Glycosphingolipid expression in pig aorta: identification of possible target antigens for human natural antibodies

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Total non-acid glycosphingolipids were isolated from the aortas of more than 80 pigs. The glycolipids were separated by HPLC, analysed by thin-layer chromatography, and tested for reactivity with monoclonal anti-blood group antibodies. The fractions were structurally characterized by NMR spectroscopy and mass spectrometry. Reactivity with both blood group A and H antibodies was seen. The major glycosphingolipid constituents were globotetraosylceramides and blood group H pentaglycosylceramides based on type 1 and type 2 core saccharide chains. Globosylsaryceramides, blood group H hexaglycosylceramides based on type 4 chain, and blood group A hexaglycosylceramides based on type 1 core chain were also present. Two structures, that may be important targets for human antibodies initiating hyperacute rejection following pig to human xenotransplantation, were present as minor constituents compared to the blood group components. These were Galα1,3Neolactotetraosylceramide and a Galα1,3Leα structure. A Leα/Y hexaglycosylceramide was also present.

Key words: glycolipids/mass spectrometry/NMR/pig aorta/xenogeneic antigens

Introduction

The lack of donor organs for human transplantation is a major problem throughout the world. The possibility to use animals as organ donors to alleviate this problem is a challenging possibility of renewed actuality. The pig is considered a possible donor species in such future human xenotransplantations (Samuelsson et al., 1993; Good et al., 1992). The similarity between the hyperacute xenorejection and the rejection seen in ABO incompatible organ transplantation was, among other things, what suggested to us that the target antigens for the preformed xenoreactive antibodies were of carbohydrate nature (Karlsson et al., 1992, 1993; Samuelsson and Cairns, 1994; Samuelsson et al., 1994).

There are now accumulating data supporting this suggestion (Good et al., 1992; Cooper et al., 1993; Sandrin et al., 1993; Rydberg et al., 1994; Satake et al., 1994a,b). With this perspective we set out to characterize the glycosphingolipid-based carbohydrate antigen expression in different pig organs, and so far the glycosphingolipid expression in pig kidneys has been reported (Holgersson et al., 1990).

In view of the fact that glycosphingolipid expression is not only species- and organ-specific but also cell-specific, we wanted to characterize the target cells of the antibody-mediated immune attack, the vascular endothelial cells (Platt et al., 1990). In order to get sufficient numbers of endothelial cells to be able to perform a structural characterization of the glycosphingolipid composition, analysis of an endothelial cell line is a possibility. However, it is well known that an immortalized cell line may not in all respects mimic the original cell type. Primary cultures of endothelial cells on the other hand do not give a sufficient amount of cells. We therefore decided to use pig aorta as a simple means of getting large numbers of pig endothelial cells, well aware of the fact that large masses of tissue of nonendothelial origin will be included. This article will describe the expression of non-acid glycosphingolipids in aortas from at least 80 pigs.

Results

TLC immunostaining

The total non-acid and the polar non-acid glycosphingolipid fractions from pig aorta (Figure 1, lane A and B, respectively) were analyzed by thin layer chromatography (TLC) followed by immunostaining with monoclonal antibody overlay in order to identify blood group reactive compounds (Figure 2). The antibodies used and their origin, specificity, and reactivity on the TLC plate are listed in Table 1. Antibody data will be further discussed in the context of individual fractions.

HPLC separation of porcine aortic glycolipids into subfractions

In view of the TLC pattern, the subfractions obtained following high performance liquid chromatography (HPLC) separation of the polar glycolipid fraction were pooled into 19 fractions (Figure 1, lanes 2–19). The fractions were subjected to NMR and MS analysis.

Fraction 2

The FAB mass spectra of fraction 2 (not shown) contain molecular and fragment ions derived from tri- and tetrahexosylceramides. As judged from the TLC plate (Figure 1) fraction 2 contains mainly triglycosylceramides and traces of glycolipids with longer carbohydrate chains.
Fig. 1. Thin-layer chromatographic analysis of the total non-acid glycolipid fraction isolated from the aortas of more than 80 pigs (lane A). This fraction was fractionated by HPLC silica gel chromatography into a nonpolar (not shown) and a polar fraction (lane B), where the latter was again subfractionated by HPLC into 19 fractions (lanes 2–19). The numbers to the left indicate the number of sugars in the carbohydrate chain. The solvent was chloroform/methanol/water, 60/35/8 (by volume) and the plate was visualized by a chemical reagent, anisaldehyde. All bands, except the band labeled with an “X”, were stained green indicating the presence of carbohydrate. The structures of the glycolipids in fractions 2–14 are listed in Table II.

