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Simultaneous expression by porcine aorta endothelial cells of glycosphingolipids bearing the major epitope for human xenoreactive antibodies (Galα1-3Gal), blood group H determinant and *N*-glycolylneuraminic acid

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Glycosphingolipids were isolated from primary cultures of porcine endothelial cells labelled with ^{14}C-galactose or ^{14}C-glucosamine. They were characterized by their mobility on thin layer chromatogram, their sensitivity to exoglycosidases, and their labelling with antibodies. In addition to the major glycosphingolipids, globotetra- and globotriaosylceramide, minor ones were identified as penta- and heptaglycosylceramide of the neolactoseries terminated by either Galα1-3Gal- (xenoreactive epitope) or Fucα1-2Gal- (H determinant). Two gangliosides were found, GM3 and GD3, and *N*-glycolylneuraminic acid was their major sialic acid. Therefore, porcine endothelial cells differ from human endothelial cells by expression of glycosphingolipids that are absent in man: two Galα1-3Gal-terminated glycolipids recognized by human natural antibodies, and two *N*-glycolylneuraminic acid-terminated gangliosides which are potent immunogens.

Keywords: porcine endothelial cells, glycosphingolipids, xenoantigens

Abbreviations: HPTLC, high performance thin-layer chromatography; GSL, glycosphingolipid; NeuAc, *N*-acetylneuraminic acid; NeuGc, *N*-glycolylneuraminic acid; PAEC, porcine aorta endothelial cell.

Introduction

Xenotransplantation of porcine organs to man is considered as a possible way to alleviate the present shortage of human organs, at least temporarily while waiting for a compatible human graft. A major obstacle to xenotransplantation is the occurrence of hyperacute rejection which is the consequence of the binding of naturally occurring antibodies of host plasma to antigens of the endothelium of the graft and activation of the complement cascade leading to alteration of the endothelium integrity. Therefore, it is important to identify antigens of endothelial cells which can be recognized by human xenoreactive natural antibodies. It is also relevant to characterize immunogens which could trigger the host immune system when hyperacute rejection is brought under control. Strong experimental evidences have suggested that the major target of human xenoreactive natural antibodies is the disaccharide determinant Galα1-3Gal- [1–3] which is expressed in porcine endothelial cells [4]. It is a human blood group type determinant corresponding to an unfucosylated blood group B (afucoB).

The current concept is that porcine endothelium expresses α3-galactosyltransferase and is devoid of α2-fucosyltransferase activity [5], whereas the reverse situation is observed in human endothelium which thus expresses blood group H, A or B determinant, depending on the individual ABO blood group [6]. Induction into transgenic pig of the expression of α2-fucosyltransferase competing for the same substrate as α3-galactosyltransferase has appeared as a means for decreasing the expression of xenoreactive antigens and humanizing

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porcine endothelium [7, 8]. However, pigs are known to express the α2-fucosyltransferase gene in epithelial cells which synthesize either the H or A determinant, depending on pig A/non-A polymorphism [5].

In the present investigation, glycosphingolipids (GSL) of porcine aorta endothelial cells were analysed after metabolic labelling of primary cultures with radioactive galactose and glucosamine. It was found that major neutral glycolipids belonged to the globoseries, whereas minor ones were penta- and heptaglycosylceramide of the neolactoseries terminated with the afucoB determinant (Galα1-3Gal-), and also with the blood group H determinant (Fucα1-2Gal-). Gangliosides were mainly GM3 and GD3 sialylated either with *N*-acetyl- or *N*-glycolylneuraminic acid. This latter sialic acid, when present in gangliosides, has been previously identified as the immunogen reacting with the Hanganutziu-Deicher heterophile antibodies [9–11].

