No simultaneous increase in type 1, 2 and 3 chain based A antigens was found.

Materials and methods

Glycolipid preparation and HPLC fractionation

Part of a kidney (62.6 g), was obtained at autopsy from a woman of blood group A_1Le(a−b+) p individual who died of pancreatic cancer. Macroscopically the kidney showed no morphological abnormalities, and the small ureter tissue was collected separately. Total neutral glycosphingolipid fractions were prepared from the lyophilized kidney (10.9 g) and ureter (95 mg) tissues as described [14, 15]. A neutral glycolipid fraction from the kidney of a blood group A_1Le(a−b+) P_1 individual [12] was used for comparison.

Part of the kidney total neutral glycolipid fractions were separated by HPLC (LKB, Bromma, Sweden) on a silicic acid column (Polygosil, 5 µm particles, Genetec, Sweden), using a chloroform:methanol:water gradient (80:20:1 to 40:40:12, by vol). The separation was monitored by thin-layer chromatography and the fractions were combined into pure or partly pure subfractions as shown in Fig. 1I, lanes 1a–e and 2a–f respectively. The amount of glycolipid in each subfraction was estimated by weighing and by densitometric scanning of the thin-layer plate using a Shimadzu CS930 scanner (Shimadzu, Japan), and serial dilution of globotetraosylceramide as in [12].

Analytical thin-layer chromatography and immunostaining

Thin-layer chromatography was performed using HPTLC plates (Merck, Darmstadt, Germany and Whatman International Ltd, Madistone, UK). Solvent used was chloroform:methanol:water (60:35:8, by vol). For chemical detection the anisaldehyde reagent [15] was used and immunostaining of the plates was performed as described [12]. The mouse monoclonal blood group antibodies used and their antigen specificity are listed in Table 1.

Proton NMR spectroscopy

Proton NMR spectroscopy was performed on the native glycolipids dissolved in 0.5 ml dimethylsulfoxide-d_6 containing 2% D_2O, at a probe temperature of 30 °C. The analyses were performed at 300, 400 or 500 MHz using either a Varian VXR 300 or XL 400 instrument (Varian, USA) or a Bruker AMX 500 apparatus (Bruker, FRG). Chemical shifts are given relative to tetramethylsilane.

Mass spectrometry

Mass spectrometry of the permethylated-reduced glycolipids [12, 14] was performed using a double focusing magnetic sector instrument of EBE type (VG Analytical Ltd, Manchester, UK). A direct probe method [16] was used in the positive EI mode to acquire spectra. The spectra were recorded over the mass range 2500–100 at 8 kV accelerating potential, 70 eV ionization energy, a trap current of 300 µA and a source temperature of 250 °C. The probe temperature was increased from 50 to 550 °C. The instrument was pre-calibrated using CsI (Merck, Germany) in the positive LSI mode prior to use.

Results

Total amount of neutral glycolipids isolated from the kidney and ureter was 40.6 mg and 0.24 mg, corresponding to 3.7 and 2.5 mg g^{-1} dry tissue weight. Previous studies have revealed the amount of neutral glycolipids ranging from 3.6 to 7.9 mg g^{-1} dry tissue weight for kidney tissue [12, 13] and 2.5 mg g^{-1} for a ureter specimen [12]. The results of thin layer chromatographic/densitometric analysis of the ureter glycolipid fraction is shown in Table 2. This fraction was not analysed further. Figure 1I shows the thin layer chromatogram of the total neutral glycolipid fractions from the blood group p kidney (lane 1), and a reference blood group P_1 kidney (lane 2). Only small amounts of glycolipids with three and four sugar residues are present in the p kidney, together with a concomitant increase of shorter and longer chain glycolipids (Fig. 1 and Table 2). The chromatographic bimodal distribution of individual glycolipids is caused by a heterogeneity in the ceramide part with regard to chain length and hydroxyl groups. The increase of the