Table I. Antigen specificity of the monoclonal antibodies used in the immunostaining experiments on TLC

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Specificity</th>
<th>Code no.</th>
<th>Reactivity</th>
<th>Binding regiona</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti H</td>
<td>H, mainly type 2</td>
<td>Dakopatts A583</td>
<td>+</td>
<td>5, 7–8, 10–12</td>
<td>Holgersson et al., 1990b</td>
</tr>
<tr>
<td>Anti A all types</td>
<td>Terminal A trisaccharide</td>
<td>Dakopatts A581</td>
<td>+</td>
<td>4, 6, 7–8</td>
<td>Breimer and Samuelsson, 1986</td>
</tr>
<tr>
<td>Anti A type 1</td>
<td>A type 1, monofucosyl</td>
<td>AH-21</td>
<td>+</td>
<td>6, 7–8, 10–12</td>
<td>Abe et al., 1984</td>
</tr>
<tr>
<td>Anti A type 2</td>
<td>A type 2, monofucosyl</td>
<td>HH-4</td>
<td>(-)b</td>
<td></td>
<td>Clausen et al., 1986</td>
</tr>
<tr>
<td>Anti A type 3</td>
<td>A type 3</td>
<td>TH-1</td>
<td>-</td>
<td></td>
<td>Clausen et al., 1985b</td>
</tr>
<tr>
<td>Anti A type 3 and 4</td>
<td>A type 3 and 4</td>
<td>HH-5</td>
<td>(+)b</td>
<td>6</td>
<td>Clausen et al., 1986</td>
</tr>
<tr>
<td>Anti ALeb</td>
<td>A type 1, difucosyl</td>
<td>HH-3</td>
<td>+</td>
<td>7</td>
<td>Clausen et al., 1985c</td>
</tr>
<tr>
<td>Anti Lea</td>
<td>Lea terminal</td>
<td>XALA Chembiomed</td>
<td>(-)b</td>
<td>5</td>
<td>Holgersson et al., 1990b</td>
</tr>
<tr>
<td>Anti Leb</td>
<td>Leb/H type 1 terminal</td>
<td>9ALB Chembiomed</td>
<td>(-)b</td>
<td></td>
<td>Holgersson et al., 1990b</td>
</tr>
<tr>
<td>Anti X</td>
<td>X terminals</td>
<td>SH-1</td>
<td>-</td>
<td></td>
<td>Singhal et al., unpublished</td>
</tr>
<tr>
<td>Anti Y</td>
<td>Y terminals</td>
<td>AH-6</td>
<td>+</td>
<td>6, 8, 10–12</td>
<td>Abe et al., 1984</td>
</tr>
<tr>
<td>Anti AY</td>
<td>A type 2, difucosyl</td>
<td>HH-2</td>
<td>-</td>
<td></td>
<td>Clausen et al., 1985c</td>
</tr>
</tbody>
</table>

aOn the TLC plate. The numerals indicates the number of sugars in the carbohydrate chain.
bResults in parentheses are indistinct.

Fractions 3 and 4
Fractions 3 and 4 have Rf values on the TLC plate comparable to triglycosylceramides. The FAB mass spectra (not shown) contain molecular and fragment ions derived from trihexosylceramides with different ceramide composition (Table II). Glycolipids with identical sugar chains separate on straight phase silica according to ceramide composition. Ceramides with nonhydroxylated long hydrocarbon chains (n22–24 fatty acids, d18:1 sphingosine) elute before hydroxylated, short hydrocarbon chains (h16–18 fatty acids, t18:0); thus, both chain length and degree of hydroxylation influence the chromatographic mobility.

Fractions 5 and 6
Fraction 5 contains one strong and one weaker, more fast-moving band, whereas fraction 6 appears as a single band on TLC. They all have Rf values comparable to four-sugar compounds, e.g., globoside. The EI mass spectra of the permethylated and the permethylated-reduced derivatives (not shown), are in complete
Fig. 2. Thin-layer immunostaining of the total non-acid glycolipid fraction (lane A) and the total polar glycolipid fraction (lane B) from pig aorta (see Figure 1). Lane R contains the reference compounds indicated to the right of the chromatograms at their respective Rf-value. Detection was accomplished by autoradiography after overlaying the plates with monoclonal antibodies, the specificities of which are indicated above each plate, and secondary 125I-labeled antibodies. The fine specificities of the antibodies used are listed in Table I.

accompany with that of globotetraosylceramide with sphingosine as long chain base and with 22 and 24 carbon nonhydroxylated fatty acids in fraction 5 and hydroxylated fatty acids in fraction 6. The proton NMR spectrum of fraction 6 contains four doublets in the anomeric region. Three β-anomeric signals at δ 4.17 ppm (Glc), 4.27 ppm (Gal), and 4.52 ppm (GalNAc), and one α-anomeric signal at 4.80 ppm (Gal). There is also one N-acetamido methyl signal at 1.85 ppm. This spectrum is in complete accordance with a globotetraosylceramide structure (Dabrowski et al., 1980).

**Fraction 7**

Fraction 7 migrates on the thin-layer plate with an Rf value comparable to glycosphingolipids with four sugars in the carbohydrate chain. The EI mass spectra of the permethylated and permethylated-reduced derivatives contain peaks that are in accordance with a globotetraosylceramide structure with 16 carbon, hydroxy and nonhydroxy fatty acids. The anomeric part of the NMR spectrum is identical with the one of fraction 6 except for an additional doublet at δ5.18 ppm. The position and coupling constant of this peak indicates the presence of a fucose, but as judged by the MS spectrum there is nothing supporting this. Furthermore, there is no corresponding methyl signal arising from fucose in the NMR spectrum. Even though there is no obvious explanation for this resonance, we believe it is due to a contamination because it has appeared in some other spectra run in our lab.
Fraction 8

