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A novel glycosphingolipid expressed in pig kidney: Galα1-3Lewis^x hexaglycosylceramide

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Immunodetection of thin layer chromatograms of neutral glycosphingolipids of pig kidney cortex with a polyclonal antibody directed against the Galα1-3Gal determinant revealed several glycosphingolipids reacting with different intensities. A minor glycosphingolipid was isolated by preparative high performance thin layer chromatography. It was characterized as a type 2 hexaglycosylceramide with the following structure Galα1-3Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glcβ1-Cer by fast atom bombardment- and desorption-chemical ionization-mass spectrometry, methylation analysis and hydrolysis with α-galactosidase followed by immunostaining with an anti-Lewis^x monoclonal antibody. The proton NMR spectrum was found compatible with the proposed structure. Two other glycosphingolipids carrying the new determinant were partially characterized as an octa- and a branched-dodecaglycosylceramide. The expression of the Galα1-3Lewis^x determinant appeared to be developmentally regulated as it increased with age. The characterization of Galα1-3Le^x in pig kidney indicates a new epitope capable of recognition by human natural antibodies in the context of xenotransplantation of pig organs to man. It also adds new members to the family of Le^x-based glycolipids.

***Keywords*: glycosphingolipids, kidney, Lewis^x, nuclear magnetic resonance, mass spectrometry, pig, xenotransplantation**

***Abbreviations*: HPTLC, high performance thin layer chromatography; FAB-MS, fast atom bombardment mass spectrometry; DCI, desorption-chemical ionization; Me_2SO-d_6, hexadeuterated dimethyl sulfoxide**

Introduction

Research on swine antigens recognized by human natural antibodies stems from the fact that pig appears as a potential organ donor to man [1]. Feasibility of xenotransplantation is presently reinvestigated as a means to alleviate the shortage of human organ donors [2]. The first issue to be addressed is the hyperacute rejection occurring after the grafting of pig organs to primates [3]. Naturally occurring antibodies of the host bind to antigens of the donor endothelium, initiating activation of the classical complement pathway which, in turn, damages the endothelium [4]. Ultimately, the damaged endothelium triggers the coagulation cascade, leading to thrombosis of capillaries within 15–90 min following the grafting. Strong experimental evidences have suggested that the major target of human xenoreactive natural antibodies is the disaccharide determinant Galα1-3Gal [5–7]. This epitope was termed afucoB in the present report as it is structurally a non-fucosylated blood group B determinant. The lack of afucoB epitope in man originates in the inactivation of the α-3-galactosyltransferase gene in the Hominidae lineage 20 million years ago [8, 9]. Its counterpart is the presence in man of natural antibodies directed against the widespread epitope Galα1-3Gal. One per cent of human B lymphocytes are committed to synthesizing anti-Galα1-3Gal antibodies [10]. Thus depletion of host xenoantibodies has appeared as a way of preventing hyperacute rejection [11]. Another possible approach is the genetic manipulation of donor pigs [6, 12, 13].

Investigation of Galα1-3Gal-terminated glycosphingolipids in porcine organs likely to be transplanted, such as kidney, was initiated as a first step to elucidate the molecular specificity of human xenoantibodies. A pentaglycosylceramide carrying the Galα1-3Gal-epitope (afucoB-5), first described in rabbit erythrocytes [14, 15], has been characterized in pig kidney [16]. It has also been shown in porcine aorta endothelial cells, together with a heptaglycosylceramide afucoB-7 [17], previously described in rabbit [18] and bovine erythrocytes [19]. The present investigation was aimed at characterizing in kidney the structure of afucoB-terminated glycosphingolipids more complex than afucoB-5, and thus probably more antigenic. A series of novel glycolipids combining afucoB and Le^x epitopes was discovered.

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Materials and methods

Animals

Kidneys were collected at a local slaughterhouse and kept on ice for transport and dissection. Cortex was separated from medulla, minced, and lyophilized.

Purification of glycosphingolipids

Lipids were extracted from the lyophilized cortex of individual pigs as already described [20]. Glycolipids were purified by chromatography of the acetylated lipid extract on a Florisil column [21]. The column was eluted successively with 1-2-dichloroethane (10 ml g^{-1} Florisil), 1-2-dichloroethane/acetone (93:7) (10 ml g^{-1} Florisil) and 1-2-dichloroethane/acetone (1:1) (20 ml g^{-1} Florisil). The glycolipid fraction obtained from the last elution step was deacetylated and desalted before separation of neutral and acid glycolipids on DEAE-Sephadex A-25 (acetate form) [22].