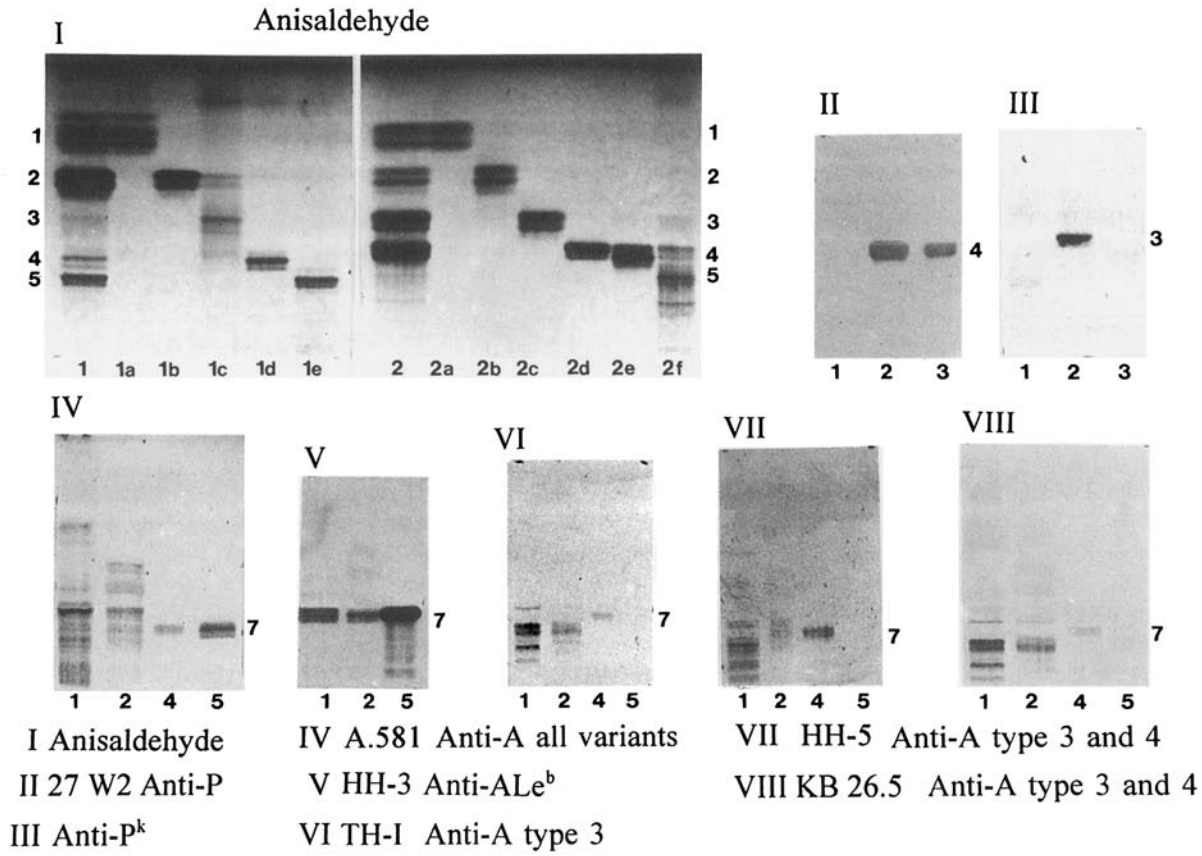

Figure 1. Thin-layer chromatographic analysis of neutral glycolipid fractions isolated from kidneys of blood group A_1Le(a−b+) p (lane 1), and A_1Le(a−b+) P_1 (lane 2) individuals. Parts of the total neutral glycolipid fractions were subfractionated by HPLC (lanes 1a–e and 2a–f, respectively). The plates were stained by a chemical reagent (plate I) and by immunostaining (plates II–VIII) using various monoclonal blood group antibodies listed in Table 1. Reference glycolipids applied were in lane 3; globotetraosylceramide, in lane 4; Globo-A (A-7-4) and in lane 5; ALe^b heptaglycosylceramide (A-7-1). Developing solvent was chloroform:methanol:water (60:35:8, by vol) and 40 µg of the total fractions (lanes 1 and 2) were applied in chart I, and 20 µg for immunostaining (plates II–VIII). The arabic numerals to the right and left indicate number of sugar residues in each glycolipid band.