This fraction migrates as a single band on the TLC plate and has an Rf value in concordance with a glycosphingolipid carrying five sugar residues in the carbohydrate chain (Figure 1). The mass spectrum (not shown) of the permethylated derivative contains a peak at m/z 189 corresponding to a deoxyhexose. There are no peaks corresponding to fragments derived from a terminal hexose or hexosamine. Peaks at m/z 638 and 606 indicate the presence of a terminal trisaccharide comprised of one deoxyhexose, one hexose, and one hexosamine. The peak at m/z 842 is explained by a terminal tetrasaccharide containing one additional hexose unit. Peaks derived from sphingosine (d18:1) and nonhydroxy fatty acids with 16–24 carbon atoms in their aliphatic chain are also present (Karlsson et al., 1976; Karlsson et al., 1982). The mass spectrum of the permethylated-reduced derivative shows intense immunonium ions (fatty acid plus complete carbohydrate chain) at m/z 1330–1442 explained by fragments derived from a pentaglycosylceramide containing three hexoses, one hexosamine, and one deoxyhexose and with C16 to C24 nonhydroxy fatty acids. The proton NMR spectrum (not shown) contains four β-anomeric doublets at 4.17 (Glc), 4.26 (Gal), 4.34 (Gal), and at 4.63 (GlcNAc) ppm. One α-anomeric signal is present at 5.04 ppm (Fuc). All anomeric signals are of equal intensity. The presence of one α-fucose is confirmed by the methyl doublet at 1.06 ppm, and the GlcNAc is confirmed from the N-acetamido methyl signal at 1.82 ppm. The spectrum is in all major aspects identical to a spectrum of a blood group H type 2 chain pentaglycosylceramide (Dabrowski et al., 1981; Kannagi et al., 1983) (Table II). Blood group H activity was also detected in the five sugar region on the TLC plate using the AS53 monoclonal antibody (DAKO, Glostrup, Denmark), which primarily detects type 2 chain blood group H determinants (Figure 2, Table I).

Fraction 9

This fraction appears as a single band on the thin layer plate and has an Rf value corresponding to a five-sugar compound (Figure 1). The mass spectrum of the permethylated and permethylated-reduced fraction 9 is identical to the spectrum of fraction 8 except that the predominant fatty acid is nonhydroxy C16 fatty acid (Smith et al., 1975; Breimer et al., 1981). The NMR spectrum indicates a mixture of two different compounds. There is a blood group H pentaglycosylceramide based on the type 1 carbohydrate chain with one α-anomeric signal at 4.99 ppm (Fuc), three β-hexose signals at 4.17 (Glc), 4.26 (Gal), and 4.44 (Gal) ppm, and one β-HexNAc signal at 4.63 ppm (GlcNAc) (Dabrowski et al., 1981; Kannagi et al., 1983). Methyl signals from one fucose are seen at 1.04 ppm and from the HexNAc at 1.82 ppm. The other compound is a blood group H pentaglycosylceramide based on the type 2 carbohydrate chain with one α-anomeric signal at 5.04 ppm (Fuc) and β-signals at 4.17, 4.26, 4.33 (Gal), and 4.58 ppm (GlcNAc), and one β-HexNAc signal at 4.63 ppm (GlcNAc). Methyl signals are seen at 1.06 ppm for the fucose and at 1.81 ppm for the GlcNAc. The NMR data are in complete agreement with the MS data because the mass spectrum gives very little information with regard to binding positions and can thus not distinguish between the H-5-1 and H-5-2 isomers (Breimer et al., 1981). The ratio between the two compounds is approximately 45% H-5-1 and 55% H-5-2. The antibody binding data presented in Figure 2, using the anti-H (AS53) monoclonal antibody is compatible with the presence of a type 2 blood group H pentaglycosylceramide.

Fraction 10

This fraction migrates as a single band in the five- to six-sugar region upon TLC analysis. Several different monoclonal anti-blood group antibodies bind in this region, indicating a complex composition (Figures 1 and 2, Table I). The NMR spectrum and
the mass spectra of the permethylated and permethylated-reduced derivatives (not shown) strongly suggested the presence of at least four different compounds. By MS/MS analysis (not shown) the following composition was determined: 40% of a pentaglycosylceramide with a Galα1,3Galβ1,4GlcNAc terminal sequence (Galα1,3nLc4), 20% of the globopentaosylceramide structure, 20% of a blood group H pentaglycosylceramide based on a type 1 carbohydrate chain, and 20% of a blood group H pentaglycosylceramide with a type 2 carbohydrate chain. Details of the analysis of fraction 10 is given in another paper (E. C. Hallberg et al., Xenotransplantation, in press).

Fraction 11

The mass spectrum of permethylated fraction 11 is shown in Figure 3. The masses and composition of the terminal sequence ions are listed in Table III. The peak at m/z 1149 may be interpreted as 1148 + 1, i.e., an ion composed of four hexoses and one hexosamine plus part of the ceramide (O-CH2-CH-N-CH3 + H). The major ceramide species is d18:1-n16:0 as given by m/z 548. The ion at m/z 1202 is probably this ceramide together with the two most proximal hexoses and one hexosamine. This ion is intense due to the predominance of this ceramide species and the fact that the fragment is common to several structures which share the same core. The mass spectrum indicates a glycolipid mixture with at least three different compounds.