Thin layer chromatography

Neutral glycolipids were chromatographed on HPTLC silica gel 60 aluminium plates (Merck) developed in chloroform/methanol/water (60:35:8). Immunostaining of chromatograms was done as already described [23] with anti-type 2 H (MR3-517, Institut National de la Transfusion Sanguine, Paris, France), anti-A (NaM87-1F6, Centre Régional de Transfusion Sanguine, Nantes, France), anti-Le^x (CD15) (8-OH5, Immunotech, Marseilles, France), and anti-type 2 lactosamine (1B2) [24] murine monoclonal antibodies. An anti-Galα1-3Gal polyclonal antibody was obtained from eggs of hens immunized with rabbit erythrocyte membranes, and affinity-purified on Synsorb Galα1-3Gal (Chembiomed) [25]. Mouse monoclonal antibodies bound to glycolipids on the chromatogram were detected by reaction with sheep biotinylated anti-mouse immunoglobulins (Amersham, 1:500 dilution) followed by labelling with streptavidin-horseradish peroxidase (HRP) conjugate. Immobilized hen anti-Galα1-3Gal antibodies were detected with HRP-labelled rabbit anti-chicken antibodies (Sigma Immunochemicals, 1:500 dilution) and human natural antibodies with HRP-labelled sheep anti-human immunoglobulins (Amersham, 1:500 dilution). In both cases, visualization was obtained by chemiluminescence using the ECL-Western blotting kit (Amersham) and short exposure (5 min) to a blue-light sensitive autoradiography film (Hyperfilm ECL, Amersham). For structural analysis, glycolipids were separated by preparative HPTLC on Silica gel 60 glass plates (Merck) in chloroform/methanol/water (60:35:8). Visualization was done with ultraviolet light after spraying the plate with a 0.05% solution of primulin in acetone/water (4:1). Each glycolipid was scraped off the plate and extracted from the gel in chloroform/methanol/water (30:60:8). Primulin was removed by chromatography of the purified glycolipid on DEAE Sephadex A-25 (acetate form).

Degradation by α-galactosidase

Cleavage of terminal α-galactose was obtained by hydrolysis of an aliquot of purified glycolipid (15–20 nmol) with 50 mU of α-galactosidase from coffee beans (Boehringer) in 50 mM citrate buffer (pH 4). After overnight incubation at 37 °C, the solution was submitted to reverse phase chromatography on Sep-Pak C_{18} cartridge (Waters).

Quantitative measurement

Quantities of glycolipids were determined by measurement of sphingosine content according to a procedure described earlier [26], either in glycolipid mixtures or in suspensions of individual glycolipid scraped off the preparative chromatogram after visualization with primuline.

Methylation analysis

The purified glycolipid was permethylated by the method of Ciucanu and Kerek [27]. The partially methylated glycolipid was submitted to acetolysis, reduction and acetylation [28]. Gas chromatography of the partially methylated alditol acetates was done on a 25 m × 0.32 mm fused silica capillary column wall-coated with 0.2 μm of OV-1. Analyses were performed on a Hewlett Packard 5890 gas chromatograph equipped with a flame ionization detector and operated in constant flow mode. Carrier gas was helium at a velocity of 40 cm s^{-1}. Samples dissolved in hexane were injected (1 μl) on column at an oven temperature of 60 °C. After 0.5 min, the oven temperature was raised to 125 °C at a rate of 20 °C min^{-1}, then up to 250 °C at a rate of 5° min^{-1}.

Mass spectrometry

The purified glycolipid was analysed as permethylated derivative. In FAB-MS, the sample dissolved in methanol was mixed on the probe with 3-nitrobenzoyl alcohol supplemented with 10% trifluoroacetic acid, and bombarded with xenon atoms at 3 keV. In DCI mode, the ionization gas was isobutane. The platinum filament carrying the sample was progressively heated, and spectra were recorded when the filament current reached 0.7 mA. Analyses were performed on a Jeol JMS SX-102 mass spectrometer.

^{1}H-NMR spectroscopy

The native glycolipid was equilibrated three times in deuterated methanol and dried. It was dissolved in 0.5 ml of Me_2SO-d_6 containing 2% D_2O. Spectra were recorded at 400 MHz with 0.4 Hz digital resolution on a Bruker ARX-400 spectrometer. The probe temperature was 55 °C. Chemical shifts are given relative to tetramethylsilane.

Results

Porcine kidney expresses either blood group A- and H-active glycolipids or only blood group H-active glycolipids [29, 30] according to the A/non-A genetic polymorphism of pigs [31]. Therefore glycolipids were purified

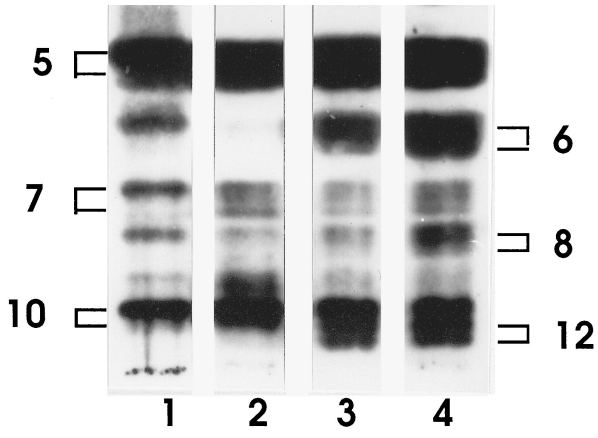

Figure 1. HPTLC and immunostaining with anti-Gal*α*1-3Gal antibody of neutral glycolipids of pig kidney cortex. Lane 1, standard sample from rabbit erythrocyte membranes; lanes 2–4, glycolipids from cortex of 3-week- (lane 2), 6-month- (lane 3) and 2-year-old (lane 4) pigs. Glycolipids were analysed in chloroform/methanol/water (60:35:8), and immunostained with affinity-purified anti-Gal*α*1-3Gal IgY from hens. Numbers in the left margin indicate the position of Gal*α*1-3Gal-terminated penta-, hepta-, and decaglycosylceramide identified in rabbit erythrocytes by Egge *et al.* [18]. Numbers in the right margin give the position of putative hexa-, octa-, and dodecaglycosylceramide which were purified and analysed in the present report.

from kidney cortex of individual pigs. Presence of A-active glycolipids was tested by immunostaining of thin layer chromatograms of cortex neutral glycolipids with a monoclonal anti-A antibody. Non-A glycolipid samples were selected for the present investigation in order to diminish as much as possible the complexity of the glycolipid mixtures.