Table 1. Antigen specificity of the mouse monoclonal antibodies used in the immunostaining experiments

Antibody	*Fig. 1*	*Code*	*Specificity*	*Reference*
Anti-P	Plate II	27 W 2	P terminal	17
Anti-P^k	Plate III		P^k terminal	18
Anti-A all variants	Plate IV	Dakopatts A 581	Terminal A trisaccharide	19
Anti-Le^b	Plate V	HH-3	A type 1, difucosyl	20
Anti-A type 3	Plate VI	TH-1	A type 3	21
Anti-A types 3 and 4	Plate VII	HH-5	A types 3 and 4	22
Anti-A types 3 and 4	Plate VIII	KB-26.5	A types 3 and 4	19
Anti-ALe^x		SH-1	Le^x terminal	12
Anti-Le^y		AH-6	Le^y terminal	23
Anti-ALe^y		HH-2	A type 2 difucosyl	20

diglycosylceramides in the p kidney is most pronounced for the faster moving band containing long chain fatty acids, which makes up 77% of the diglycosylceramides, compared to 40% in the reference kidney. A similar ceramide distribution has been found in erythrocytes of p individuals [5]. Part of the total glycolipid fraction was separated by HPLC into subfractions as shown in Fig. 1I lanes 1a–e and 2a–f respectively. The subfractions from the p kidney were structurally characterized as described below, and the structures concluded are listed in Table 2

Table 2. Neutral glycolipid structures identified in the kidney and ureter of a blood group A_1Le(a−b+) p human individual

Glycolipid structure[a]	*Kidney*	*Ureter*
Hex-Cer	1.0[b] (28)[c]	0.7 (26)
Galβ1-4Glcβ1-1Cer	2.4 (64)	1.8 (71)
GlcNAcβ1-3Galβ1-4Glcβ1-1Cer	+ (0.5)	+ (1)
Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer	0.1 (2)	+ (1)
Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glcβ1-1Cer	0.2 (5)	+ (1)
GalNAcα1-3(Fucα1-2)Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer	+	
	3.7 (100)	2.5 (100)

[a]The kidney glycolipid structures are based on thin-layer chromatography, mass spectrometry, proton NMR spectroscopy and antibody binding. In addition, several minor blood group glycolipids were identified by immunostaining of thin layer chromatrograms seen in Fig. 1. For the ureter glycolipids, only thin layer chromatographic/densitometric analysis was done.
[b]Expressed as $mg\,g^{-1}$ dry tissue weight.
[c]Relative amount expressed as percentage.

Fraction 1b

This fraction migrates identically to lactosylceramide by thin layer chromatography. Proton NMR spectroscopy (not shown) was identical to that of lactosylceramide [24], with two β anomeric proton signals at 4.18 and 4.23 ppm respectively.