The mass spectrum of the permethylated-reduced derivatives shows, as expected, a complex pattern due to the mixture of different glycolipids (Figure 4). However it is known that immonium ions are especially abundant in spectra of permethylated-reduced derivatives (Samuelsson, 1986; Samuelsson et al., 1990). The peaks in the spectrum which are assigned to be immonium ions are listed in Table IV together with interpretations. The peak at m/z 1360 could be derived from immonium ions of a glycolipid with a carbohydrate chain of one deoxyhexose, three hexoses, and one hexosamine in combination with a hydroxy C16 fatty acid, or by a chain with four hexoses and one hexosamine in combination with a nonhydroxy C16 fatty acid. This is due to the fact that the mass difference between hydroxy and nonhydroxy fatty acids and between hexose and deoxyhexose is the same, i.e., 30 atomic mass units. To resolve this, a daughter ion spectrum of the m/z 1360 immonium ion was run (not shown). This spectrum has a peak at m/z 624 which shows the presence of a terminal trisaccharide with one hexose, one deoxyhexose, and one hexosamine. This supports the former interpretation, i.e., one deoxyhexose, three hexoses, and one hexosamine on a hydroxy C16 fatty acid. The immonium ions at m/z 1561 and 1562 can be interpreted in two alternative ways and was therefore also analyzed by MS/MS (not shown). MS/MS indicated that these peaks are explained by two structures, where the presence of one of them is supported by a peak at m/z 799 indicating a tetrasaccharide terminal with two deoxyhexoses, one hexose, and one hexosamine. The presence of a terminal tetrasaccharide composed of one deoxyhexose, one hexose, and two hexosamines is indicated by a peak at m/z 856. The presence of these two structures is further supported by the positive reactivity with the anti-Leα and the anti-A antibodies, respectively (Figure 2).
Table III. Terminal sequence ions in the mass spectrum of the permethylated derivatives of fraction 11 of pig aorta glycolipids.

<table>
<thead>
<tr>
<th>m/z</th>
<th>Number and type of saccharide residue</th>
<th>Possible origin/terminal saccharide</th>
</tr>
</thead>
<tbody>
<tr>
<td>189</td>
<td>Terminal deoxyhexose</td>
<td></td>
</tr>
<tr>
<td>219</td>
<td>Terminal hexose</td>
<td></td>
</tr>
<tr>
<td>260</td>
<td>Terminal hexosamine</td>
<td></td>
</tr>
<tr>
<td>464</td>
<td>1 hexose, 1 hexosamine</td>
<td>Gb₄, Lac₄, nLc₄</td>
</tr>
<tr>
<td>638, 606 (638–32)</td>
<td>1 hexose, 1 hexosamine, 1 deoxyhexose</td>
<td>H, Le⁺/X trisaccharides</td>
</tr>
<tr>
<td>668, 636 (668–32)</td>
<td>2 hexoses, 1 hexosamine</td>
<td>Gal₃, nLc₄ trisaccharide, Gb₅ trisaccharide</td>
</tr>
<tr>
<td>812</td>
<td>1 hexose, 1 hexosamine, 2 deoxyhexoses</td>
<td>Le⁺/Y tetrasaccharides</td>
</tr>
<tr>
<td>842</td>
<td>2 hexoses, 1 hexosamine, 1 deoxyhexose</td>
<td>Le⁺/X, blood group B tetrasaccharides, H-6–4 tetrasaccharide</td>
</tr>
<tr>
<td>872</td>
<td>3 hexoses, 1 hexosamine</td>
<td>Gal₃, nLc₄ tetrasaccharide, Gb₅ trisaccharide</td>
</tr>
<tr>
<td>883</td>
<td>1 hexose, 2 hexosamines, 1 deoxyhexose</td>
<td>Blood group A trisaccharide</td>
</tr>
<tr>
<td>1016</td>
<td>2 hexoses, 1 hexosamine, 2 deoxyhexoses</td>
<td>Le⁺/Y tetrasaccharides</td>
</tr>
<tr>
<td>1046</td>
<td>3 hexoses, 1 hexosamine, 1 deoxyhexose</td>
<td>Le⁺/X tetrasaccharides, H-6–4 tetrasaccharide</td>
</tr>
<tr>
<td>1087</td>
<td>2 hexoses, 2 hexosamines, 1 deoxyhexose</td>
<td>Blood group A tetrasaccharide</td>
</tr>
</tbody>
</table>

Table IV. Immonium ions, and their possible composition, in the mass spectrum of the permethylated-reduced fraction 11 of pig aorta glycolipids

<table>
<thead>
<tr>
<th>m/z</th>
<th>Fatty acid</th>
<th>Number and type of saccharide residue</th>
<th>Probable glycolipid structure</th>
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<tbody>
<tr>
<td>1360*</td>
<td>h16:0</td>
<td>3 hexoses, 1 hexosamine, 1 deoxyhexose</td>
<td>H-5, Le-5, X-5</td>
</tr>
<tr>
<td>1390</td>
<td>h16:0</td>
<td>4 hexoses, 1 hexosamine</td>
<td>Gal₃,nLc₄</td>
</tr>
<tr>
<td>1561*</td>
<td>n16:0</td>
<td>3 hexoses, 2 hexosamines, 1 deoxyhexose</td>
<td>A-6</td>
</tr>
<tr>
<td>1562*</td>
<td>h18:0</td>
<td>3 hexoses, 1 hexosamine, 2 deoxyhexoses</td>
<td>Le-6</td>
</tr>
<tr>
<td>1589</td>
<td>n18:0</td>
<td>3 hexoses, 2 hexosamines, 1 deoxyhexose</td>
<td>A-6</td>
</tr>
<tr>
<td>1616</td>
<td>n24:0</td>
<td>3 hexoses, 1 hexosamine, 2 deoxyhexoses</td>
<td>Le-6</td>
</tr>
<tr>
<td>1646</td>
<td>h24:0</td>
<td>3 hexoses, 1 hexosamine, 2 deoxyhexoses</td>
<td>Le-6</td>
</tr>
</tbody>
</table>