By HPTLC-immunostaining of neutral glycolipids with the anti-Galα1-3Gal IgY antibodies raised in hens immunized with rabbit red blood cell membranes [25], pig kidney cortex was found to express multiple neutral glycolipids containing the Galα1-3Gal (afucoB) determinant at the non-reducing end of carbohydrate chains (Fig. 1). The shorter element of the series, and the most abundant (3.6 mol/100 mol neutral glycolipids), was a pentaglycosylceramide (afucoB-5) already characterized in the whole kidney by Jalali-Araghi and Macher (16) and Samuelsson *et al.* (30), and previously found in rabbit [14, 15, 18] and bovine erythrocytes [19]. In the present study, a more polar glycolipid was also detected with the anti-Galα1-3Gal IgY antibodies. It was named GL-6 according to its mobility on thin layer chromatogram.

Characterization of a hexaglycosylceramide terminated with the Galα1-3Le^x determinant

GL-6 was isolated by preparative thin layer chromatography, permethylated, and analysed by FAB-MS (Fig. 2, upper trace). The ion at *m/z* 842 could be interpreted as coming from a terminal tetrasaccharide fragment containing two hexoses, one hexosamine and one deoxyhexose. It was the same signal as for a blood group B determinant [32]. Ions for a terminal hexose (*m/z* 219 and 187) and a terminal deoxyhexose (*m/z* 189 and 157) were present in the region of lower masses. The pentasaccharide ion was seen at *m/z* 1046. Series of ceramide ions were seen for sphingosine bound to fatty acids C_{16} (*m/z* 548) to C_{24} (*m/z* 660). The molecular mass region of the spectrum was better seen in DCI than in FAB mode (Fig. 2, lower trace). It indicated a hexaglycosylceramide containing four hexoses, one N-acetylhexosamine, one deoxyhexose, one sphingosine and one fatty acid with 16–24 carbon atoms. The C_{22} species was the most abundant one, as attested by the molecular ion (*m/z* 1873) and the ceramide ion (*m/z* 604). The diagnosis of a blood group B-active glycolipid given by the MS spectrum was surprising because the antibodies used for immunostaining were specific of the afucoB determinant and did not cross-react with the blood group B determinant. GL-6 was submitted to methylation analysis (Fig. 3, upper trace). Absence of peaks of 4,6-di-OMe-Gal (-2,3Gal1-) and 3,6-di-OMe-GlcNAc (-4GlcNAc1-) from the gas chromatography profile ruled out the presence of a blood group B determinant. Actually, there were two residues of 2,4,6-tri-OMe-Gal (-3Gal1-) and one residue of 6-OMe-GlcNAc (-3,4GlcNAc1-) for one residue of 2,3,6-tri-OMe-Glc (-4Glc1-), indicating a Le^a or Le^x core terminated with a galactose residue 1-3-linked to the penultimate galactose. Reactivity upon HPTLC-immunostaining with an anti-Le^x (anti-CD15) monoclonal antibody of the five sugar chain glycolipid resulting of the α-galactosidase hydrolysis of GL-6 indicated unambiguously a Le^x core (Fig. 4, panel B, lane 4). Therefore GL-6 was a novel structure:

Galα1-3Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glcβ1-Cer

The 400 MHz spectrum displayed in the anomeric region seven proton signals with approximately the same intensity (Fig. 5, upper trace). Signals were assigned by comparison with the spectrum of the afucoB-5 obtained from the same cortex glycolipid sample as GL-6 (Fig. 5, lower trace), and related Le^x- and Le^y-active glycolipids (Table 1). The α-anomeric signal at 4.843 ppm was attributed to the terminal Galα1-3 residue as in afucoB-5. The other α-anomeric signal, at 4.889 ppm, could be assigned to the Fucα1-3 residue. The chemical shift of the H-1 of Fucα1-3 has been reported to be unaffected by fucosylation (Le^y-6) or sialylation of the Le^x determinant [33]. However, the H-1 signal of Fuc VI was shifted 0.013 ppm downfield from the position described for Le^x-active pentaglycosylceramide [33], closer to the position described for B-Le^y-active heptaglycosylceramide [34] (Table 1). The signal at 4.785 ppm could be assigned to the GlcNAc III and the signal at 4.354 ppm to the Gal IV. They were shifted to higher frequencies compared to their position in afucoB-5. Similar shifts have been observed for these protons when Fucα1-3 is added to nLc_4Cer or to $IV^2FucnLc^4Cer$ [33]. The doublets at 4.169