Materials and methods

Primary culture of porcine endothelial cells

Endothelial cells were isolated from pig thoracic aorta by treatment with 0.4% collagenase A (Boehringer) in PBS buffer pH 7.4 for 15 min at 37 °C. Isolated cells were recovered in RPMI 1640 culture medium (GIBCO) containing 20% newborn calf serum, 50 mg ml^{-1} penicillin and streptomycin, 2 mM L-glutamine. They were seeded on 2% porcine gelatin in 250 ml flasks (2×10^5 cells per flask). Cultures were done in the medium described for cell recovery. Cells were serially passaged with 0.25% trypsin/0.09% EDTA. Cells at confluence in a flask were used to seed three subsequent flasks. Under these conditions, PAEC kept stable characteristics up to the 12th passage. They were identified by their cobblestone morphology, uptake of acetylated LDL, reactivity with anti-von Willebrandt factor antibody, and absence of reactivity with anti-α-actinin antibody. Cells at passage 7 tested positive for lipopolysaccharide-induced E-selectin gene expression. Consequently, labelling experiments were done on cells at the 7th passage.

Radiolabelling of primary culture of porcine aorta endothelial cells

Metabolic radiolabelling of glycosphingolipids was performed by addition of 10 μCi of D-(1-^{14}C)galactose (55 mCi mmol^{-1}) or D-(1-^{14}C)glucosamine (52 mCi mmol^{-1}) (Amersham) to the culture medium at the end of the exponential growing phase. Cells were cultured for 48 h until confluence (about 2×10^6 cells), and then harvested by treatment with trypsin/EDTA and centrifuged. The cell pellet was washed with saline and lyophilized.

Glycosphingolipid isolation

The lyophilized material (10 mg) was submitted to lipid extraction by four incubations at 70 °C for 30 min first with methanol followed three times with chloroform:methanol (1:2), as already described [12]. The combined extracts were evaporated under nitrogen. Dry lipids were acetylated with pyridine:acetic anhydride (2:1.5). Acetylated glycolipids were purified according to Saito and Hakomori [13] by chromatography on a Florisil column (1 g) with 20 ml of 1-2-dichloroethane:acetone (1:1) after removal of neutral lipids with 1-2-dichloroethane. After deacetylation and desalting, neutral glycolipids were separated from acid glycolipids by chromatography on DEAE-Sephadex A-25 (acetate form, 0.5 g) according to Ueno *et al.* [14]. Neutral and acid glycolipids purified from 2×10^6 cells were both solubilized in 100 μl of chloroform:methanol (2:1).

Exoglycosidase degradation

Enzymatic reactions were performed with 10 μl of the neutral or acid glycolipid solution purified from 2×10^6 cells. Cleavage of terminal α-galactose was obtained with 50 mU of α-galactosidase from coffee beans (Boehringer) in 50 mM citrate buffer pH 4. Cleavage of terminal β-galactose was done in the same buffer, with 30 mU of β-galactosidase from jack beans (Sigma) in the presence of 50 μg of sodium taurodeoxycholate. Cleavage of terminal β-*N*-acetylgalactosamine was done with 45 mU of β-*N*-acetylglucosaminidase from jack beans in the same conditions, except that citrate buffer was at pH 5.5. Cleavage of terminal fucose was done with 160 mU of α-fucosidase (Boehringer) in 100 mM acetate buffer pH 4.6, in the presence of 50 μg of taurodeoxycholate. Terminal sialic acids were cleaved from acid glycolipids with 0.1 U of *Vibrio cholerae* neuraminidase (Behringwerke). For each enzymatic hydrolysis, after overnight incubation at 37 °C, the solution was submitted to reverse phase chromatography on Sep-Pak C_{18} cartridge (Waters) as already described [12]. Sodium taurodeoxycholate, when present, was removed by chromatography on DEAE-Sephadex A-25 (acetate form, 0.1 g). Sialic acids were purified from the Sep-Pak flow-through fraction by chromatography on Dowex-50.