*Structure verified by EI-MS/MS

The anomeric region of the NMR spectrum of this fraction is shown in Figure 5. The α-signals at 5.07 and 4.92 ppm and the β-signals at 4.55 and 4.50 ppm can be assigned to a type 1 chain blood group A hexaglycosylceramide. The α-signals at 4.98 and 4.83 ppm together with β-signals at 4.67 and 4.39 ppm arise from a six-sugar Le⁻ structure. The presence of Gal₃,nLc₄ is indicated by an α-signal at 4.84 and β-doublets at 4.67 and 4.29 ppm. The α-anomeric signal of low intensity at 5.04 and two weak β-signals at 4.59 and 4.31 ppm are most probably derived from a blood group H type 2 chain pentaglycosylceramide. The α-anomeric signal at 4.81 ppm, typical of a globo-core, would indicate, when compared with the previous fraction, the presence of the globopenta glycolipid. In the methyl signal regions there are signals present which are derived from the analysis of the anomeric region, but assignments are not possible due to the low resolution caused by severe signal overlap (Dabrowski et al., 1981; Kannagi et al., 1983; Hanfland et al., 1984; Clausen et al., 1985a). By summarizing the data obtained from the analysis of this fraction, the following conclusions can be drawn: the fraction contains Gal₃,nLc₄, a globopentosylceramide structure, a blood group A type 1 chain hexaglycosylceramide, and a type 2 chain difucosyl hexaglycosylceramide containing one deoxyhexose, one hexose, and one hexosamine, and a terminal tetrasaccharide containing one deoxyhexose, two hexoses, and one hexosamine, respectively. A small fragment at m/z 1046 indicates the presence of a terminal pentasaccharide structure of low abundance containing three hexoses, one deoxyhexose, and one hexosamine. The mass spectrum of the permethylated-reduced derivative shows immonium ions at m/z 1589, 1617, and 1646 derived from a carbohydrate structure composed of four hexoses, one hexosamine, and one deoxyhexose in combination with 20, 22, and 24 carbon, nonhydroxy fatty acids, respectively.

The NMR spectrum of fraction 12 (not shown) contains two α-anomeric signals at 4.95 (Fuc) and 4.81 (Gal) ppm and four β-anomeric doublets at 4.48 (Gal), 4.46 (GalNAc), 4.26 (Gal), and 4.17 (GlC) ppm. One fucose methyl signal is present at 1.08 ppm and one N-acetamido methyl signal at 1.82 ppm. This is in very good agreement with the spectrum of a blood group H hexaglycosylceramide based on the type 4 chain (Holgersson et al., 1991).

Fraction 13

The mass spectrum of the permethylated derivative indicates the presence of a terminal deoxyhexose at m/z 189 and terminal hexosamine at m/z 260. There is no evidence of a terminal hexose. Peaks at m/z 638 and 843 are derived from a terminal trisaccharide containing one deoxyhexose, one hexose, and one hexosamine, and a terminal tetrasaccharide containing one deoxyhexose, two hexoses, and one hexosamine, respectively. A fragment at m/z 1046 indicates the presence of a terminal pentasaccharide structure of low abundance containing three hexoses, one deoxyhexose, and one hexosamine. The mass spectrum of the permethylated-reduced derivative shows immonium ions at m/z 1589, 1617, and 1646 derived from a carbohydrate structure composed of four hexoses, one hexosamine, and one deoxyhexose in combination with 20, 22, and 24 carbon, nonhydroxy fatty acids, respectively.

The NMR spectrum of fraction 12 (not shown) contains two α-anomeric signals at 4.95 (Fuc) and 4.81 (Gal) ppm and four β-anomeric doublets at 4.48 (Gal), 4.46 (GalNAc), 4.26 (Gal), and 4.17 (GlC) ppm. One fucose methyl signal is present at 1.08 ppm and one N-acetamido methyl signal at 1.82 ppm. This is in very good agreement with the spectrum of a blood group H hexaglycosylceramide based on the type 4 chain (Holgersson et al., 1991).

Fraction 13

The mass spectrum of permethylated fraction 13 contains peaks derived from a terminal deoxyhexose at m/z 189 and from a terminal hexosamine at m/z 260. Terminal trisaccharide fragments are seen at m/z 638 and 688. Terminal tetrasaccharide fragments are seen at m/z 842, 872, and 883, terminal pentasaccharide fragments at m/z 1046 and 1087 and terminal hexasaccharide fragments at m/z 1250. This suggests the presence of a mixture of blood group H and A hexaglycosylceramides. The
spectrum does not exclude a Lea/X determinant, however the migration of fraction 13 on the TLC plate (Figure 1) supports a hexaglycosylceramide structure.