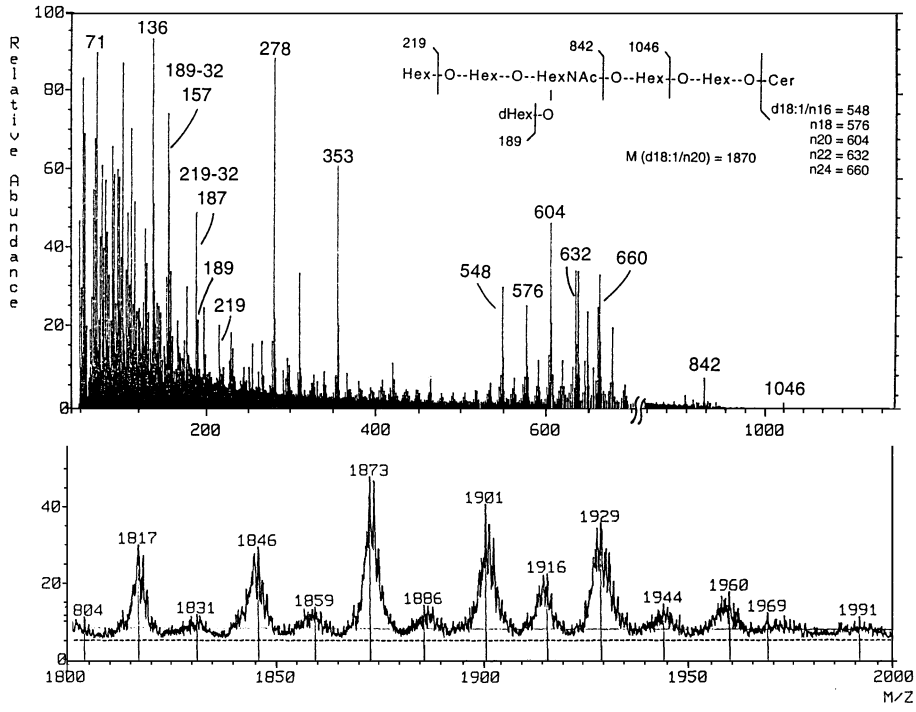

Figure 2. Mass spectrum of the permethylated Gal*α*1-3Gal-terminated hexaglycosylceramide. Upper trace, FAB-mass spectrum. Numbers on the fragmentation scheme and on the spectrum refer to nominal masses. Lower trace, molecular mass region of the DCI-mass spectrum. The largest peak of each cluster was directly labelled by the data system according to the instrument calibration. Labelling corresponds to real masses of $(M + H)^+$ ions for C_{16} (*m/z* 1817) to C_{24} (*m/z* 1929) fatty acid-containing molecules. Labelled masses for C_{18} (*m/z* 1846) and C_{23} (*m/z* 1916) fatty acid-containing glycolipids exceed real masses by 1 mass unit. The drawn spectrum results from the accumulation of 28 scans between *m/z* 600 and 2200, and is reproduced without magnification.

and 4.290 ppm are characteristic of H-1 β-protons of Glc I and Gal II, respectively. The seventh proton signal (4.578 ppm) with a large coupling constant was interpreted as a quartet arising from the H-5 of Fucα1-3, coupled to the H-6 methyl protons of the same residue, the doublet of which was seen at 1.031 ppm (Table 1). In conclusion, the proton NMR spectrum fully supported the proposed structure for GL-6.

Expression of GL-6 was studied by HPTLC-immunostaining of neutral glycolipids isolated from the kidney cortex of pigs at different ages, from three weeks to two years, with hen anti-Galα1-3Gal antibodies (Fig. 1, lanes 2–4). It was almost undetected in 3-week-old pigs (Fig. 1, lane 2), increased with age, and was more abundant at 2 years (Fig. 1, lane 4) than at 6 months (Fig. 1, lane 3), whereas expression of afucoB-5 appeared stable with development (Fig. 1, lanes 2-4, GL-5). The contribution of GL-6 to the cortex of a 240 g kidney was estimated at 100 nmol (0.66 mol per 100 mol neutral glycolipids). Three longer chain Galα1-3Gal-terminated glycolipids also appeared developmentally regulated (Fig. 1, lanes 2–4). The less polar one, with a HPTLC mobility slower than that of a Galα1-3Gal-terminated heptaglycosylceramide (Fig. 1, lane 1, GL-7) characterized in rabbit erythrocytes [18], bovine erythrocytes [19] and porcine aorta endothelial cells [17], was termed GL-8. The two others appeared with a HPTLC mobility slower than a Galα1-3Gal-terminated decaglycosylceramide characterized in rabbit erythrocytes (Fig. 1, lane 1, GL-10) [18]. They were initially termed GL-12.

Partial structural characterization of three elongated lactoseries glycolipids bearing the Galα1-3Le^x determinant

Although GL-8 and GL-12 occurred in very low amounts (less than 10 nmol each per cortex of a 240 g kidney), they were successfully isolated (Fig. 4, panel A, lanes 2, 3). However, the two components of GL-12 could not be separated. Isolated glycolipids were submitted to α-galactosidase hydrolysis. The degradation products were less polar on HPTLC than the initial products and were immunostained with the anti-Le^x antibody (Fig. 4, panel B, lanes 5, 6). This finding indicated that GL-8 and GL-12 were both terminated with the Galα1-3Le^x determinant. Methylation analysis was essayed. GL-8 occurred in such a low quantity that signals for carbohydrate residues had often a lower intensity than contaminants. However, it was possible to detect the same carbohydrates as for GL-6, particularly a distinct signal for -3,4GlcNAc1- (not shown). The most probable, although tentative, structure for GL-8 was thus