Detection of GSL after thin-layer chromatography

Neutral GSL were chromatographed on HPTLC silica gel 60 aluminum plates (Merck) developed in chloroform:methanol:water (60:35:8). Gangliosides were chromatographed on HPTLC silica gel 60 glass plates developed in chloroform:methanol:2.5 M ammonia containing 0.25% KCl (50:40:10). Purified sialic acids were chromatographed on Silica gel 60 HPTLC plates developed in 1-propanol:2.5 M ammonia (7:3). Radioactive GSL and sialic acids were directly visualized by exposure of the dry

chromatography plate to Hyperfilm-MP (Amersham) at −80 °C for 3 days. Immunostaining of neutral GSL chromatograms was done according to published techniques [15, 16] with mouse monoclonal anti-type 2 H antibody (MR3-517, Institut National de la Transfusion Sanguine, Paris, France) and anti-Galα1-3Gal polyclonal antibody purified on Synsorb Galα1-3Gal (Chembiomed) from immunoglobulins isolated from eggs of hens immunized with rabbit erythrocyte membranes. Mouse monoclonal antibodies bound to GSL on the chromatogram were detected by reaction with sheep biotinylated anti-mouse immunoglobulins (Amersham, 1:500 dilution) followed by labelling with streptavidin-horseradish peroxidase conjugate. Immobilized anti-Galα1-3Gal antibodies were detected with horseradish peroxidase-labelled anti-chicken antibodies (Sigma Immunochemicals, 1:1000 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).

Results

Neutral glycosphingolipids of PAEC

Metabolic labelling of PAEC with radioactive carbohydrates yielded radioactive GSL which appeared as double bands on the autoradiography of chromatograms (Fig. 1). Glucosamine is a precursor of the biosynthesis of *N*-acetylhexosamines and sialic acids, whereas galactose is preferentially incorporated into hexoses [17].

Labelling with ^{14}C-glucosamine yielded radioactive GSL at the level of tetrahexosylceramide and longer chains GSL (Fig. 1, lane 1). ^{14}C-Galactose labelled neutral GSL from monohexosylceramide to at least heptaglycosylceramide (Fig. 1, lane 2). The glycolipid with the mobility of a trihexosylceramide (GL-3), present on the ^{14}C-galactose autoradiogram (Fig. 1, lane 2) and not visible on the ^{14}C-glucosamine autoradiogram, generated a radioactive dihexosylceramide after hydrolysis with α-galactosidase (Fig. 2, lane 2). Such a change of chromatography mobility indicated that GL-3 was most probably globotriaosylceramide (Gb_3Cer) (Table 1). After β-*N*-acetylhexosaminidase hydrolysis, the ^{14}C-glucosamine-labelled GL-4 disappeared without appearance of a shorter chain radioactive glycolipid, whereas the ^{14}C-galactose-labelled GL-4 was replaced by a trihexosylceramide with the same mobility as Gb_3Cer (not shown). These results indicated that the GL-4 of PAEC was most probably globoside (Gb_4Cer) (Table 1).

Structures of longer chain glycolipids were demonstrated with exoglycosidase hydrolysis and immunostaining with specific antibodies. Among GSL more polar than Gb_4Cer, two double band glycolipids GL-5b and

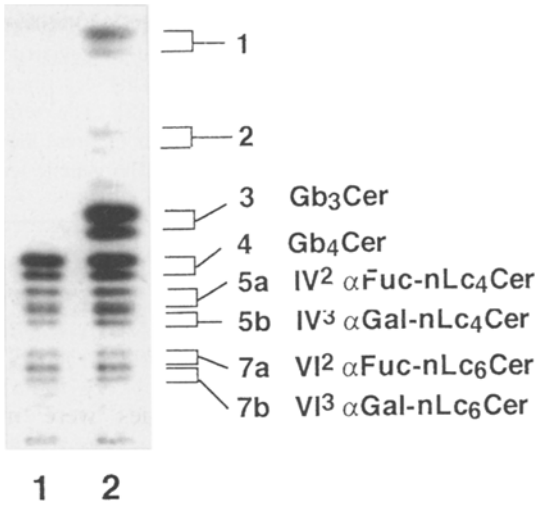

Figure 1. Neutral GSL profile of metabolically labelled PAEC visualized by autoradiography after HPTLC. Lane 1, metabolic labelling with ^{14}C-glucosamine; lane 2, metabolic labelling with ^{14}C galactose. Numbers in the margin indicate the number of sugar residues of the carbohydrate chain of the corresponding glycolipids which were termed GL-1 to GL-7b in the text. Structures of the oligosaccharide chains defined in the present study are also indicated. Solvent of thin-layer chromatography: chloroform:methanol:water (60:35:8).