The NMR spectrum of this fraction has the H-6-4 compound as the predominant structure (Holgersson et al., 1991).

**Fraction 14**

Electrospray MS of native fraction 14 (Figure 6) indicates a mixture of different hexaglycosylceramide structures and possibly a heptaglycosylceramide. The molecular ion at m/z 1575 most probably originates from a blood group H hexaglycosylceramide, but this peak was subjected to tandem MS for further elucidation (Figure 7). The analyses were run in the presence of sodium creating sodium adduct ions, and thus 23 mass units heavier fragments. A composition of four hexoses, one hexosamine, and one deoxyhexose in combination with a 16 carbon nonhydroxy fatty acid on a trihydroxy base would give rise to a molecular ion peak at m/z 1574. The peaks at m/z 739 and 901 are explained by the ceramide together with one and two hexoses, respectively. A terminal tetrasaccharide composed of two hexoses, one hexosamine, and a deoxyhexose gives rise to the peak at m/z 696. The peak at m/z 534 is explained by a terminal trisaccharide with one deoxyhexose, one hexose, and one hexosamine in accordance with a blood group H determinant. The rest of the structure, i.e., three hexoses and the ceramide explain the peak at m/z 1063. These two fragments are the only ones consistent solely with a blood group H hexaglycosylceramide of this molecular weight. A small peak at m/z 185 indicates a terminal hexose and a peak at m/z 347 a terminal disaccharide with two hexoses. These two peaks together with the somewhat more intense peak at m/z 550 indicate a terminal trisaccharide with two hexoses and one hexosamine, a sequence consistent with the recently discovered Galα1,3Lewis structure (Bouhours et al., 1997). The other peaks in the daughter ion spectrum originates in fragments common to both structures (Figure 7, top chart). The molecular ion at m/z 1616 (Figure 6) may originate from a blood group A hexaglycosylceramide with the same ceramide as above, i.e., a 16 carbon nonhydroxy fatty acid on a trihydroxy base. The peaks at m/z 1644, 1672, 1700, and 1728 are the corresponding molecular ions for 18, 20, 22, and 24 carbon nonhydroxy fatty acids. This is supported by the positive reaction with the anti-A and anti-A type 1 antibodies in the six sugar region on TLC (see Figure 2). The addition of a deoxyhexose to the latter structure giving an ALexheptaglycosylceramide could explain the molecular ion at m/z 1874 (Figure 6, top chart). The binding of an antibody specific for the ALeX-determinant (see Table I) supports this interpretation. Regrettably, we did not have enough
Fig. 5. The anomic region of the 300 MHz proton NMR spectrum recorded from fraction 11 of pig aorta glycolipids. Data were collected at a probe temperature of 30°C with a digital resolution better than 0.3 Hz/point using 90° pulses. A signal with a small coupling constant (J < 5 Hz) is derived from α-configured hydrogens of the first carbon (the glycosidic bond carbon), and is here called an α-anomeric signal. In the same way, a signal with a large coupling constant (J > 5 Hz) is derived from a β-configured hydrogen at the first carbon, and is referred to as a β-signal.

Substance to run MS/MS analyses on m/z 1616 and 1874 or to carry out any NMR analysis.

Fraction 15–19

The glycosphingolipid content of these fractions is too small to enable any structural characterization. However, the presence of a Galβ1,3Gal terminal in fraction 15 has been indicated by the reactivity with human hyperimmune sera from patients transplanted with pig islet cells (Rydberg et al., 1994).

Semiquantification of globo- and lacto-series glycolipids

An estimation of the distribution of the glycolipids between globo- and lacto-series shows an approximate ratio of 10:1 among the total neutral glycolipids and 1:1 among the polar glycolipids. This is in agreement with the situation in human aorta (Prokazova et al., 1986).

Discussion

The glycolipid expression in pig aorta is very similar to the expression in pig kidney. The major components in both types of tissue are tri- and tetrahexosylceramides of the globoseries (Holgersson et al., 1990b; Jalali-Araghi and Macher, 1994). Blood group A and H hexaglycosylceramides as well as H pentaglycosylceramides are also abundant constituents (Holgersson et al., 1990b; Bouhours et al., 1997). Human blood group H determinant has also been detected by immunostaining on primary aortic endothelial cells in culture (Bouhours et al., 1996). These findings are in contrast to a study in which immunofluorescent staining of different pig tissue failed to detect blood group determinants in blood vessels (Oriol et al., 1993). The discrepancy could be explained by different expression of glycolipids in different individuals and on different cell types in the tissue. It is possible that isolation and chemical characterization of glycolipids of an extensively vascularized organ, e.g., placenta or lung, would give a more relevant picture of the antigen expression of endothelial cells. The question raised is whether or not the same glycolipids are expressed on a particular cell type in vessels derived from different tissue (Holgersson et al., 1990a) and if cultured cells in all respects mimic cells in vivo. The need to do both biochemical and immunohistochemical analyses of the tissue expression of possible antigens is thus obvious, as these techniques are complementary to each other. Conclusions reached by antibody recognition are dependent on the characterization of the antibody binding epitope. Antibody binding data does not necessarily conclude new structures or give information on carrier saccharide chain type, nor will genetic mapping do in a foreseeable future. Chemical characterization, however, can give the complete structure, including the ceramide type of a glycosphingolipid. Unfortunately, chemical characterization requires that quite a lot of material be available, which makes it difficult to characterize the glycolipid expression on a specific cell type of low abundance in a particular tissue.
Fig. 6. The result of positive ion mode ESI of the native glycolipid fraction 14 from pig aorta. The accelerating voltage was set at 4 kV with a scan time of 5 s/decade in the mass range of 100–2600 m/z. The sample was dissolved in chloroform/methanol/water 39/60/1 (by volume) with 0.5 nmol/jl NaCl. The proposed fragmentation of the molecular ions at m/z 1616 and 1874 is shown at the top. Figures in parenthesis shows fragments that are not represented as peaks in the spectrum. The tentative structural assignment is based on mass spectrometry data (number and type of sugars and ceramide) and TLC antibody binding data.