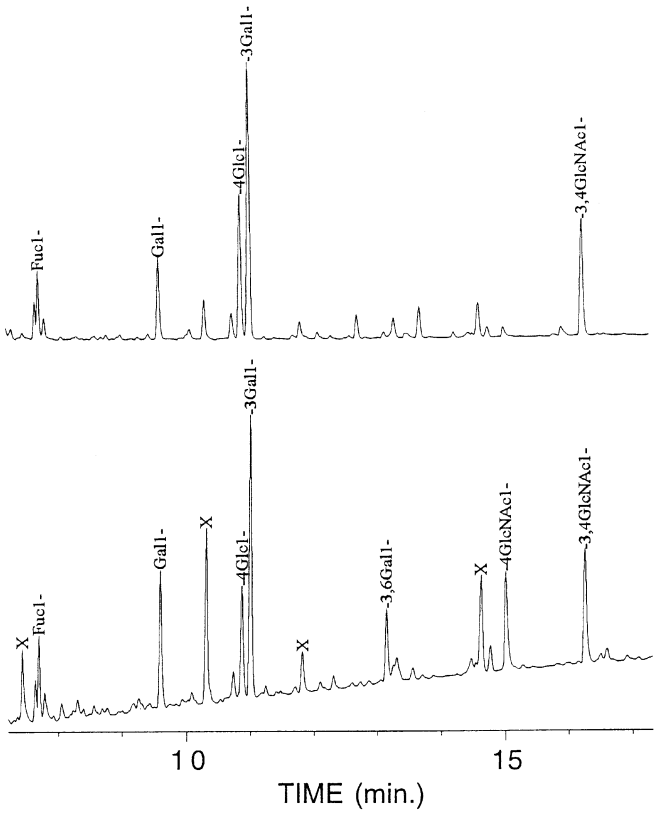

Figure 3. Gas chromatography of the partially methylated alditol acetates of Galα1-3Gal-terminated hexaglycosylceramide (upper trace) and dodecaglycosylceramide (lower trace) from pig kidney cortex. Peaks labelled X are non-carbohydrate contaminants.

$V^3FucVI^3GalnLc_6Cer$ (Table 2, GL-8). The gas chromatography profile of the partially methylated alditol acetates of GL-12 (Fig. 3, lower trace) yielded one -4Glc1- for three -3Gal1-, and signals for -4GlcNAc1- and -3,6Gal1-, indicating a branched type 2 lactoseries core structure. The signals for the component carbohydrates of Galα1-3Le^x determinant, although not quantitative, were compatible with two -3,4GlcNAc1-, two Gal1- and two Fuc1- upon comparison with the GL-6 spectrum (Fig. 3, upper trace). It was concluded that GL-12 was probably a dodecaglycosylceramide with a type 2 lacto core structure and two branches both containing a Galα1-3Le^x determinant. However, two Le^x-active glycolipids were generated by α-galactosidase hydrolysis of GL-12, at the level of ten and nine carbohydrate chains (Fig. 4, panel B, lane 6). Furthermore, the nine carbohydrate chain glycolipid was also reactive with an anti-type 2 lactosamine antibody (not shown). These results suggested that GL-12 actually contained two distinct branched Galα1-3Le^x-terminated glycolipids: a dodecaglycosylceramide bearing two Galα1-3Le^x determinants (Table 2, GL-12) and an undecaglycosylceramide (GL-11) with a Galα1-3Le^x determinant on one branch and an afucoB determinant on the other branch.

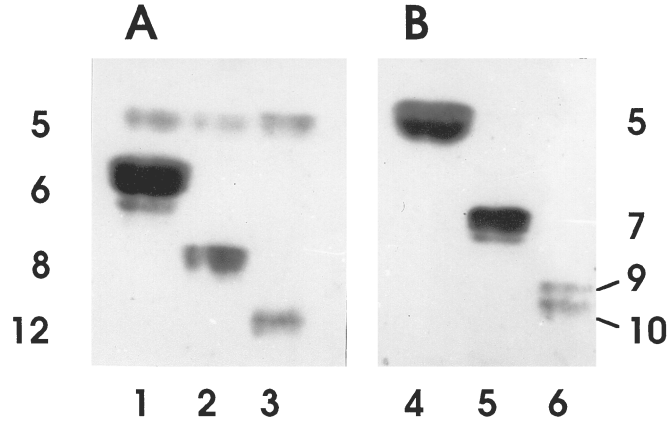

Figure 4. HPTLC and immunostaining of Galα1-3Gal-terminated glycolipids of pig kidney cortex before and after α-galactosidase hydrolysis. Purified hexa-, octa-, and dodecaglycosylceramide were submitted to α-galactosidase hydrolysis. Glycolipids before hydrolysis (panel A, lanes 1–3) and after hydrolysis (panel B, lanes 4–6) were analysed on the same thin layer plate. Panel A, immunostaining of native glycolipid chromatogram with affinity-purified anti-Galα1-3Gal immunoglobulins from hens; panel B, immunostaining of hydrolysed glycolipid chromatogram with anti-Le^x mouse monoclonal antibody.

Reactivity of human natural xenoreactive antibodies with GL-6

The purified Galα1-3Le^x-terminated hexaglycosylceramide was visualized upon HPTLC-immunostaining with a human serum with a high titre of anti-Galα1-3Gal antibodies attested by ELISA with neutral glycolipids of rabbit erythrocytes (Fig. 6). This result indicated that human natural anti-Galα1-3Gal antibodies recognized the Galα1-3Le^x epitope.