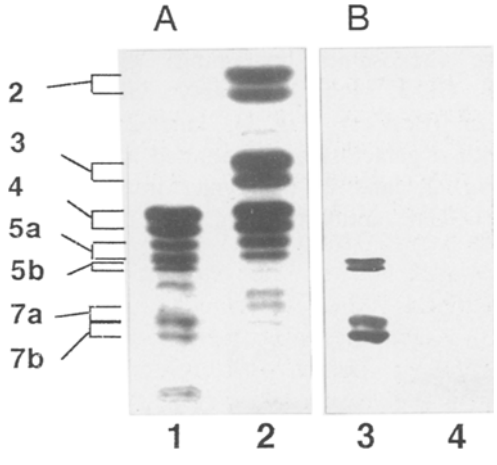

Figure 2. Autoradiography and immunostaining of PAEC neutral GSL chromatogram after α-galactosidase hydrolysis. Panel A, autoradiography; panel B, immunostaining of the same chromatogram with anti-Galα1-3Gal antibody. Lanes 1 and 3, native ^{14}C-glucosamine-labelled GSL; lanes 2 and 4, ^{14}C-galactose-labelled GSL after hydrolysis by α-galactosidase. In the left margin, same numbers as in Fig. 1. In lane 1, the faster band of GL-7a was displaced toward GL-5 by a non glycolipid impurity. In lane 2, the double band of GL-6 yielded by hydrolysis of GL-7b was located just ahead of native GL-7a.

GL-7b disappeared from the autoradiogram after hydrolysis with α-galactosidase of the ^{14}C-galactose labelled GSL (Fig. 2, lane 2) as well as the ^{14}C-glucosamine-labelled GSL (not shown). GL-5b and GL-7b were both

Table 1. Structure of glycosphingolipids mentioned in the text. In addition to the structure, systematic abbreviated names were given for globoseries glycolipids. H-active glycolipids, first described in human erythrocytes, were termed according to Watanabe *et al.* [18]. The term afuco-B was chosen by reference with asialo-gangliosides in order to stress the structural similarity with the blood group B and the absence of fucose. The genetic term afuco-B was followed by the number of glycosyl residues in the molecule

Galα1-4Galβ1-4Glcβ1-Cer	Gb_3Cer
GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-Cer	Gb_4Cer
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
Fucα1-2Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-Cer	H_1
Fucα1-2Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-Cer	H_2
Fucα1-2Galβ1-4GlcNAcβ1-6 Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-Cer Fucα1-2Galβ1-4GlcNAcβ1-3	H_3

immunostained with anti-Galα1-3Gal antibody (Fig. 2, lane 3 and Fig. 3, lane 3). They had the same mobility as rabbit erythrocytes afucoB-5 and afucoB-7, respectively (Fig. 3, lane 4). Indeed, the immunostaining reactivity disappeared after hydrolysis with α-galactosidase (Fig. 2, lane 4). Hydrolysis of the ^{14}C-glucosamine-labelled GL-5b with α- and β-galactosidase led to the appearance of a radioactive (hexosamine-containing) glycolipid at the level of GL-3, most probably GlcNAcβ1-3Galβ1-4Glcβ1-Cer (Lc_3Cer) (Fig. 4, lane 2). Hydrolysis of GL-7b with α-galactosidase generated a hexaglycosylceramide (Fig. 2, lane 2) no longer immunostained with anti-Galα1-3Gal antibody (Fig. 2, lane 4). It was concluded that GL-5b and GL-7b had oligosaccharide chains identical with afucoB-5 and afucoB-7 of rabbit erythrocytes, which are known to be Galα1-3nLc_4Cer and Galα1-3nLc_6Cer [19].