The antigens that are detected on endothelial cells by lectins and antibodies in vitro are probably instantly recognized by circulating antibodies, for example, following xenotransplanta-

tion, thereby initiating a hyperacute rejection. However, structures on deeper lying cell layers might be equally accessible for antibodies if the superficial layers are destroyed by an immune
Fig. 7. The positive ion mode ES-TOF daughter ion spectrum of the precursor resolved molecular ion of the native glycolipid fraction 14 prepared from pig aorta. The low energy collision of the molecular ion was made using argon gas at a lab frame collision energy of 400 eV. The proposed fragmentation of the molecular ions is shown at the top. Figures in parenthesis shows fragments that are not represented as peaks in the spectrum. The structures are suggested on the basis of earlier published data.

attack. Even though not proven to be expressed on endothelial cells, structures such as $\text{Gal}^{\alpha 1,3}\text{Le}^\alpha$ identified from whole porcine aorta may contribute to such a rejection process (Bouhours et al., 1996; this article).
Pig aorta glycosphingolipids

The GaAl1,3nLc3 has previously been described in pig kidney (Jalali-Araghi and Machet, 1994; Bouhours et al., 1997) and is in this article identified in aorta by solid structural characterization. Bouhours and collaborators (Bouhours et al., 1996) have by antibody labeling and exoglycosidase treatment tentatively shown the presence of this structure in aortic endothelial cells. Longer, GaAl1,3-terminated structures have been described previously (Bouhours et al., 1997), some of which are fucosylated. When human hyperimmune sera from patients transplanted with pig islet cells were characterized, binding of antiallo antibodies and exoglycosidase treatment tentatively identified in aorta by solid structural characterization. This article identified in aorta by solid structural characterization. This fraction contained galactosamines and disaccharides, including Galβ1,3GalNAc and Galβ1,4GlcNAc. Galβ1,3Gal and Galβ1,4GlcNAc in fraction 15 have previously been described in pig kidney (Jalali-Araghi and Machet, 1994; Bouhours et al., 1997). This fraction was rerun on the same HPLC column with a linear gradient of C/M/W 70/30/2 to 40/40/12 (by volume). The fractions obtained were analyzed by TLC and pooled, resulting in 19 fractions which were subsequently analyzed by NMR and MS.

Analytical thin-layer chromatography

Thin-layer chromatography was performed on HPTLC plates (Si-60, Merck, Darmstadt, Germany) with C/M/W, 60/35/8 (by volume), as solvent. Detection was accomplished by a chemical reagent, anisaldehyde (Karlsson, 1987), or by autoradiography after immunostaining the plates with monoclonal antibodies followed by 125I-labeled secondary antibodies (Samuelsson, 1987).

Proton NMR spectroscopy

1H NMR spectroscopy was performed on deuterium-exchanged purified native glycolipids dissolved in 0.5 ml of dimethyl sulfoxide-d6 containing 2% D2O. Spectra were recorded at 300 MHz using a Varian VX300 (Varian, Palo Alto, CA). Data were collected at a probe temperature of 30°C with a digital resolution better than 0.3 Hz/point using 90° pulses, but no relaxation delay. Chemical shifts are given relative to tetramethylsilane. The data were processed off line, using the NMR1 software (New Methods Research Inc., Syracuse, NY). Resolution enhancement was achieved using either a Lorentz-to-Gauss transformation or Maximum Entropy calculations as implemented in NMR1.

Mass spectrometry

Mass spectrometry of the permethylated or permethylated-reduced glycolipid fractions was performed on a ZAB-HF (VG Analytical, Manchester, UK) magnetic sector instrument operated in positive electron impact ionization (EI) mode.

The permethylated-reduced sample of a single fraction (fraction 11) was analyzed by tandem mass spectrometry (AutoSpec-ETOFPD, Micromass, Manchester, UK). This instrument has a configuration with a magnetic sector as the first mass spectrometer and an orthogonal accelerated time of flight as the second (TOF-MS). The sample was loaded on a Pyrex sample holder probe tip and inserted into the ionization source held at a temperature of 250°C. The temperature of the probe was increased at a rate of 25°C/min from 50 to 350°C. Spectra were recorded in the mass range 100–3100 m/z at a scan time of 10 s/decade and at a resolution of 2000 (at 10% valley definition). Daughter ion spectra of selected monoisotopically resolved immonium precursor ions were recorded at 800 eV lab frame collision energy (Bateman et al., 1995) using argon as collision gas. Liquid secondary ion mass spectrometry (LSIMS) of Csl (Merck, Germany) was used to precalibrate the instrument prior to EI-MS analyses.