Fraction 1c

Thin layer chromatography of this fraction (Fig. 1I, lane 1c) revealed a mixture of di- and triglycosylceramides and trace amounts of tetraglycosylceramides. EI mass spectrometry of the permethylated-reduced derivative (not shown) revealed immonium ions derived from a Hex-Hex-Ceramide structure with mainly non-hydroxy 22:0, 23:0, 24:1 and 24:0 fatty acids at *m/z* 805, 819, 831 and 833, together with hydroxy 22:0, 23:0 and 24:0 fatty acids at *m/z* 835, 849 and 863. A series of immonium ions originating from HexNAc-Hex-Hex-Ceramide compound with non-hydroxy 16:0 to 24:0 fatty acids was found at *m/z* 952–1064. A terminal HexNAc sugar was present at *m/z* 246. In addition, trace amounts of a tetraglycosylceramide with one hexosamine and three hexoses were found by immonium ions of the non-hydroxy 22:0, 23:0 and 24:0 fatty acid at *m/z* 1240, 1254 and 1268. The proton NMR spectrum of fraction 1c is shown in Fig. 2. A group of β-anomeric signals is seen between 4.1 and 4.3 ppm together with a β-anomeric signal at 4.61 ppm. These data show the presence of lactotriaosylceramide, and the greater intensity of the down field β-anomeric signals are due to lactosylceramides present in the sample (Fig. 1I, lane 1c). Note the absence of the α anomeric proton Galα1-4 signal at 4.8 ppm, present in the spectra of globoseries compounds [24]. The relatively low amount of glycolipids and the presence of non-glycolipid contaminants in the fraction (seen in the front of the thin-layer plate in Fig. 1I, lane 1c) gives a low signal to noise ratio and weak signals of non-carbohydrate origin (compare spectrum 1e). The absence of globotriaosylceramide in the p kidney fraction is further supported by the lack of staining in the three sugar region, using an antibody specific for the P^k antigen (Fig. 1III, lane 1).

Fraction 1d

The weight of the fraction was 0.3 mg and it migrated as one band in the four sugar region by thin-layer chromatography (Fig. 1I, lane 1d). The mass spectrum (not shown) of the permethylated-reduced fraction showed a series of immonium ion peaks at *m/z* 1156–1268, derived from a tetraglycosylceramide containing one hexosamine, three hexoses and non-hydroxy 16:0–24:0 fatty acids. Small amounts of the corresponding hydroxy 16:0 to 24:0 fatty acid species were present at *m/z* 1186–1298. Terminal saccharide peaks were found at *m/z* 219 (Hex-) and 450 (Hex-HexNAc-). Since no peak was found at *m/z* 246 (terminal HexNAc), the carbohydrate sequence is assigned as Hex-HexNAc-Hex-Hex-Ceramide. The proton NMR spectrum of fraction 1d is shown in Fig. 2. Four β-anomeric signals are found at 4.66 ppm ($J_{1,2}$ = 8.7 Hz), 4.26 ppm ($J_{1,2}$ = 6.6 Hz) and at 4.15 and 4.22 ppm, which are in accordance with a neolactotetraosylceramide [24]. The sharp signal at 4.56 is a contaminant. Note that no signal is seen at 4.8 ppm (Galα1-4), similar to fraction 1c. The P antibody did not react with the tetraglycosylceramide band present in the total glycolipid fraction (Fig. 1II, lane 1). The structure of the four sugar compound is concluded to be neolactotetraosylceramide.

Fraction 1e

Fraction 1e (1 mg) contains glycolipid components migrating below the four sugar region by thin-layer chromatography (Fig. 1I lane 1e), with a pentaglycosylceramide as the major component. Mass spectrometry of the permethylated reduced fraction 1e is shown in Fig. 3. The major component is a pentaglycosylceramide with one deoxyhexose, one hexosamine, three hexoses and non-hydroxy 16:0–24:0 fatty acids as shown by the intense immonium ions at *m/z* 1330–1442, and small amounts of