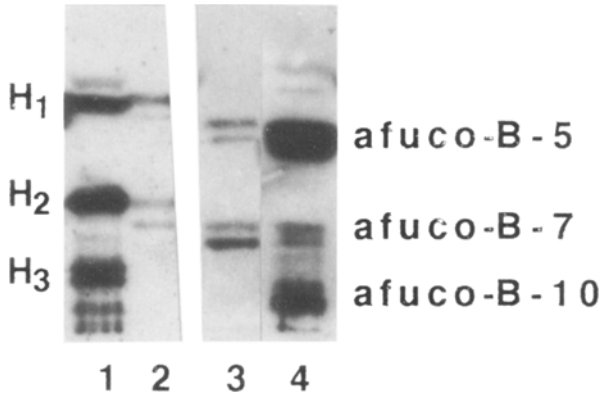

Figure 3. HPTLC-immunostaining of PAEC neutral GSL of a non-A pig. Lanes 1–2, mouse anti-type 2 H monoclonal antibody; lanes 3–4, hen anti-Galα1-3Gal polyclonal antibody. Lane 1, neutral GSL of human blood group O erythrocyte membranes; lanes 2 and 3, neutral GSL of PAEC; lane 4, neutral GSL of rabbit erythrocyte membranes. Immunostainings were done separately after developing GSL on the same chromatogram in the solvent chloroform:-methanol:water (60:35:8).

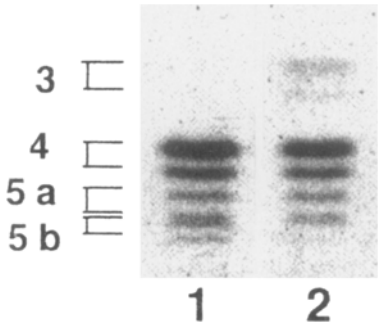

Figure 4. Autoradiography after HPTLC of PAEC neutral GSL metabolically labelled with ^{14}C-glucosamine. Lane 1, native GSL; lane 2, after hydrolysis with α- and β-galactosidase.

GL-5a and GL-7a were immunostained as two doublets with a monoclonal antibody specific to human blood group type 2 H determinant (Fucα1-2Galβ1-4GlcNAcβ-) (Fig. 3, lane 2). The faster band of GL-5a had the same mobility as Fucα1-2nLc_4Cer (H_1) and that of GL-7a as Fucα1-2nLc_6Cer (H_2) of human blood group O erythrocytes [18] (Fig. 3, lane 1) (Table 1). No signal was obtained either with anti-type 1 H or anti-Le^x antibodies. GL-5a and GL-7a disappeared from the autoradiogram and were no longer immunostained after hydrolysis with α-fucosidase (not shown). It was concluded that GL-5a and GL-7a were H-active glycolipids with the same neolacto core structure as the corresponding afucoB glycolipids. Comparison of the respective immunostainings of the fucosylated and Galα1-3Gal-terminated GL-5

and GL-7 (Fig. 3) gave the keys to understanding the complex pattern observed on the autoradiogram (Fig. 1): the slower band of GL-5a and GL-7a tended to merge respectively with the faster band of GL-5b and GL-7b. Autoradiography of the metabolically labelled glycolipids (Fig. 1) indicated that the fucosylated glycolipids occurred in the same range of quantity as the afucoB glycolipids.

PAEC gangliosides

Labelling with ^{14}C-galactose or ^{14}C-glucosamine gave the same chromatography profile, i.e. two double bands of GM3 and two double bands of GD3 (Fig. 5, lane 1). GM3 appeared as the major ganglioside. Ammonia-containing solvents usually segregate the NeuGc-bearing glycolipids in the slower migrating double band, whereas the NeuAc-bearing glycolipids migrate faster. However, the ceramide composition can modify this type of distribution. Thus, gangliosides were submitted to the action of *V. cholerae* neuraminidase in order to cleave the terminal sialic acids. Most of the gangliosides were hydrolysed and replaced by lactosylceramide (Fig. 5, lane 2). A residual double spot slightly more polar than GM3(NeuGc) was produced from ^{14}C-galactose and ^{14}C-glucosamine-labelled gangliosides. It could be interpreted either as a neutral glycolipid containing *N*-acetylhexosamine (generated from a sialylated lactoseries glycolipid), or a neuraminidase-resistant ganglioside such as GM1. The structure of this minor product was not elucidated further. These results indicated that the main gangliosides of PAEC were built on a lactosylceramide core which was either monosialylated (GM3) or disialylated (GD3). The sialic acids purified after hydrolysis were analyzed by comparison with standard sialic acids. *N*-glycolylneuraminic acid appeared by far the major sialic acid of gangliosides of PAEC after chemical detection (Fig. 6, lane 2) and autoradiography (Fig. 6, lane 3).