Another fraction (fraction 14) was analyzed by electrospray ionization tandem mass spectrometry. The glycosphingolipid sample was dissolved in C/M/W 39/60/1 (by volume) containing 0.5 mmol/μl NaCl. The sample solution was delivered into the ES source from a loop injector (50 μl) at a flow rate of 5–7 μl/min using an HPLC-pump (JASCO PU-980, Tokyo, Japan). The resolution of the instrument (i.e., 2500 at 10% valley) was obtained from a singly charged cluster ion of Csl (10 ng/μl). Positive ion spectra were collected in a profile mode over the mass range of 100–2600 at a scan time of 5 s/decade at 4 kV ion acceleration voltage. This spectrum was used to calibrate the instrument. The orthogonal accelerated time of flight mass

Materials and methods

Tissue specimens

Pig aortas (from more than 80 different animals of a local cross-breed of Yorkshire, Hampshire, and a Swedish native breed) were collected from the local abattoir. The tissue was dissected free from fat and connective tissue before it was cut into small pieces, frozen, and freeze-dried. No ABO blood group typing was performed.

Glycolipid preparation

A total non-acid glycolipid fraction was prepared as described (Karlsson, 1987; Holgersson et al., 1990b). This fraction was purified by HPLC (Pharmacia-LKB, Sweden) using a 22 × 300 mm silicic acid column (POLYGOSIL, 10 μm particles; Skandinaviska Genetec, Sweden) with a linear gradient of chloroform/methanol/water (C/M/W) 80/20/1 to 40/40/12 (by volume) over 280 min with a flow rate of 2 ml/min. Eluted fractions were analyzed by TLC and pooled according to TLC migration. A glycolipid fraction was obtained containing the more polar compounds having carbohydrate chains with more than three sugar residues. This fraction was rerun on the same HPLC column with a linear gradient of C/M/W 70/30/2 to 40/40/12 (by volume). The fractions obtained were analyzed by TLC and pooled, resulting in 19 fractions which were subsequently analyzed by NMR and MS.

The identification of GaAl1,3nLc3 has previously been described in pig kidney (Jalali-Araghi and Machet, 1994; Bouhours et al., 1997) and is in this article identified in aorta by solid structural characterization. Bouhours and collaborators (Bouhours et al., 1996) have by antibody labeling and exoglycosidase treatment tentatively shown the presence of this structure in aortic endothelial cells. Longer, GaAl1,3-terminated structures have been described previously (Bouhours et al., 1997), some of which are fucosylated. When human hyperimmune sera from patients transplanted with pig islet cells were characterized, binding of antiallo antibodies and exoglycosidase treatment tentatively identified in aorta by solid structural characterization. This fraction contained galactosamines and disaccharides, including Galβ1,3GalNAc and Galβ1,4GlcNAc. Galβ1,3Gal and Galβ1,4GlcNAc in fraction 15 have previously been described in pig kidney (Jalali-Araghi and Machet, 1994; Bouhours et al., 1997). This fraction was rerun on the same HPLC column with a linear gradient of C/M/W 70/30/2 to 40/40/12 (by volume). The fractions obtained were analyzed by TLC and pooled, resulting in 19 fractions which were subsequently analyzed by NMR and MS.
sputrometer was operated in the positive ion mode at a lab frame collision energy of 400 eV using argon as collision gas. The resolution of MS-1 was preset to 1000 at 10% valley definition, before the ions entered the collision cell. The TOF mass spectrometer was optimized at a resolution of ~600 at 50% full width half maximum using the m/z 2215 ion peak of the Cal cluster ion produced by ESI-MS. The calibration of the TOF-MS made from the daughter ion spectrum of the same precursor ion. In addition, spectra were averaged over several scans, smoothed, background subtracted and peak detected using the Opus software facilities (Micromass, Manchester, UK).

A few samples (fractions 2–5) were analyzed as native glycolipids by LSIMS in negative ion mode. For more details about the technical conditions and interpretation of spectra, see references (Breimer et al., 1979, 1980; Samuelsson, 1986; Lindström et al., 1992).

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Abbreviations
EI, electron ionization; ESI, electrospray ionization; FAB, fast atom bombardment; MS, mass spectrometry; NMR, nuclear magnetic resonance. The sugar types are abbreviated Hex for hexose, HexNAc for N-acetylhexosamine, dHex for deoxyhexose. The ceramide types are abbreviated n for nonhydroxy fatty acid, h for hydroxy fatty acid, d for dihydroxy base and t for trihydroxy base. In the shorthand designation for blood group glycolipids, the letter(s) indicates blood group determinant, the first numeral the number of sugar residues and the second numeral the type of carbohydrate chain blood group antigen and N-glycolylneuraminic acid.

Identification of several blood group type glycolipids in the small intestine of porcine aorta endothelial cells of glycosphingolipids bearing the major carbohydrate chain blood group A determinant. Accommodation: a working paradigm for progressing toward clinical discordant xenografting. Transplant Proc., 23, 205–207.