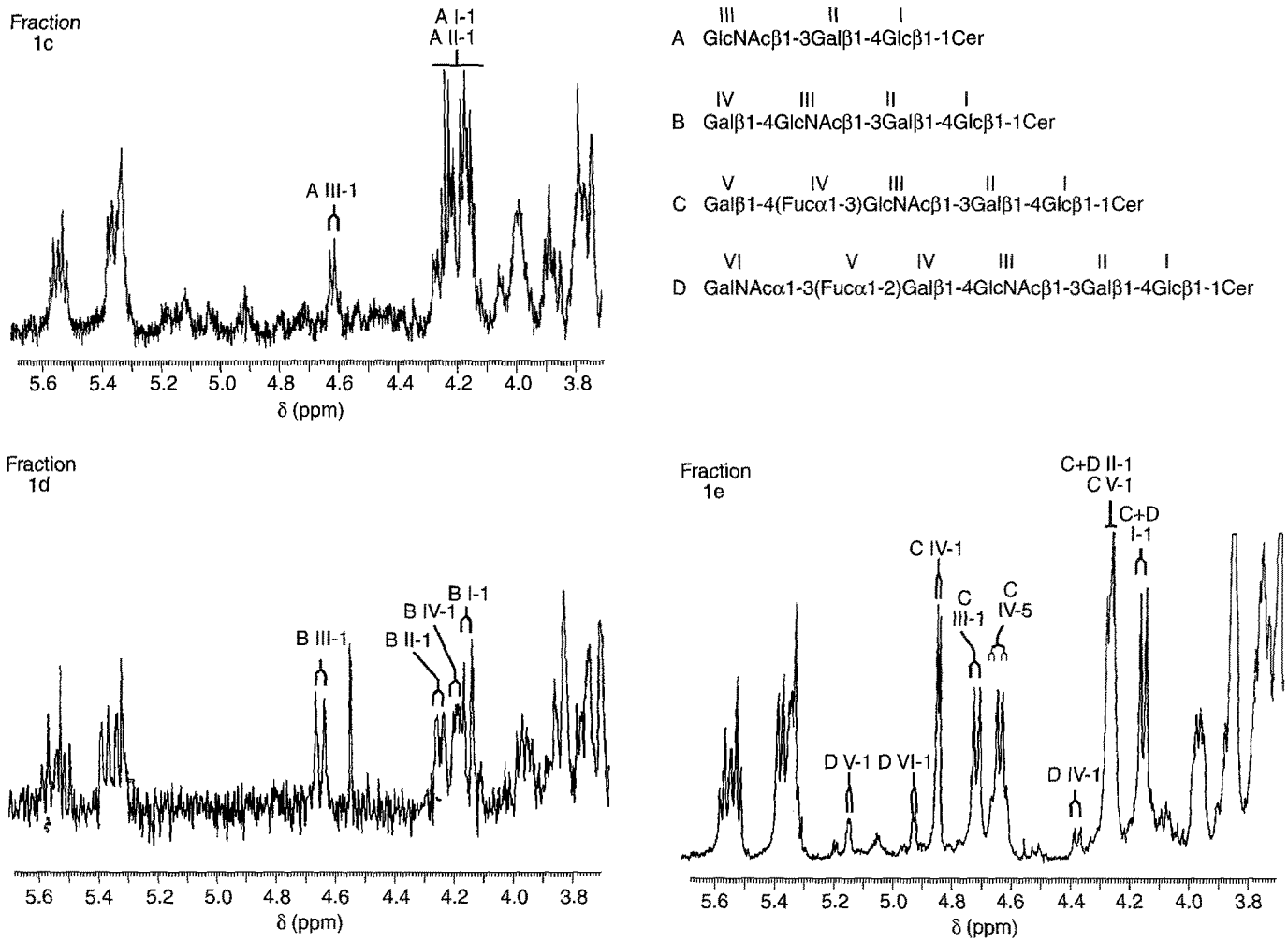

Figure 2. Anomeric regions of the proton NMR spectra of fractions 1c, 1d and 1e isolated from the kidney of a blood group A_1Le(a−b+) p individual. Sample amounts were 100 μg (1c), 50 μg (1d) and 150 μg (1e) respectively. Note the absence of the globoseries Galα1-4 signal at 4.8 ppm in all spectra.

hydroxy fatty acid seen at *m/z* 1472 for the 24:0 species. Carbohydrate sequence ions are seen at *m/z* 624, 640, 828, 1033 as indicated in the formula, and the fragment at *m/z* 1106 (1105 + 1) contains the whole saccharide chain and part of the ceramide [25]. The peaks at *m/z* 707–819 originate from the fatty acid and the inner two hexoses as indicated in Fig. 3. Small amounts of the tetraglycosylceramide are seen at *m/z* 1156–1268. The series of peaks at *m/z* 1562–1674 originate from hexaglycosylceramide having one deoxyhexose, two hexosamines, three hexoses and non-hydroxy 16:0–24:0 fatty acids. Ions originating from the terminal tetrasaccharide of the hexaglycosylceramide are seen at *m/z* 855.