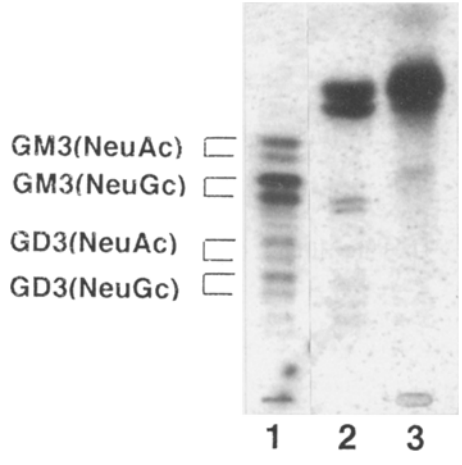

Figure 5. Autoradiography after HPTLC of PAEC gangliosides metabolically labelled with ^{14}C-galactose. Gangliosides before (lane 1) and after (lane 2) desialylation by *V. cholerae* neuraminidase; lane 3, standard tritiated lactosylceramide. Solvent system: chloroform:methanol:2.5 M aqueous ammonia containing 0.25% KCl (50:40:10).

Discussion

Glycosphingolipids are quantitatively minor cellular components although their preferential segregation in the outer leaflet of the plasma membrane makes them functionally important cell components. Their study in porcine endothelial cells appeared inescapable for a better understanding of the reactivity of natural human xenoreactive antibodies with porcine tissues. As endothelial cells are very minor contributors to the aorta, they are unaccessible to classical structural characterization after isolation, as already acknowledged in a report on the GSL composition of pig aorta [20]. Primary culture of PAEC which grows as an adherent monolayer, did not expand the endothelial cell population sufficiently to reach a GSL yield amenable to classical chemical analysis. Metabolic labelling of PAEC by addition of ^{14}C-galactose or ^{14}C-glucosamine, combined to exoglycosidase hydrolysis of GSL mixtures and immunostaining of GSL chromatogram with specific antibodies, made possible the determination of their GSL profile. It also led to a probably accurate characterization, although tentative with respect to the techniques available, of the GSL structures which could be recognized by human natural antibodies in endothelial cells.

Porcine aorta endothelial cells resembled human endothelial cells with regard to their major neutral glycosphingolipids, globotri- and globotetraosylceramide [21]. Most significant was the characterization of two GSL, a penta- and a heptaglycosylceramide of the neolactoseries with terminal Galα1-3Gal- epitope. The

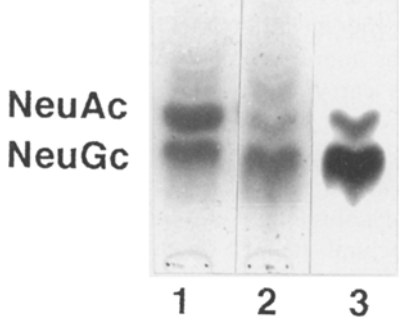

Figure 6. Thin layer chromatography of sialic acids of PAEC gangliosides. Sialic acids were purified from hydrolysis by *V. cholerae* neuraminidase of total gangliosides of PAEC metabolically labelled with ^{14}C-glucosamine. Lanes 1 and 2, chemical visualization with resorcinol/HCl of standard sialic acids (lane 1) and sialic acids from PAEC gangliosides (lane 2); lane 3, autoradiography of lane 2. Solvent system: 1-propanol:2.5 M aqueous ammonia (70:30).