Discussion

Immunostaining of thin layer chromatograms of glycolipids of pig kidney cortex with a polyclonal anti-Galα1-3Gal antibody revealed at least seven molecular species of different polarity and different apparent concentration (Fig. 1). In a previous study of pig kidney glycolipids, immunostaining with a murine monoclonal anti-Galα1-3Gal antibody revealed only one reactive glycolipid that has been characterized as afucoB-5 [16]. Detection of the minor species which were studied in the present investigation could be achieved only because a potent anti-Galα1-3Gal antibody had been previously purified from hen eggs [25]. Human natural anti-Galα1-3Gal antibodies are poor analytical reagents, and human plasma contains numerous anti-carbohydrate antibodies of other specificities. Unfortunately, affinity purification of human anti-Galα1-3Gal antibodies does not increase their titre because they are partially inactivated upon elution of affinity columns. High titre antisera cannot be obtained from most conventional animals, as the expression of the afucoB epitope is ubiquitous among mammals [8]. In

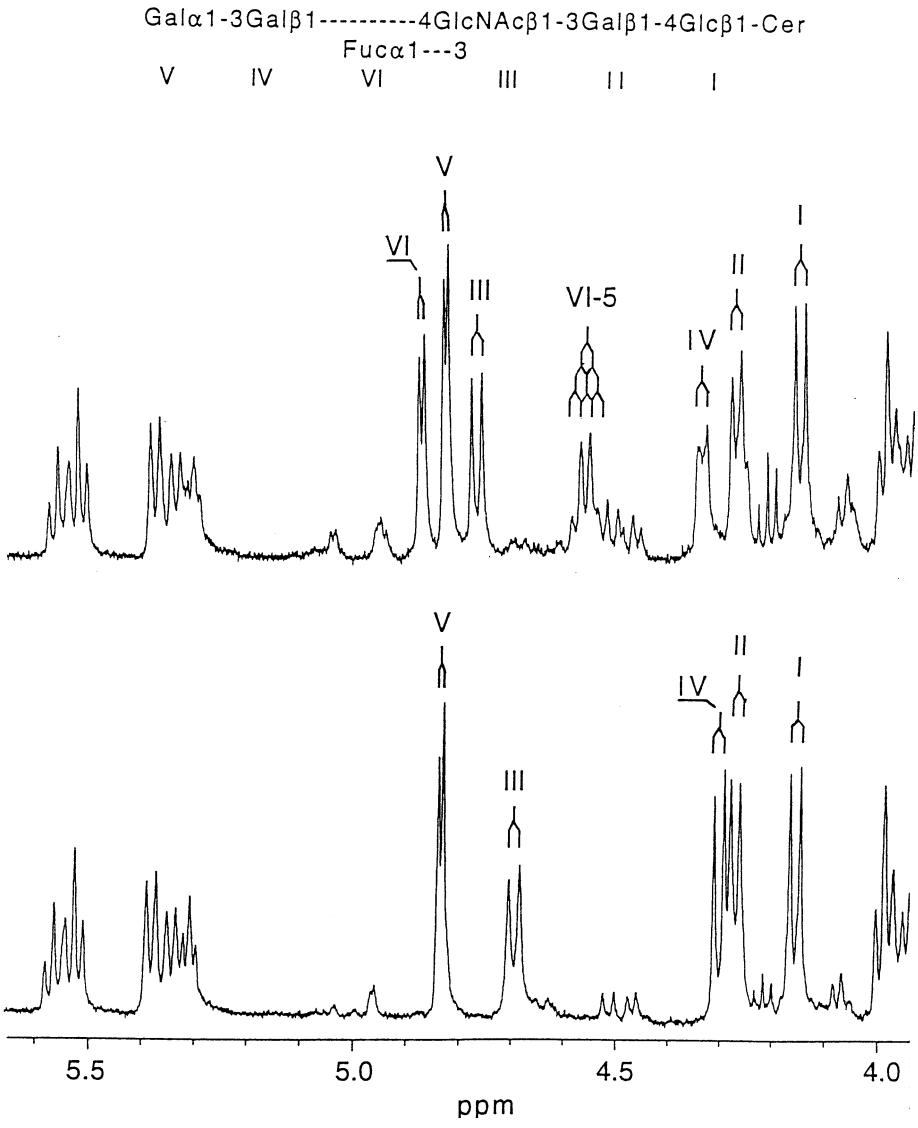

Figure 5. Anomeric region of the 400 MHz spectrum of the Galα1-3Gal-terminated penta- and hexaglycosylceramide from pig kidney cortex. Glycolipid samples of penta- (650 µg) and hexaglycosylceramide (290 µg) were deuterium exchanged, and dissolved in 0.5 ml of Me_2SO-d_6 containing 2% D_2O and 0.03% of tetramethylsilane. Upper trace, spectrum of hexaglycosylceramide. Lower trace, spectrum of pentaglycosylceramide. Spectra were recorded at 55 °C and resulted from the accumulation of 256 pulses. Shifts were calculated by reference to the tetramethylsilane signal. Signals were assigned to anomeric protons of sugar residues labelled in roman numerals as indicated on the scheme. VI-5 refers to the H-5 proton of fucose residue VI.

order to get a potent antibody, hens, which are known not to express the afucoB epitope as all birds [8], were immunized with rabbit erythrocyte membranes which express large amounts of afucoB-active glycolipids. Subsequently, IgY immunoglobulins were recovered from egg yolks and purified with an excellent yield on Galα1-3Gal affinity column (Synsorb) [25].