The anomeric region of the proton NMR spectrum of fraction 1e (Fig. 2) contains one α-anomeric signal at 4.84 ppm ($J_{1,2}$ = 3.3 Hz), two β-anomeric signals at 4.72 ppm ($J_{1,2}$ = 7.2 Hz), and 4.17 ppm ($J_{1,2}$ = 7.8 Hz), as well as two β-anomeric signals around 4.27 ppm. These signals are in accordance with the X-5 pentasaccharide [26] (reference spectrum recorded at 55 °C). In the spectrum two α-anomeric signals at 5.14 ppm ($J_{1,2}$ = 3.6 Hz), and 4.92 ppm ($J_{1,2}$ = 4.2 Hz) and one β-anomeric signal at 4.38 ppm are seen which originate from the terminal trisaccharide of a blood group A type 2 chain structure. The pentaglycosylceramide band on the thin layer plate was strongly stained by the X antibody (SH-I) (not shown). The conclusion is that the major component of fraction 1e is the X-5 glycolipid Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glcβ1-1Cer, together with small amounts of blood group A type 2 chain compound GalNAcα1-3(Fucα1-2)Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer. In addition, several other blood group active glycolipids are detected by immunostaining of the total glycolipid fractions (Fig. 1 IV–VIII), but the amount of substance was too low to be resolved by mass spectrometry and NMR spectroscopy.