Galα1-3Gal- determinant has been identified as the major antigenic determinant of the pig vascular endothelium to react with naturally occurring xenoreactive IgM of primate blood [1–3], inducing the hyperacute rejection process. Man and the closest primates do not express this determinant [4] because the gene for the α3-galactosyltransferase, which is active in all other mammals, has become inactive during the evolution [22, 23]. As a consequence in man, 1% of B lymphocytes are committed to synthesizing anti-Galα1-3Gal antibodies [24]. The presence of the Galα1-3Gal- epitope in endothelial cells of porcine tissues has been well documented by histological studies using the *G. simplicifolia 1* lectin [5]. However, structures bearing this epitope in endothelial cells have never been characterized. A Galα1-3nLc_4Cer (Table 1, afucoB-5) has been characterized in whole porcine kidney [20, 25] and has been reported to be present in whole aorta [20]. The present work described the same structure in PAEC. In addition, a heptaglycosylceramide (Table 1, afucoB-7), previously found in erythrocyte membranes of rabbit [19] and cow [26], was characterized for the first time in pig. Passive absorption of GSL antigens from the culture medium was ruled out as metabolic labelling demonstrated their *de novo* synthesis.

In this report, the Galα1-3Gal- epitope has been termed afucoB by reference to its structural similarity with the blood group B determinant (Table 1). The term afucoB also takes into account the specificity of each determinant biosynthesis. The afucoB epitope is synthesized by an α3-galactosyltransferase acting on a non-fucosylated *N*-acetyllactosamine acceptor substrate [27], whereas the blood group B determinant is synthesized by an α3-galactosyltransferase which transfers galactose only to a fucosylated acceptor, Fucα1-2-*N*-acetyllactosamine [28]. PAEC were found to express penta- and hexaglycosylceramide carrying the afucoB epitope. It cannot be excluded that endothelial cells of vessels of transplantable organs express more complex GSL with two or more antennae also carrying the afucoB epitope, a structural configuration that might favour the binding of anti-Galα1-3Gal IgM. Similarly, multiple afucoB epitopes are likely to be carried at the nonreducing ends of N-glycan chains of glycoproteins of endothelial cells [3, 29], although structural details of these glycans still await elucidation. Another important finding brought by the present study is the expression of human blood group H determinant in porcine endothelial cells. Studies using immunofluorescence techniques with lectins on histological sections failed to detect human blood group determinants in pig vessels [5]. The present work not only characterized $IV^2FucnLc_4Cer$ in porcine aorta endothelial cells, as found by Gillard *et al.* in human endothelial cells [21], but also an elongated structure, $IV^2FucnLc_6Cer$, not described in human endothelial cells, analogous to the heptaglycosylceramide with terminal afucoB. It has been established that a genetic A/non-A polymorphism exists in the pig species [5]. Actually, the pig from which the aorta endothelial cells were derived was of the non-A type, as assessed by the sole expression of H-glycolipids in kidney, pancreas and lung (not shown). These tissues were found to express A-active glycolipids in other animals.

One strategy to overcome hyperacute rejection would be to prevent the synthesis of the Galα1-3Gal- epitope by engineering pigs with a knock-out of the α3-galactosyltransferase gene if gene knock-out techniques become available for the pig [7]. Characterization of fucosylated GSL in porcine endothelial cells with the same core structure as Galα1-3Gal- terminated GSL, and occurring within the same range of quantity, suggests an alternate solution. Directing overexpression of the pig's own α2-fucosyltransferase in endothelial cells would diminish the expression of the afucoB epitope. However, this strategy would be meaningful only in non-A pigs in order not to induce overexpression of the A determinant which could occur in A-expressing pigs.

The finding of *N*-glycolylneuraminic acid, hydroxylated form of the widespread *N*-acetylneuraminic acid, in the gangliosides of porcine endothelial cells was in accordance with what has already been described in porcine erythrocytes [30]. The human species is the only species, with birds, not to express NeuGc. Natural antibodies against this epitope are very rare and thus not threatening at the time of the graft. However, NeuGc is a potent immunogen. The Hanganutziu-Deicher heterophile antibodies acquired after administration of animal serum have been demonstrated to recognize gangliosides bearing NeuGc [9–11]. It is highly probable that, after the hyperacute rejection linked to the Galα1-3Gal- epitope is overcome, the NeuGc-containing gangliosides will be a further obstacle to xenotransplantation. The enzyme responsible for NeuAc hydroxylation has been identified [31], opening the way to another type of transgenic pig engineering. Alternatively, pigs naturally defective for NeuAc hydroxylation might exist as it was found for cats and dogs [32, 33], and thus provide organs less immunogenic to humans.

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