In addition to a potent anti-Galα1-3Gal antibody, reduction of the complexity of the glycolipid mixtures was necessary for isolating glycolipids characterized in the present study. Two means were used for this purpose. First, purification of glycolipids from cortex instead of total kidney led to a better yield of Galα1-3Gal-terminated complex glycolipids. Second, only non-A pigs were selected for this study. In agreement with a previous study [29], it was observed by immunostaining with anti-A and anti-type 2 H monoclonal antibodies that cortex glycolipid mixtures from pigs expressing blood group A antigens contained several A-active glycolipids in addition to faintly expressed H-active glycolipids penta- and heptaglycosylceramide (H-5 and H-7) (not shown). Our study of the glycolipid composition of porcine aorta endothelial cells has shown

Table 1. Chemical shifts (ppm from tetramethylsilane) and $^3J_{1,2}$ coupling constant (Hz) of glycosidic H-1 of glycolipids as well as H-5 and H-6 of Fucose α1-3.

	Galα1----------3Galβ1---------4GlcNAcβ1-3Galβ1----4Glcβ1-Cer							Fuc VI		References
		Fucα1--------2		Fucα1-------3						
	V	VII	IV	VI	III	II	I	H-5	H-6	
GL-6	4.843 (3.1)		4.354 (6.9)	4.889 (3.7)	4.785 (7.5)	4.290 (6.9)	4.169 (7.8)	4.578	1.031	This Work
afucoB-5	4.847 (3.4)		4.316 (7.4)		4.705 (8.1)	4.286 (7.1)	4.170 (7.8)			This work
afucoB-5	4.83 (2.9)		4.32 (7.8)		4.72 (8.3)	4.29 (7.8)	4.16 (7.8)			Ref. 16
Le^x-5			4.295 (7.3)	4.876 (4.3)	4.748 (7.9)	4.280 (7.3)	4.206 (7.3)	4.590	1.020	Ref. 33
Le^y-6		4.973 (4.3)	4.404 (6.7)	4.875 (3.7)	4.712 (7.9)	4.276 (7.3)	4.223 (7.9)	4.644	1.060	Ref. 33
B-Le^y-7	4.980 (4.0)	5.104 (3.8)	4.462 (7.5)	4.881 (3.8)	4.719 (7.5)	4.278 (7.0)	4.224 (7.8)	4.659	1.085	Ref. 34

Spectra were recorded at 55 °C. Reference spectra of afucoB-5 (16) and B-Le^y-7 (34) were recorded at 50 °C and 65 °C, respectively.

Table 2. Structure of glycosphingolipids mentioned in the text.

Galα1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-Cer	afucoB-5
Galα1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-Cer	afucoB-7
Galα1-3Galβ1-4GlcNAcβ1-6 Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-Cer Galα1-3Galβ1-4GlcNAcβ1-3	afucoB-10
Galα1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-Cer Fucα1-3	GL-6
Galα1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-Cer Fucα1-3	GL-8
Fucα1-3 Galα1-3Galβ1-4GlcNAcβ1-6 Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-Cer Galα1-3Galβ1-4GlcNAcβ1-3 Fucα1-3	GL-12

Structure of GL-8 and GL-12 are tentative. GL-11 is likely to be similar to GL-12 and to contain only one fucose residue. The generic term afuco-B was followed by the number of glycosyl residues in the molecule.

that H-5 and H-7 chromatograph slightly faster than afucoB-5 and afucoB-7, respectively, and that H-7 is not contiguous to afucoB-5 [17]. Galα1-3Le^xGL-6 precisely chromatographed between H-7 and afucoB-5 on thin layer plates. The strategy of using glycolipids from cortex of non-A pigs led to isolation of GL-6 by preparative thin layer chromatography, reasonably devoid of contaminants as attested by methylation analysis and proton NMR spectroscopy.

The biochemical characterization in porcine kidney of a series of anti-afucoB-specified Le^x glycolipids adds novel members to both the family of afucoB- and the family of Le^x-based glycolipids. The first afucoB-active glycolipid was characterized as afucoB-5 in rabbit erythrocytes [14, 15]. AfucoB-active glycolipids with elongated linear [18, 19] or branched [18] oligosaccharides were later discovered in bovine and rabbit erythrocytes. The Le^x-active pentaglycosylceramide was the first to be described [28] in

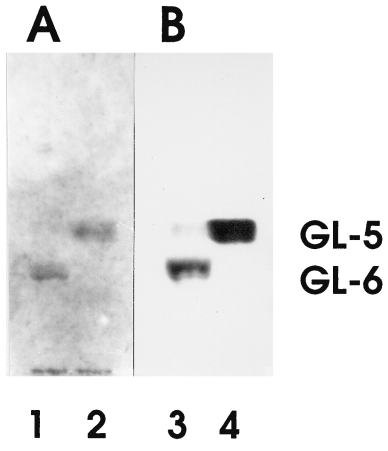

Figure 6. HPTLC and immunostaining with human serum of purified GL-5 and GL-6 xenoantigens from pig kidney cortex. Panel A, immunostaining with a human serum with a high titre of anti-Galα1-3Gal natural antibodies (dilution 1:100). Panel B, immunostaining with hen anti-Galα1-3Gal immunoglobulins. HPTLC was performed with 1 nmol of purified Galα1-3Le^x-terminated hexaglycosylceramide (GL-6) (lanes 1 and 3) and 1 nmol of purified afucoB-5 (GL-5) (lanes 2 and 4).

a series of glycolipids from human tumours or leukocytes having exclusively linear structures with 1–3 lactosamine units [35–37]. In pig kidney, neither Le^x nor Le^y determinants could be detected by HPTLC-immunostaining of purified neutral glycolipids, and the Galα1-3Le^x epitope appeared to terminate linear and branched lactoseries carbohydrate chains alike, in the same way as human ABH blood group determinants [38]. It has been previously shown that the Le^x trisaccharide is not a substrate for the α-3-galactosyltransferase and that the afucoB trisaccharide is a substrate for the α-3-fucosyltransferase [39]. Therefore, it is likely that GL-6, GL-8 and GL-12 are synthesized from afucoB-5, 7 and 10, respectively. It is not known whether α-3-fucosylation of a branched structure such as afucoB-10 follows priority rules as do α-2-fucosylation [40] and α-3-sialylation [41] of isoneolactooctaosylceramide. The precise determination of which branch of GL-11 is fucosylated would bring insights into the sequence of the last biosynthesis steps of GL-12. However, the very small amounts that could be obtained for GL-11 precluded further structural investigations.