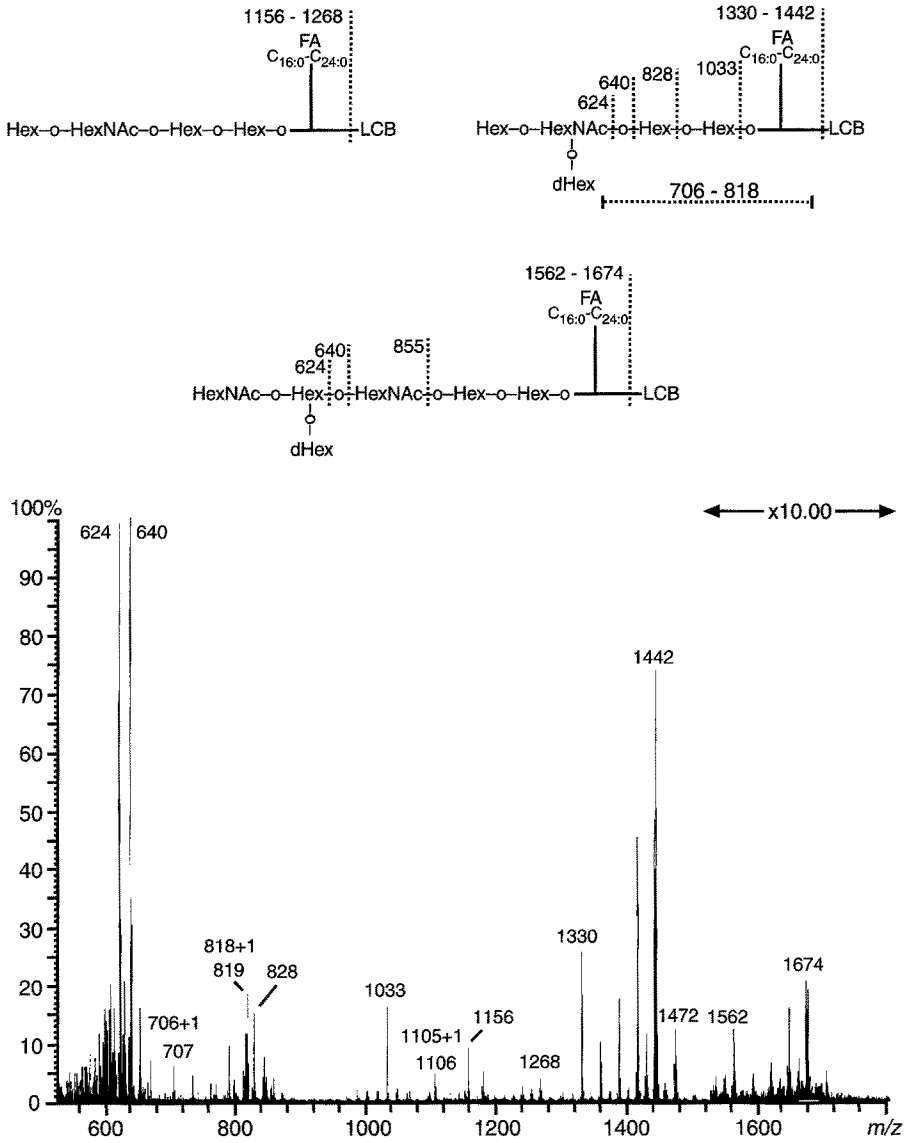

Figure 3. Mass spectrum of the permethylated-reduced fraction 1e isolated from the kidney of a blood group A_1Le(a−b+) p individual. The spectrum is a summary of 60 scans recorded continuously during the sample distillation in the ion source.

Blood group A glycolipids

The total glycolipid fractions of the p and reference kidneys were immunostained by several blood group A antibodies (Fig. 1 IV–VIII). Compared with the reference kidney, more blood group A active bands are seen in the p kidney. These are probably due to a contamination of blood cells and plasma in the organ collected at autopsy. The reference kidney was obtained for transplantation purpose and the vascular tree had been perfused with salt solution. The major blood group A glycolipids in human kidneys is the type 4 chain structure or 'Globo-A' [14]. The p kidney fraction stained with an antibody reacting with all A determinants (Fig. 1 IV lane 1) shows staining in the six sugar region and down to the sample origin. A weak band is seen in the seven sugar region which is strongly stained by anti-ALe^b (HH-3) (Fig. 1 V). No specific antibody for the A type 4 determinant is available, but several antibodies reacting with both A type 3 and 4 structures exist. Three of these antibodies (TH-1, HH-5 and KB 26.5) react with the p kidney fraction (Fig. 1 VI–VIII lane 1) and the A-7-4 reference sample (lane 4). However, the seven sugar region of the p kidney is not stained by these antibodies, indicating that this blood group A structure is lacking in the p kidney tissue.

Discussion

The structural study of neutral glycolipids of the kidney tissue from a blood group p individual has shown the

absence of globoseries glycolipids. Kidney is the human organ which contains the highest level of extended globoseries compounds, with five to seven sugar residues, including the blood group A type 4 chain structure [12, 14]. As expected, these structures could not be detected in the p kidney. The presence of a blood group X pentaglycosylceramide and an A type 2 chain hexaglycosylceramide was concluded by analyses of the slow migrating glycolipid mixture containing several compounds. Since these glycolipids are the major compounds in the fraction these structures could be safely assigned by combined MS and NMR analyses. Several minor blood group glycolipids are also found but can only be partially structurally defined due to the small amount present. The total amount of glycolipids (per mg of dry tissue) is similar to the amount found in P kidneys [14]. In addition to an accumulation of lactosylceramides, the total amount of lactotriaosyl-, neolactotetraosyl- and X-pentaglycosylceramides is also increased. The increase of lactosylceramides with longer fatty acyl chains has also been observed for p erythrocytes [5]. This is probably an effect of the block in the biosynthesis of globotriaosylceramides [5], since globotetraosyl- and globotriaosylceramides in the P kidney are dominated by the long chain species [14].

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