It must be mentioned that the Galα1-3Lex determinant has been previously shown to occur at the non reducing end of long carbohydrate chains of mucins of cobra venom, indicating possible coexpression of active α-3-galactosyltransferase and α-3-fucosyltransferase in cells of reptiles [42].

Immunohistological observations of porcine kidney have demonstrated that the afucoB epitope is strongly expressed in epithelial cells (brush borders and cytoplasm) of proximal convoluted tubules and vascular endothelial cells, whereas Le^x antigens are only detected in a few epithelial cells of distal convoluted tubules that do not express the afucoB epitope [31]. These data, together with the characterization in the present work of a series of Galα1-3Le^x-active glycolipids with absence of detectable Le^x-active glycolipids, indicate that most of the cells expressing an α-3-fucosyltransferase activity also express an α-3-galactosyltransferase activity. Therefore, the biosynthesis of the Galα1-3Le^x determinant, which was previously carried out *in vitro* with heterologous α-3-galactosyltransferase and α-3-fucosyltransferase [39], is actually performed *in vivo* by normal cells of porcine kidney. The obvious result of both enzyme activities is a Galα1-3Le^x determinant that is still recognized by anti-afucoB antibodies but does not react with anti-Le^x antibodies.

While this work was submitted for publication, it was reported that murine teratocarcinoma cells change their phenotype from Le^x to Galα1-3-Le^x upon retinoic acid-induced differentiation [43]. In this case, although α-3-fucosyltransferase is active during the whole differentiation process, Le^x epitopes are 'masked' from antibody recognition by differentiation-activated α-3-galactosylation. In the present study, the expression of glycolipids carrying the Galα1-3Le^x epitope increased with animal age, whereas the afucoB-terminated glycolipids were equally expressed in 3 week-old and in older animals. Therefore, in pig kidney, Galα1-3Le^x might be considered as a developmentally regulated antigen under the control of α-3-fucosyltransferase.

As a member of the Le^x antigen family, the Galα1-3Le^x determinant characterized in porcine kidney might be involved in cell to cell interactions in pig, and also in the interactions between human blood cells and porcine tissues after a xenotransplantation. In man, Le^x, sialylated-Le^x(SLe^x), and Le^x-related determinants expressed on glycoproteins and glycolipids at the surface of leukocytes are ligand of P and E selectins, and therefore play a role in the trafficking of leukocytes [44]. Previous studies of pig neutrophils with monoclonal antibodies directed against Le^x, SLe^x, dimeric Le^x, Le^y and unsubstituted lactosamine failed to detect any of these type 2 chain epitopes which are abundantly expressed in human cells [45, 46]. A possibility is that, in pig, Le^x epitopes are substituted by Galα1-3 in neutrophils as efficiently as in kidney, preventing reactivity with anti-Le^x-related antibodies. SLe^x is not the natural E and P selectin ligand of pig as well as several other species [46]. However, SLe^x is efficient at inhibiting leukocyte adhesion at sites of inflammation in animal experimental models [47, 48]. Such data suggest a possible structural relationship to SLe^x of the natural ligands of non-human selectins [44]. The Galα1-3Le^x determinant is a good candidate for fulfulling the role of selectin ligand in pig.

The historic finding of SLe^x was made by Rauvala who characterized the epitope as part of a minor ganglioside of human kidney [49], a long time before its role as E selectin ligand was discovered [50, 51]. Likewise, the Galα1-3Le^x epitope presently characterized in minor glycolipids of porcine kidney might turn out to be ligand for E selectin in pig.

SLe^x and Galα1-3Le^x are structurally related, as previously noticed by Joziasse *et al.* [39]. Both epitopes have a free hydroxyl group at C-2 and a terminal residue α-linked to the C-3 of β-galactose. Such a configuration does not occur in Le^y, ALe^y or BLe^y. A new study is necessary to evaluate the potential physiological role of the Galα1-3Le^x epitope in pig.

In the prospect of grafting pig organs to human, the Galα1-3Le^x epitope is expected, as variant of the afucoB epitope, to bind human natural antibodies (Fig. 6), and participate in the hyperacute rejection process. In order to avoid the immediate graft rejection, two types of approach are under study: treatment of the host or treatment of the donor. Absorption of natural anti-Galα1-3Gal antibodies of the host plasma on immobilized Galα1-3Gal disaccharide has proven its efficiency in the transplantation of pig kidneys to *Cynomolgus* monkeys [11]. Treatment of the donor consists in modifying pig genome in order to decrease or suppress the expression of α-3-galactosyltransferase [6, 12, 13]. Considering the existence and the possible function of the Galα1-3Le^x determinant, such projects might have unexpected effects, first, on the survival of pigs, and second, on the cellular response of the human or primate host toward the graft endothelium.

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